Antibacterial Applications of Clove Essential Oil & Its main Constituents

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Abstract

Xanthomonas citri subsp. *citri* has become an increasingly prevalent pathogen in areas where citrus fruits are produced. This bacterium causes growths on the exterior of citrus fruits known as citrus canker. These growths make the fruit ineligible for being sold, and trees infected with it need to decontaminated according to guidelines set forth by integrated management measures. We tested the efficacy of a naturally occurring compound as a bactericidal agent, namely clove essential oil, alongside some of the most abundant compounds present in clove essential oil, namely Eugenol, Isoeugenol, and β-caryophyllene. Due to ML.2 certification restriction, *E. coli* was used as model organism, instead of X. citri. The oils themselves were shown to be effective at concentrations of 2.5% or higher, which is consistent with the results for isolated Eugenol. Isoeugenol was shown to be effective at a lower concentration, inhibiting growth at a concentration of 1.25%. β-caryophyllene was not shown to have any bactericidal characteristics. All effective treatments have in common that they work by disrupting membrane integrity, causing nearly every cell in a permeability assay to show up as having membrane damage after only 15 minutes of treatment with the compounds at half of the Minimum Inhibitory Concentration (MIC). Assuming that *X. citri* subsp. *citri* behaves similarly to *E. coli*, this shows that clove oil can be used as an alternative to the harsh pesticides currently in use.

1. Introduction

Currently, over 60% of the global supply of orange juice is produced from oranges grown in Brazil. The orchards where these oranges are grown are being threatened by a phytopathogen that causes Asiatic Citrus Canker (ACC), *Xanthomonas citri* subsp. *citri* (Gottwald et al. 2002). The method that was used until recently to fight off this pathogen was burning down infected trees. While effective, this causes great monetary losses to those responsible for the orchard. To not have to resort to this option of fighting *Xanthomonas citri* subsp. *citri*, farmers opt for a less destructive approach. They spray fruits of all infected trees, as well as the neighbouring trees in the orchards with pesticides in the form of copper salts. This approach does come with its own sacrifices, however, in that the pathogens are developing resistance to these pesticides (Behlau et al. 2011), as well as the fact that this persistent copper usage causes accumulation of copper in the soil, which is harmful to the environment (Nunes et al. 2016).

Due to the problems previously described, it is imperative that other, less harmful, antimicrobial agents are discovered and tested for their efficacy. One such potential candidate, is clove essential oil. Morão et al. (2020) highlight the fact that many phenolic compounds are effective at eliminating bacteria. Clove essential oil has already been widely studied as an antimicrobial agent. A study performed by Soraggi Battagin et al. (2021) showed that washing citrus fruits with clove oil was effective at killing *X. citri*. Research done for this paper focuses on which main constituent of clove oil (Eugenol, Isoeugenol, or β-caryophyllene (Jirovetz et al. 2006)) is the most active agent.

This research presented in this paper highlights that, on *Escherichia coli*, of these three compounds present in clove oil, Isoeugenol was the most effective constituent, being bactericidal at half the concentration at which Eugenol starts becoming bactericidal. β-caryophyllene ended up not being active. The compounds that were found to be bactericidal were further analysed for their mode of action, the results which pointed towards membrane disruption.

2. Materials and methods

2.1 Organism studied

While the research as outlined in the introduction calls for *Xanthomonas citri* subsp. *citri* to be used, this was not possible due to not being certified for ML-2 research. As such, *Escherichia coli* was chosen as a gram-negative model organism, grown in pre-mixed Carl Roth LB-medium (Lennox). A tandem study was performed by a colleague performing the same experiments on gram-positive *Bacillus subtilis* to compare the results on gram-negative and gram-positive.

2.2 Clove essential oil (CEO) constituents to be studied

A mass spectrometry of Clove essential oil performed by Soraggi Battagin et al. (2021) revealed that a list of 16 compounds were identified as present to a reasonable degree of certainty. This, combined with the study on CEO composition by Jirovetz et al. (2006) lead to the selection of three compounds for use in this study: Eugenol, Isoeugenol and β-caryophyllene, as these three compounds are the most abundant in clove oil. These compounds were acquired from Sigma Aldrich at 98% purity. In addition to the selection of compounds from clove essential oil, the bactericidal effects of clove essential oil in its entirety were also studied. Two brands of clove essential oil were put under study and given arbitrary names: Physalis Eugenia Caryophyllata, under pseudonym Jeff, and De Tuinen natural care Kruidnagel, under pseudonym Bob. A stock was then prepared for both CEOs of 5 parts clove oil to 1 part DMSO.

2.3 Antimicrobial test of CEO

To determine at what concentration these compounds exhibit antibacterial activity, an antimicrobial test was performed in both glassware, and on a 96-well plate. For the experiment using glassware, an overnight culture of *E. coli* was used to inoculate glass tubes containing various concentrations of clove oil: 0.25%, 0.5%, 0.75%, 1%, 2%, 4%, and 5%. This clove oil came from stock of 5 parts CEO to 1 part DMSO, meaning that the highest concentration of DMSO present in any tube did not exceed 1%. The controls for this experiment were kanamycin as a positive control, no treatment as a negative control, and DMSO 1% to confirm that that concentration of DMSO does not interfere with the results.

For the antimicrobial test using the 96-well plate, filling of the wells was done according to table 1.

Table 1: scheme for the filling of the 96-well plate for clove oil analysis.

		Jeff			Bob						Genta	negative	DMSO
		1	2	3	4	5	6	7	8	9	10	11	12
B. Subtilis	A	5%	5%	5%	5%	5%	5%						
	B	2,50%	2,50%	2,50%	2,50%	2,50%	2,50%						
	C	1,25%	1,25%	1,25%	1,25%	1,25%	1,25%						
	D	0,63%	0,63%	0,63%	0,63%	0,63%	0,63%						
E. Coli	E	5%	5%	5%	5%	5%	5%						
	F	2,50%	2,50%	2,50%	2,50%	2,50%	2,50%						
	G	1,25%	1,25%	1,25%	1,25%	1,25%	1,25%						
	H	0,63%	0,63%	0,63%	0,63%	0,63%	0,63%						
			10 µL of bacterial culture was added										
			10 µL of medium was added										

Every filled well had a final volume of 100µL. Columns 7, and 9 were left empty, column 8 contained the same serial dilution as columns 1-3, but starting from D and H for *B. subtilis* and *E. coli* respectively. This was done to attempt to explain unexpected results in 3.2. Columns 10, 11, and 12 were the controls, 20 µg/ml gentamycin as positive control, LB medium as negative control, and 1% DMSO as another negative control. The wells that did not have bacterial culture added were used as blanks for relevant treatments. Rows A, B, C, and D belong to the tandem study and can be disregarded in the context of *E. coli*.

This plate was then incubated in a Tecan infinite 200Pro plate reader at 30°C for 24 hours, taking OD_{600} measurements every 15 minutes. Data was processed in Excel.

This was followed by a minimal bactericidal concentration (MBC) assay that was performed by inoculating a set of LB plates with 5µL of liquid from every well, which were then incubated overnight at 30°C and inspected for growth.

2.4 Antimicrobial test of clove oil substituents

The same steps were taken for this assay as for the clove oil test using the 96-well plate, using the three compounds instead of the two clove oil brands. Filling for this plate was done according to table 2.

Table 2: scheme for the filling of the 96-well plate for compound analysis.

	Eugenol			Isoeugenol			β-caryophyllene			Genta	negative	DMSO
	1	2	3	4	5	6	7	8	9	10	11	12
A	5%	5%	5%	5%	5%	5%	5%	5%	5%			
B	2,50%	2,50%	2,50%	2,50%	2,50%	2,50%	2,50%	2,50%	2,50%			
C	1,25%	1,25%	1,25%	1,25%	1,25%	1,25%	1,25%	1,25%	1,25%			
D	0,625%	0,625%	0,625%	0,625%	0,625%	0,625%	0,625%	0,625%	0,625%			
E	0,32%	0,32%	0,32%	0,32%	0,32%	0,32%	0,32%	0,32%	0,32%			
F	0,16%	0,16%	0,16%	0,16%	0,16%	0,16%	0,16%	0,16%	0,16%			
G	0,08%	0,08%	0,08%	0,08%	0,08%	0,08%	0,08%	0,08%	0,08%			
H	0,04%	0,04%	0,04%	0,04%	0,04%	0,04%	0,04%	0,04%	0,04%			

10 µL of bacterial culture was added

10 µL of medium was added

All wells that had bacterial culture added, as well as the wells for the controls that did not have bacterial culture added, had a final volume of 100µL. The remaining wells had a final volume of 90µL. Due to this, those wells could not be used as a reliable blank for the relevant wells, and thus, the DMSO control wells that did not have bacterial culture added served as a blank for this entire experiment. Columns 10, 11, and 12 were the controls, 20 µg/ml gentamycin as positive control, LB medium as negative control, and 1% DMSO as another negative control.

2.5 Mode of action

The method of action for bactericidal activity this study focuses on, is permeabilization of the membrane. This was done using a protocol for membrane permeability assay modified from Morão et al. (2020). This study limits the treatment time to only 15 minutes, has a separate negative and DMSO control, and uses Gentamycin as a positive control. The concentration of the compounds that was used for this experiment are half of their respective MIC. After treatment, the samples were centrifuged, washed, and centrifuged again. The pellet was then resuspended in saline solution containing SYTO9 and propidium iodide. 10µL of every sample was then introduced to microscope slides mounted with agarose pads. The microscope used was Nikon Ti microscope equipped with an ORCA-flash 4.0 camera (Hamamatsu) and filters FITC and TRITC. Images were acquired with NIS Elements 4.10 software and processed using FIJI ImageJ.

2.6 Reactive Oxygen Species (ROS) formation assay

A ROS assay was performed. This was done by growing a culture of *E. coli* in 10mL LB medium overnight, which was then diluted to an OD_{600} of 0.1 and left to grow into exponential phase to an OD_{600} of ~0.3. When exponential phase was reached, 1mL of bacterial culture was added to 4 individual 2mL Eppendorf tubes. These Eppendorf tubes were then centrifuged at 10000 rpm for 5 minutes, after which the supernatant was discarded, and the pellet resuspended in wash buffer. After another round of centrifugation, the supernatant was discarded again, and the pellet resuspended in 1mL of LB media. A black-lined 96-well plate was then prepared the same way as in section 2.4. This plate was then deposited in the same Tecan plate reader, this time measuring fluorescence at an excitation wavelength of 488 and an emission wavelength of 530nm for one hour, taking measurements every 5 minutes.

3. Results and discussion

3.1 Antimicrobial test of clove oils

3.1.1 test in glassware

After introducing colonies of *E. coli* to various concentrations of Clove Essential Oil (CEO) in liquid medium, these tubes were left to incubate for 24 hours at 30°C, after which the growth of in these tubes was assayed by inoculating plates of LB agar. Preliminary analysis of this assay showed that CEO does not significantly limit the growth of *E. coli,* as turbidity was observed in all tubes except the tube that contained the positive control. Photos of these tubes can be found in figure 1.

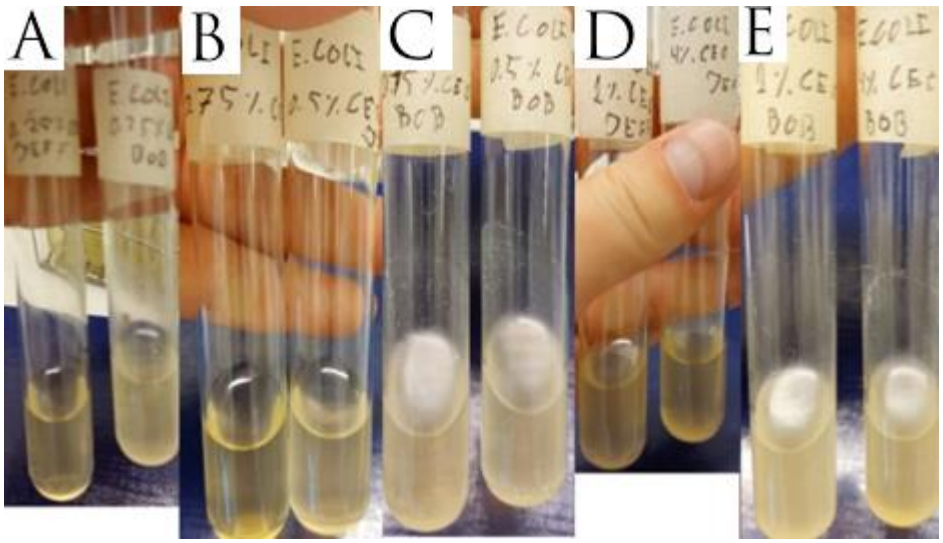


Figure 1: photos of the tubes that were not plated after being retrieved from the incubator. All of these tubes are turbid to some degree, indicating bacterial growth in each of them. These photos also show the formation of a solid compound at the edge of the liquid level with the angle of the incubator. **(A)** from left to right: 0.25% Jeff, 0.25% Bob **(B)** 0.75% Jeff, 0.5% Jeff **(C)** 0.75% Bob, 0.5% Bob **(D)** 1% Jeff, 4% Jeff **(E)** 1% Bob, 4% Bob.

At first, the unknown solid discussed in figure 1 was thought to be bacterial growth that had solidified, but this was later put into question. The presence of DMSO then came to mind as a possible curdling agent for the oily compounds in CEO, and as such, an experiment was performed to test that hypothesis. This experiment consisted of incubating the same mixture as the 5% CEO mixture as before, but this time without adding the bacteria. This experiment did not show formation of any solid on the walls of the tubes. Due to this fact, the results of that experiment are not included here.

This result does come with a few caveats, however. When retrieving the tubes containing the bacteria from the incubator, all the CEO was observed to be clumped up at the bottom of the tubes with the higher concentrations of CEO. This would point to the CEO not being able to dissolve and mix with the medium, even with the addition of DMSO as an emulsifier. Another, even bigger caveat comes after an observation of the growth media used for this experiment. After the plating was done, turbidity was observed in the medium stock. This was followed by an overnight incubation of an inoculated plate with just that growth media as inoculum. This plate yielded the image in figure 2. These colonies are not consistent with the morphology of colonies of *E. coli* as compared to other plates made as preparation for this experiment. As this contaminated medium was used to make all of the dilutions, growth was observed in every treatment, even in the positive control (figure 2). As such, the results of this entire experiment can be discarded. The results of this experiment did serve as an educational piece for a change in methodology for following experiments.

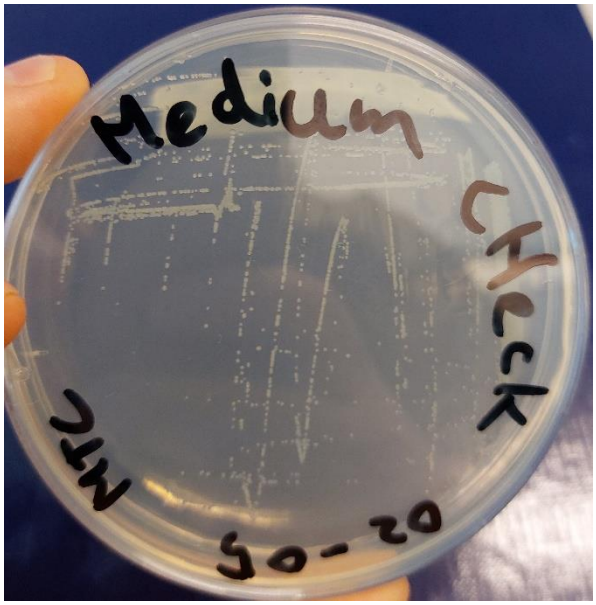


Figure 2**:** streak of growth medium suspected of being contaminated. This shows clear signs of contamination, but due to the difference in colony morphology to *E. coli*, is not of known origin, likely a contaminant from the air or bench.

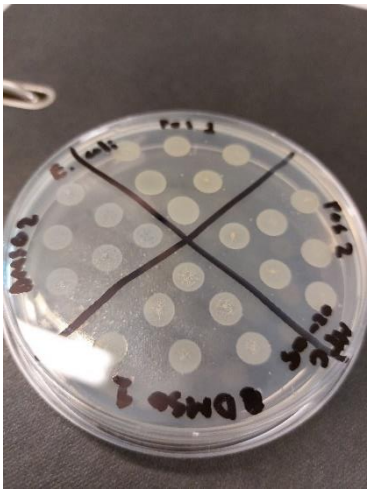


Figure 3**:** The plate containing the positive and DMSO controls. This plate shows that growth was observed even in the positive control, fortifying the conclusion that the growth media was contaminated. Another noteworthy observation about this plate is that the colonies that are growing on the DMSO 2 control are greyer in colour. This observation does not currently have any explanation and will thus be considered as an artifact.

3.1.2 Test on 96-well plate

Due to the discarded results of the glassware experiment, the decision was made to perform a broth microdilution experiment in a 96-well plate. The wells of the plate were filled following the scheme in table 1.

After measuring the growth over 24 hours, the graphs found in figure 4 were produced, showing that the lowest concentration at which there is absolutely no growth of *E. coli* is 2.5% for treatment with Jeff and Bob.

This MIC assay was followed up with an MBC assay where 5µL of every well was transferred to a set of plates of LB medium, which was incubated overnight. This confirmed the findings found with the MIC assay, showing no growth at the expected concentrations, and even showing no growth at the spot of 1.25% Bob. From this, it can be concluded that with a minimum concentration of 2.5%, CEO is bactericidal for *E. coli*.

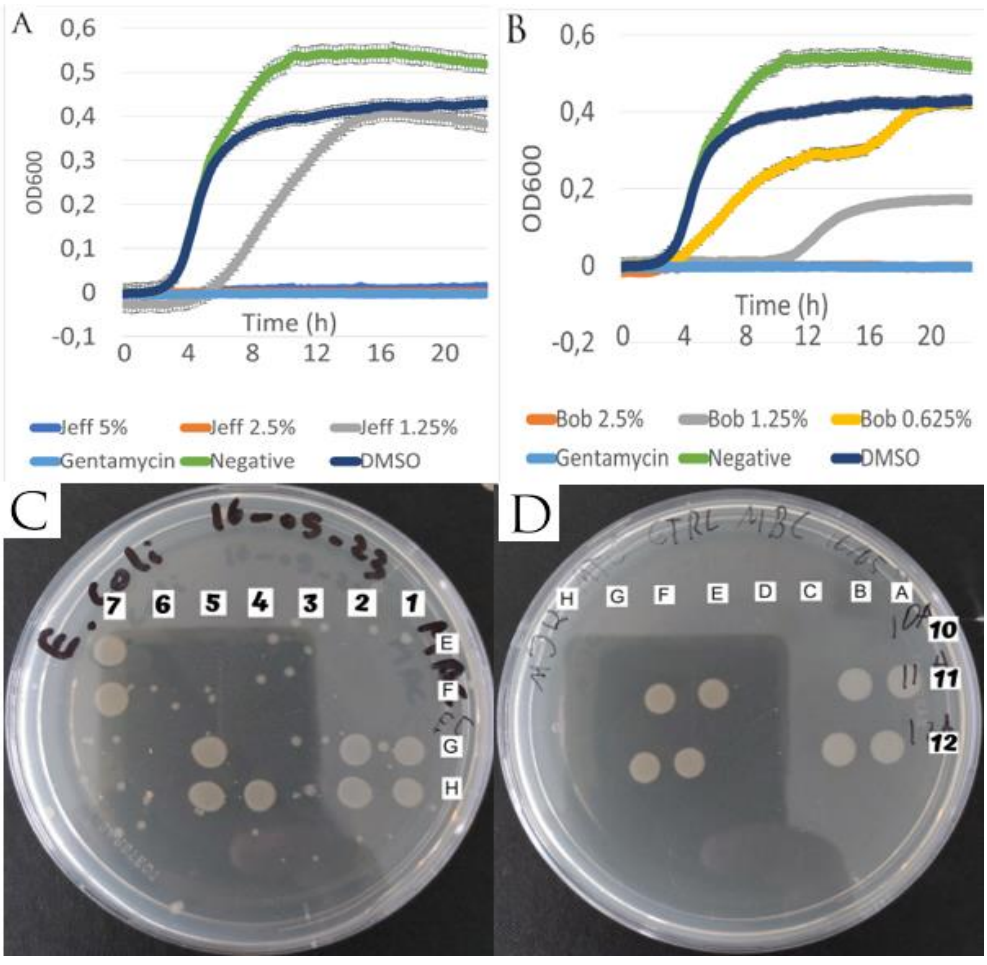


Figure 4: Growth curves for MIC assay under treatment with the two brands of CEO, as well as the following MBC assay. (**A**) Growth curve for treatment with Jeff CEO. The lowest concentration that causes absolutely no growth is observed to be 2.5%, but slowing down of growth already happens at a concentration of 1.25%, suggesting that the actual MIC lies somewhere between 2.5% and 1.25. (**B**) Growth curve for treatment with Bob CEO. The lowest concentration that causes absolutely no growth is observed to be 2.5%, but slowing down of growth already happens at a concentration of 1.25%, suggesting that the actual MIC lies somewhere between 2.5% and 1.25%. (**C**) MBC assay from the 96-well plate. Column 7 is not relevant for this experiment, as this was done to rule out machine malfunction as a possible cause for unexpected results in 3.2 and can be disregarded. (**D**) Controls for the MBC assay. The droplets for column 12 are slightly shifted to what is to the left in the image. Growth belongs to row A, B, E, and F.

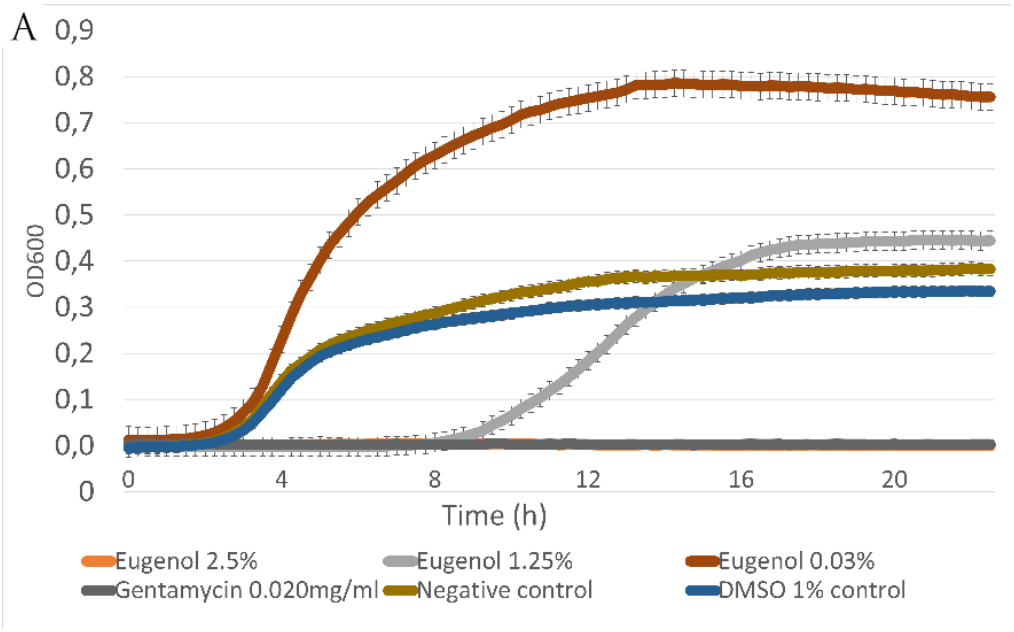
3.2 Antimicrobial test of compounds

The 96-well plate for this experiment was filled according to the scheme found in table 2.

After measuring growth over 24 hours, the graphs found in figure 5 were produced, showing that the lowest concentration at which there is absolutely no growth of *E. coli* is 2.5% for treatment with Eugenol, while under treatment with Isoeugenol, 1.25% is already enough to completely halt growth of *E. coli*. In contrast to these other two compounds, β-caryophyllene does not have any bactericidal effect.

What is of note in the growth curves for Eugenol and Isoeugenol treatments, is that the growth line for 0.03% concentration of compound is significantly higher than both the negative, and the DMSO controls. Hypotheses of what could have caused this include the possibility of artifacts in the plate reader and that these compounds could have a positive effect on growth when present in low concentrations, but due to time constraints, this was not followed up on, and was instead left as material for further study.

This MBC assay was performed with a replicator, in theory allowing a clean transfer of fluid from the 96-well plate to a large LB plate. This tool, however, was bent in such a way that the fluid from the middle wells could not be picked up. This resulted in a really messy plate that was nigh unintelligible if not for the results from the MIC assay.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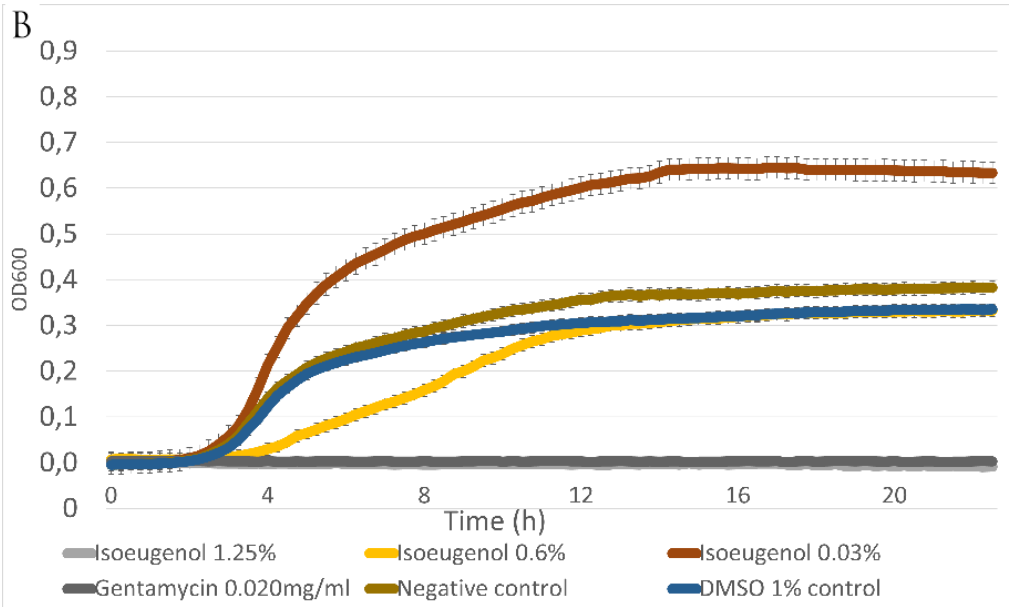
Gentamycin 0.020mg/ml
Negative control
DMSO 1% control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Time (h)
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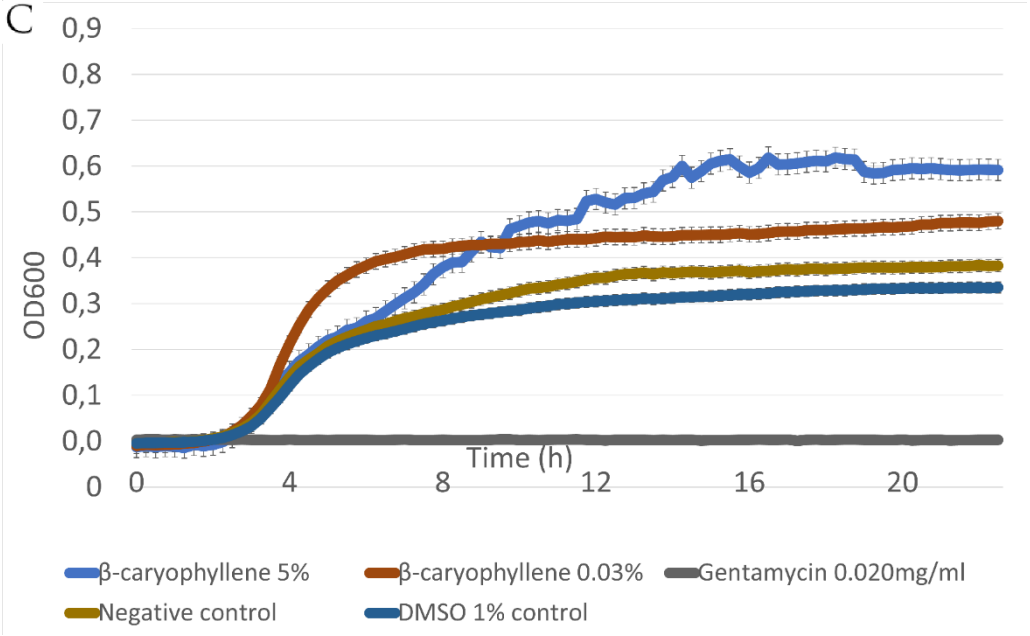
Isoeugenol 0.6%
Isoeugenol 0.03%
Gentamycin 0.020mg/ml
Negative control
DMSO 1% control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β-caryophyllene 0.03%
Gentamycin 0.020mg/ml
Negative control
DMSO 1% control

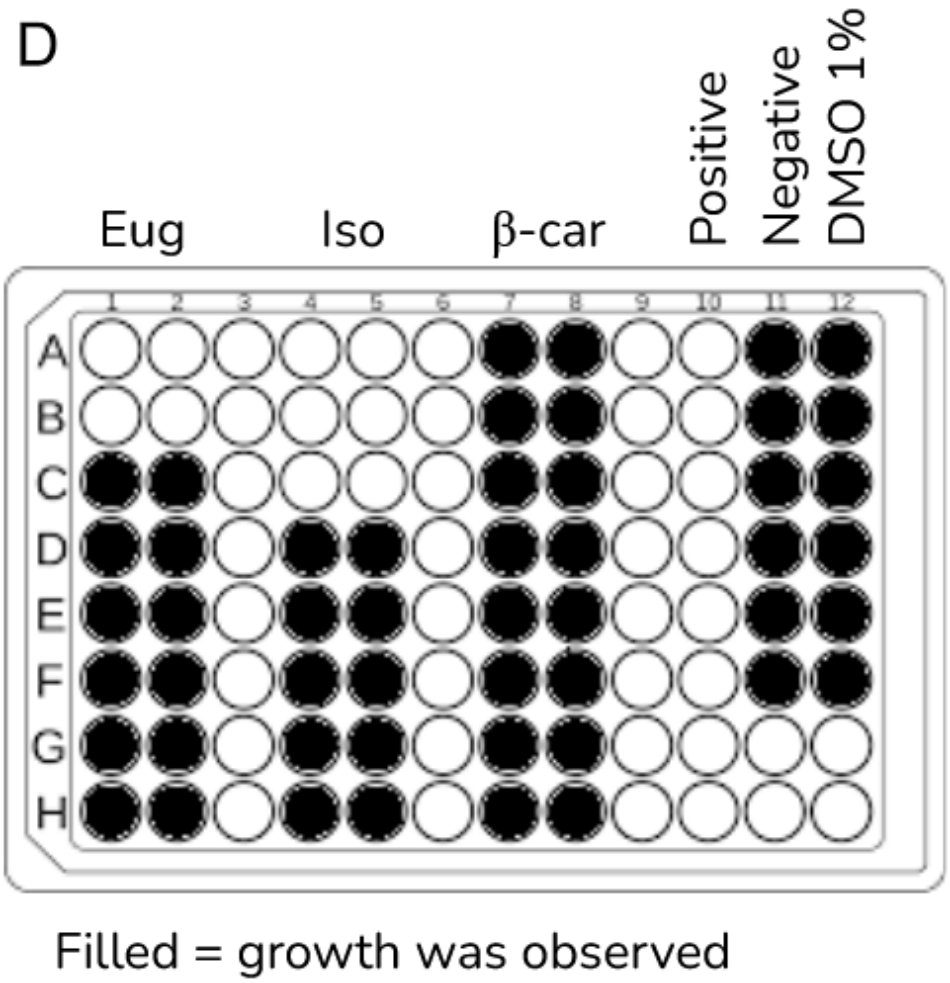

Figure 5: growth curves for MIC assay under treatments with Eugenol, Isoeugenol, and β-caryophyllene respectively **(A) (B) (C)**, as well as the following MBC assay **(D)**. An interpretation of the MBC plate is included here in lieu of the plate itself.

3.3 Mode of action analysis

Membrane permeabilization was tested by analysing samples of bacteria treated with Eugenol and Isoeugenol for 15 minutes at half MIC under a fluorescence microscope. When cells have their membranes permeabilized, influx of propidium iodide is possible, causing those cells to show up in the TRITC fluorescent field. The microscopy images can be found in figure 6. From these images, it can be inferred that treatment with Eugenol and Isoeugenol causes membrane permeabilization at a short timescale, faster than permeabilization by cell death as caused by gentamycin.

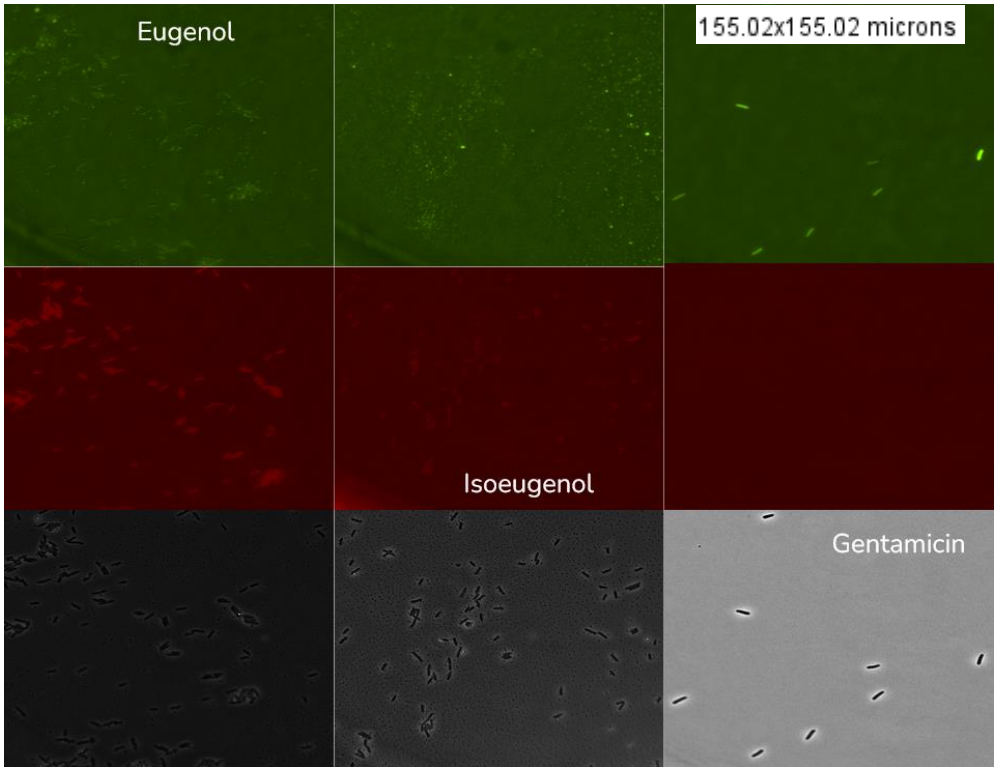

Figure 6: Fluorescence microscopy images of the two treatments, alongside the positive control of gentamycin. Every image in the same column belongs to the same treatment. Cells fully stained with green are not permeabilized, cells fully stained in red are. Treatment with the compounds leads to fast membrane permeabilization.

3.4 ROS formation assay

To test if ROS formation is what causes the membrane permeabilization, an experiment was performed to compare oxidative stress under treatment with the CEOs to oxidative stress when exposed to hydrogen peroxide. This experiment did not deliver any meaningful results, as even the highest concentration of hydrogen peroxide did not lead to a high increase in ROS production. For this reason, the results of the experiment will not be included in this paper. An idea for a future experiment could be to increase the treatment time to multiple hours to give the oils ample chance to lead to an effect, but since the mode of action acts on a fast timescale, this was not relevant to this research.

3.5 Results of the tandem study

The effects of clove oil and its main constituents is similar in gram positive *B. subtilis* and gram negative *E. coli*. The only real difference found between the two organisms is the fact that under the highest concentration of treatment, *B. subtilis* was shown to be affected in the MIC assays, but in the MBC assays, inoculated spots did show growth. This is likely because *B. subtilis* is a known spore-forming bacterium and thus does not undermine the expected efficacy of these substances on *X. citri*, as *X. citri* does not form spores.

4. Conclusions

Treatments with Clove Essential Oil (CEO), as well as pure Eugenol at concentrations of 2.5% or higher and to Isoeugenol at concentrations of 1.25% or higher are bactericidal to *E. coli*, while β-caryophyllene does not have any bactericidal effect. These treatments work by disrupting the membranes of cells at a fast timescale. This is likely not caused by the formation of ROS. The results point towards Isoeugenol being the most effective constituent of CEO for the inhibition of *E. coli* growth, proving to be more effective than CEO itself, which, assuming *X. citri* reacts similarly, could make it a suitable candidate in the prevention of widespread ACC infection as an alternative to copper salts.

5. References

Behlau, F.; Canteros, B.I.; Minsavage, G.V.; Jones, J.B.; Graham, J.H. Molecular Characterization of Copper Resistance Genes from Xanthomonas citri subsp. citri and Xanthomonas alfalfae subsp. citrumelonis. Appl. Environ. Microbiol. 2011, 77, 4089–4096. http://dx.doi.org/10.1128/AEM.03043-10

Gottwald, T.R.; Graham, J.H.; Schubert, T.S. Citrus Canker: The Pathogen and Its Impact. Plant Heal. Prog. 2002, 3, 15. http://dx.doi.org/10.1094/PHP-2002-0812-01-RV

Jirovetz, L., Buchbauer, G., Stoilova, I., Stoyanova, A., Krastanov, A., Schmidt, E. Chemical Composition and Antioxidant Properties of Clove Leaf Essential Oil , Journal of Agricultural and Food Chemistry 2006 54 (17), 6303-6307, https://dx.doi.org/10.1021/jf060608c

Morão LG, Lorenzoni ASG, Chakraborty P, Ayusso GM, Cavalca LB, Santos MB, Marques BC, Dilarri G, Zamuner C, Regasini LO, Ferreira H, Scheffers D-J. Investigating the Modes of Action of the Antimicrobial Chalcones BC1 and T9A. *Molecules*. 2020; 25(20):4596. https://doi.org/10.3390/molecules25204596

Nunes, I.; Samuel, J.; Brejnrod, A.; Holm, P.E.; Johansen, A.; Brandt, K.K.; Priemé, A.; Sørensen, S.J. Coping with copper: Legacy effect of copper on potential activity of soil bacteria following a century of exposure. FEMS Microbiol. Ecol. 2016, 92, 175. http://dx.doi.org/10.1093/femsec/fiw175

Soraggi Battagin T, Nicolas Caccalano M, Dilarri G, et al. Syzygium aromaticum (clove) essential oil: An alternative for the sanitization of citrus fruit in packinghouses. J Food Process Preserv. 2021;45:e15496. https://doi.org/10.1111/jfpp.15496