Determination of Antimicrobial Activity of Alkyloxy Benzene-1,2-diols

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Abstract

Asiatic citrus canker is a disease in orange trees, caused by the bacterium *Xanthomonas citri.* Earlier research has showed the antimicrobial effects of alkyl gallates and alkyl dihydroxybenzoates against this bacterium. Alkyloxy benzene-1,2-diols have also shown to be effective. I was interested to know whether these ethers had an antimicrobial effect against other bacterial species as well. Therefore, it was tested whether they had antimicrobial activity against *Bacillus subtilis* and *Escherichia coli*. It was found that the ethers with between 4 and 9 carbons in their carbon tail had an antimicrobial effect on *B. subtilis*, but not on *E. coli*. The highest activity was found for the ethers with 6, 7 or 8 carbons in their tail. The results for the Minimal Inhibitory and Minimal Bactericidal Concentration were the same, showing that the ethers are in fact bactericidal. Performance of a LIVE/DEAD assay showed that heptyloxy benzene-1,2-diol permeabilizes the bacterial membrane of *B. subtilis*.

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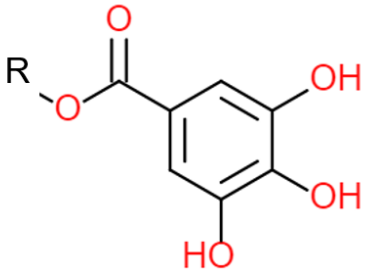
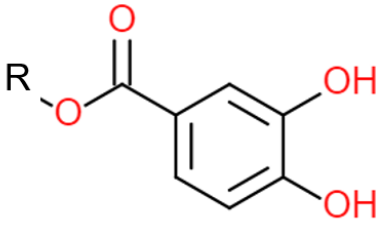
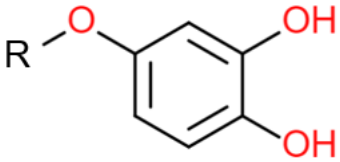
1. Introduction

The Gram negative bacterium *Xanthomonas citri subsp. citri* is the cause of a disease in orange trees called Asiatic citrus canker. Infection with this pathogen causes brown lesions on the leaves and early fruit drop. It is currently present in several countries that produce oranges, for example in the USA and Brazil. The occurrence of the disease has resulted in restrictions in the orange-fruit trade between these countries and Europe, leading to economic losses (Dewdney, Roberts, Graham, Chung, & Zekri, 2013). The current method to control the spread of citrus canker includes the use of copper sprays. Despite its effectiveness in control of the disease, there are several downsides to the use of copper: copper accumulates into the soil, it is toxic for many life forms and copper induces the development of resistance (Silva et al., 2013). Therefore, researchers started looking for an alternative to copper.

Table 1: structures of different chemical groups tested for antimicrobial activity

Chemical Group Name	Structure Formula
Alkyl gallates	
Acetylated alkyl gallate derivatives	
Alkyl dihydroxybenzoates	
Alkyloxy benzene-1,2-diols	

In 2013, Silva et al. found that alkyl gallates (see **Table 1**) have antimicrobial activity against *X. citri*. They found a positive correlation between the alkyl chain length and MIC values, with the compounds containing 9 to 11 carbons in their side chain having the highest antimicrobial activity. *X. citri* cells that were treated with alkyl gallates before application to leaves in an infection model, lost their ability to colonize orange leaves. This

showed that the gallates indeed have the potential to protect against citrus canker. The study also investigated the possible targets of the gallates and it was found that the gallates disrupt cell division and chromosome segregation (Silva et al., 2013). Król et al have further investigated the target mechanisms of the alkyl gallates by looking at its effects on the Gram positive *Bacillus subtilis*, and showed that cell division protein FtsZ is a direct target of the alkyl gallates and that alkyl gallates target membrane integrity (Król et al., 2015).

In 2018, Savietto et al. discovered that the acetylation of one of the hydroxyl groups increased the antibacterial activity. The alkyl gallate derivatives (see Table 1) have an increased lipophilicity and are therefore better able to penetrate membranes. After acetylation, the ability to act on membranes was maintained, however, the ability to interact with FtsZ was lost (Savietto et al., 2018). In that same year, Nazaré et al evaluated the antimicrobial effect of alkyl dihydroxybenzoates (see Table 1), which were shown to be effective against *X. citri*. An increased activity for heptyl dihydroxybenzoate compared to heptyl gallate was found, showing that a decrease in the amount of the hydroxyl groups increased the antimicrobial activity. Again, a combination of FtsZ inhibition and membrane permeability was identified as the cause of cell death (Nazaré et al., 2018).

In this report, I investigated another set of compounds, alkyloxy benzene-1,2-diols (see Table 1). The difference between these compounds and alkyl dihydroxybenzoates is that there is an ether bound instead of an ester bound connecting the benzene-ring and alkyl group. The reason for the interest in these compounds is that they are derived from 1,2,4-benzenetriol (BTO), which can be obtained from sugar cane bagasse. Producing antimicrobial compounds out of agricultural waste could possibly provide for a more environmental friendly option to tackle citrus canker. Our research group has shown that these compounds do show antimicrobial activity against *X. citri* as well, with the highest activity for heptyloxy benzene-1,2-diol (Cavalca, unpublished data).

The antimicrobial activity of alkyloxy benzene-1,2-diols has not been tested against other bacteria so far. I want to know whether these compounds show activity against bacterial species other than *X. citri.* Knowing the effect of these compounds is very important when attending to implement one or several of them in nature as anti-*X. citri* agent. It will also give more inside into their working mechanism. To start, the antimicrobial activity of alkyloxy benzene-1,2-diols against two different bacterial species is tested; the Gram-positive model organism *B. subtilis* and the Gram-negative model organism *Escherichia coli*.

I expected to find activity against *B. subtilis*; in the study of Król et al, *B. subtilis* was used to demonstrate the working mechanism of alkyl gallates (Król et al., 2015) and Savietto et al found that their alkyl gallate derivates are effective against *B. subtilis* (Savietto et al., 2018). It was therefore hypothesized that the ethers that have an effect on *X. citri* will have an effect on *B. subtilis* as well. For *E. coli* I did not expect to find any activity, since other similar compounds tested by our research group did not show any activity against *E. coli* as well.

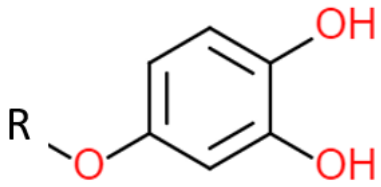
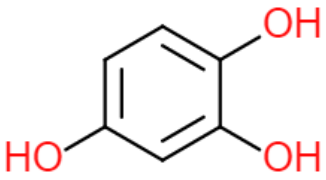
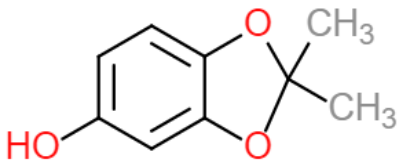
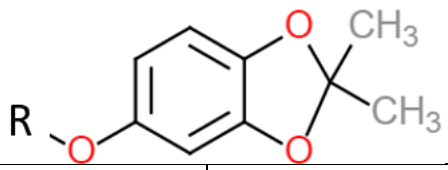
I am interested to see whether the ethers, when shown to be effective, disrupt the bacterial membrane. This because the studies described above indicate membrane permeability as one of the main targets for the studied compounds. When hypothesized that the alkyloxy benzene-1,2-diols have a similar working mechanism, it is expected to see membrane disruption when bacterial growth inhibition is present.

2. Materials and Methods

2.1. Compounds

The alkyloxy benzene-1,2-diols tested on their antimicrobial activity in this study are derived from 1,2,4-benzenetriol (BTO). Synthesis of these compounds has been performed by the Molecular Microbiology group in collaboration with the Engineering and Technology institute of the Groningen University. See **Table 2** for an overview of the different compounds used in this study and the abbreviations used in this paper to refer to them. All the compounds used in this study were diluted to a concentration of 11 mg/ml in DMSO.

Table 2: structure and naming of the compounds used in this study

Alkyloxy benzene-1,2-diols 	
Abbreviation	**Chemical name**
4C	4-(butyloxy)benzene-1,2-diol
5C	4-(pentyloxy)benzene-1,2-diol
6C	4-(hexyloxy)benzene-1,2-diol
7C	4-(heptyloxy)benzene-1,2-diol
8C	4-(octyloxy)benzene-1,2-diol
9C	4-(nonyloxy)benzene-1,2-diol
12C	4-(dodecyloxy)benzene-1,2-diol
14C	4-(tetradecyloxy)benzene-1,2-diol
BTO 	1,2,4-benzenetriol
Protected BTO (pBTO) 	2,2-dimethylbenzodioxol-5-ol
Protected BTO derived ether 	
5cp	5-(pentyloxy)-2,2-dimethylbenzodixole
7cp	5-(heptyloxy)-2,2-dimethylbenzodixole

2.2. Bacterial strains and Growth Conditions

Two different bacterial strains have been studied; *B. subtilis* wildtype 168 and *E. coli* wildtype Mg1654. These strains were taken from the strain collection of the Molecular Microbiology department of University of Groningen. Both were grown at 30 degrees Celsius, either while shaking in liquid LB Lennox medium (tryptone: 10 g/L, yeast extract: 5 g/L, NaCl: 5 g/L); or on solid LB Lennox agar medium (tryptone: 10 g/L, yeast extract: 5 g/L, NaCl: 5 g/L, agar: 15 g/L). Bacterial stocks were made at a 50% dilution with glycerol, which serves as a preserving agent. The bacteria used for the stocks were collected during exponential growth. For each strain, three stocks were made to ensure independent replicates. The stocks were stored at -80 degrees Celsius.

2.3. Growth Curves and Cell Count

Growth curves were made to determine growth behavior, and to establish the timing of the different growth phases. *B. subtilis* was grown for 20 hours in a 96-wells plate at 30 degrees Celsius under constant shaking inside a microplate reader (Biotek Powerwave). The OD at 600 nm was measured during this time period every 30 minutes. Two different volumes of bacterial dilution (100 and 200 µl) with the same starting OD were compared. As a negative control, LB-Lennox medium was used. See **Appendix A.**

E.coli was grown for 24 hours in a 96-wells plate at 30 degrees Celsius under constant shaking inside a microplate reader (Biotek Synergy MX). The OD was measured during this time period every 30 minutes. As a negative control, LB-Lennox medium was used. See **Appendix B.**

Cell count *B. subtilis:* Culture was grown overnight and diluted to an OD at 600 nm of 0,1 in the morning. At different timepoints, the OD at 600 nm of was measured using a spectrophotometer (Novaspec Plus). After each measurement, the bacterial culture was diluted 10x. Out of this 10x dilution, a dilution range from 10^{-1} till 10^{-8} in PBS was made. Out of each dilution, 100 µl was spread on a LB-Lennox agar plate.

Cell count *Escherichia coli:* Culture was grown overnight and diluted to an OD at 600 nm of 0,1 in the morning. At different timepoints, the OD at 600 nm was measured using a spectrophotometer (Novaspec Plus). After the measurement, the bacterial culture was diluted 10x. Out of this 10x dilution, a dilution range from 10^{-1} till 10^{-8} in PBS was made. Out of dilution 10^{-4} - 10^{-8}, 100 µl was spread on a LB-Lennox agar plate.

2.4. Minimal Inhibitory and Minimal Bactericidal Concentration Assay

Bacteria were grown to about 10^7 cells/ml (*B. sub*: OD ≈ 0,4, *E. coli* OD ≈ 0,65) at 30 degrees under constant shaking, and diluted 10 times. Six different compounds at a concentration of 110 µg/ml were added to a 96-wells plate and diluted using a 2-fold scheme to concentrations ranging from 12,5 to 100 µg/ml. DMSO at 1% and kanamycin at 20 µg/ml were added to the plate as solvent control and positive control. To the wells, 10 µl of bacterial dilution was added (end volume = 100 µl). Medium only containing compound, without addition of bacteria, was used as chemical control. Purely LB-Lennox medium was used as blank. The 96-wells plate was covered with a breathable membrane (Diversified Biotech Breathe-Easy Gas Permeable Sealing Membrane for Microliter Plates) and was put to grow for 24 hours at 30 degrees Celsius under constant shaking in a plate incubator. After these 24 hours the OD inside the wells was measured using a microplate reader (Biotek Powerwave).

Initially, the 96 wells-plate was incubated for a period of 4 hours inside a microplate reader. Every half hour the OD was measured during a time period of 4 hours; this was later changed to one measurement after 24 hours, see Results.

Part of the liquid inside the wells of the 96-wells plate was moved to a 15 cm petri dish filled with LB-Lennox agar medium with the means of a microplate replicator. This plate was placed in a 30 degrees incubator to allow surviving bacteria from the wells to grow overnight.

2.5. Resazurin Microtiter Assay

This method is an alternative method for establishing the Minimal Inhibitory concentration. It was used after an incubation time of 4 hours instead of 24 hours, but has later been discarded as method to establish the MIC, see 3. Results.

After moving part of the bacterial solutions inside the 96-wells plate with the microplate replicator (see 2.4), 15 µl of 1% Resazurin was added to each well. After this, the fluorescence of the bacterial solutions was measured using a microplate reader (Biotek Synergy MX). Reduction of the growth medium caused by cell viability causes the nonfluorescent dye resazurin to change into the red fluorescent die resorufin. By measuring the fluorescence of the solutions with chemicals and comparing them to the blank, the percentage of growth inhibition can be determined.

2.6. LIVE/DEAD Assay

To test the viability of the bacterial cells after treatment with compound, a LIVE/DEAD assay was performed using the LIVE/DEAD BacLight Bacterial Viability Kit K7012. In this assay, a combination of two dyes are used: SYTO 9, which is membrane permeable and penetrates all bacteria in a population, and Propidium iodide, which is membrane impermeable and therefore only penetrates bacteria with damaged membranes. Penetration of Propidium iodide causes a reduction in the fluorescence of SYTO 9. This results in bacterial cells with intact membranes to stain fluorescent green, while cells with damaged membranes stain fluorescent red (Molecular Probes, 2004).

A bacterial culture of *B. subtilis* was grown to a concentration 10^7 CFU/ml (OD ≈ 0,4) at 30 degrees Celsius in a shaking incubator. Compound was added to get the desired concentration, end volume = 500 µl. 1% DMSO is used as negative control and 5 µg/ml Nisin is used as positive control. The bacterial solutions with compound were incubated for 15 minutes at 30 degrees Celsius. After these 15 minutes the bacteria were washed using 1 mL of 0.85% NaCl solution. A mix of NaCl solution and dyes has been made: 1 µl SYTO 9 dye (3,34 mM, diluted in DMSO) + 1 µl Propidium iodide (20 mM, diluted in DMSO) + 332 µl NaCl 0,85% solution. Of this mixture, 50 µl is added to the bacterial solutions. The samples were then incubated in the dark for 15 minutes and transferred to agarose slides (1% w/v agarose in PBS). The bacteria were imaged using a Nikon Eclipse Ti Fluorescence Microscope with optic filters FITC and TRITC using Nikon Imaging Software (Nikon, n.d.).

3. Results

To test for antimicrobial activity, the minimal inhibitory and minimal bacteriostatic concentration have been determined. Different methods can be used to establish the minimal inhibitory concentration.

3.1. Minimal Inhibitory Concentration: 4 hours

The OD at 600 nm has been measured for 4 hours to establish the MIC. This time period was chosen based on the growth curve made of *B. subtilis*, see Appendix A. When establishing the degree of growth inhibition of a compound, it is ideal to measure during exponential growth. After 4 hours, the bacteria are in the middle of the exponential growth phase.
Several measurements of the OD of *B. subtilis* at 600 nm for 4 hours after treatment with the compounds 5C (5 repetitions), 6C (5 repetitions), 7C (4 repetitions), 8C (one measurement) and 9C (one measurement) have been conducted. The results showed that for the compounds 5C, 6C, 7C and 8C, the MIC is between 12,5 and 25 µg/ml, and for 9C between 50 and 100 µg/ml. These results suggest little difference in MIC between the ethers with different carbon lengths.
Looking at a longer growth curve of 18,5 hours, we could see that the bacteria of *B. subtilis* treated with compound would often start growing after a longer period than 4 hours, see **Appendix C**. Measurements using this time period give therefore a different result. They showed that the MIC of 5C and 9C are between 50 and 100 µg/ml and the MIC of 6C, 7C and 8C between 25 and 50 µg/ml.
These results show that the number for the MIC is very dependent on the chosen time period of measuring. They do however show that the ethers have antimicrobial activity against *B. subtilis*.

3.2. Resazurin Microtiter Assay

Resazurin Microtiter Assay (REMA) was performed to establish the MIC of the compounds after the 4 hour growth curve. The MIC for the ethers against *B. subtilis* was established to be between 25 and 50 µg/ml for 5C, 6C and 9C and at between 12,5 and 25 µg for 7C and 8C.
By using REMA, a more precise number for the MIC can be established than by measuring the OD. However, it is important to note that REMA is not a measure for bacterial growth, but for bacterial activity. Having little bacteria with a lot of activity can result in the same numbers as having many bacteria with little activity. This method is therefore not the best suited for measuring bacterial growth inhibition. Therefore, this assay has later been discarded as method to establish the MIC.

3.3. Minimal Inhibitory Concentration: 24 hours

Because of the difference in results based on the chosen time period, the time period for establishing the MIC was changed to 24 hours. This time period is also more in line with other research where the antimicrobial activity of compounds has been established. The OD at 600 nm has been measured after 24 hours of incubation with compound. Each result, unless stated otherwise, is based on three biological replicates. The ethers have shown to be effective against *B. subtilis*, but not against *E. coli*. The highest activity is found for the compounds 6C, 7C and 8C. For an overview of the results, see **Table 2**.

3.3.1. Bacillus subtilis

The minimal inhibitory concentration of six alkyloxy benzene-1,2-diols against *B. subtilis* has been determined. For all ethers tested, an inhibitory effect on the growth of *B. subtilis* has been found. The MIC of 4C (2 out of 3 replicates), 5C, and 9C (2 out of 3 replicates) lies between 50 and 100 µg/ml. The MIC of 6C, 7C and 8C lies between 25 and 50 µg/ml. The inhibitory effect of the precursor BTO as well as several intermediates of the reaction process has also been measured. The MIC for all of them (BTO, pBTO, 5cp & 7cp) has been established to be higher than 100 µg/ml.

3.3.2. Escherichia coli

The minimal inhibitory concentration of eight alkyloxy benzene-1,2-diols against *Escherichia coli* has been determined. I found that the MIC is higher than 100 µg/ml for all of them (4C, 5C, 6C, 7C, 8C, 9C, 12C & 14C).

The inhibitory effect of the precursor BTO as well as several intermediates of the reaction process have been measured as well. The MIC of BTO was found to be between 50 and 100 µg/ml. The MIC of the intermediates (pBTO (no replicates), 5cp & 7cp) has been established to be higher than 100 µg/ml.

3.4. Minimal Bactericidal Concentration

Following the the MIC measurement after 24 hours of incubation, the bacteria were placed onto media not containing any compound to establish the MBC. Each result, unless stated otherwise, is based on three biological replicates. Results show that the tested ethers with between 5 and 9 carbons are bactericidal against *B. subtilis*, but do not have antimicrobial effect on *E. coli*.

3.4.1. Bacillus subtilis

The minimal bactericidal concentration of six alkyloxy benzene-1,2-diols against *B. subtilis* has been determined. For all compounds tested with a carbon chain between 5 and 9, a bactericidal effect on *B. subtilis* has been found. The MBC of 5C, and 9C lies between 50 and 100 µg/ml. The MBC of 6C (2 out of 3 replicates), 7C and 8C lies between 25 and 50 µg/ml. The MBC of 4C was higher than 100 µg/ml. The inhibitory effect of the precursor BTO as well as several intermediates of the reaction process have been measured as well. The MBC for all of them (BTO, pBTO, 5cp & 7cp) has been established to be higher than 100 µg/ml.

3.4.2. Escherichia coli

The minimal bactericidal concentration of eight alkyloxy benzenediols against *Escherichia coli* has been determined. I found that the MBC is higher than 100 µg/ml for all of them (4C, 5C, 6C, 7C, 8C, 9C, 12C & 14C). The inhibitory effect of the precursor BTO as well as several intermediates of the reaction process have been measured as well. The MBC of BTO was found to be between 50 and 100 µg/ml. The MBC of the intermediates (pBTO (no replicates), 5cp & 7cp) has been established to be higher than 100 µg/ml.

Table 3: Minimal Inhibitory and Minimal Bactericidal Concentration of several compounds

	Bacillus subtilis		*Escherichia coli*	
Compound	**MIC** (µg/ml)	**MBC** (µg/ml)	**MIC** (µg/ml)	**MBC** (µg/ml)
4C	50-100*	>100	>100	>100
5C	50-100	50-100	>100	>100
6C	25-50	25-50*	>100	>100
7C	25-50	25-50	>100	>100
8C	25-50	25-50	>100	>100
9C	50-100*	50-100	>100	>100
12C	-	-	>100	>100
14C	-	-	>100	>100
pBTO	>100	>100	>100**	>100**
5cp	>100	>100	>100	>100
7cp	>100	>100	>100	>100
BTO	>100	>100	50-100	50-100

**Result found in 2 out of 3 repetitions*

***No replications for this result*

3.5. LIVE/DEAD ASSAY

LIVE/DEAD assay was used to establish whether treatment with compound permeabilizes the bacterial membrane. The percentage of *B. subtilis* cells with a permeabilized membrane has been determined for 4-(heptyloxy)benzene-1,2-diol at a concentration of 25 and 50 µg/ml. It has also been determined for 7cp at a concentration of 50 µg/ml. See **Table 4**. The numbers were based on a single treatment with no replicates. The percentage of cells with permeabilized membranes after treatment with 7C at a concentration of 25 µg/ml was 78,4%, and at a concentration of 50 µg/ml it is 80,4%. The percentage of cells with permeabilized membranes after treatment with 7cp at a concentration of 25 µg/ml was 21,9%.

Table 4: Percentage of dead *Bacillus subtilis* cells after treatment with compound

Treatment	Concentration	Percentage Permeabilized
7C	25 µg/ml	78,4
7C	50 µg/ml	80,4
7cp	50 µg/ml	21,9
DMSO	1%	0,9*
Nisin	5 µg/ml	98,8*

n: min = 315, max = 1578. Numbers for 7C and 7cp based on a single treatment
**Average based on two treatments*

4. Conclusions and Discussion

It was shown that several alkyloxy benzene-1,2-diols have an antimicrobial effect on *Bacillus subtilis*, but no effect has been found for *Escherichia coli*. The number established for the minimal inhibitory concentration are the same as the ones for the minimal bactericidal concentration, showing that the tested ethers are bactericidal against *B. subtilis.*

The LIVE/DEAD assay has shown that heptyloxy benzene-1,2-iol disrupts the bacterial membrane. This makes it likely that the ethers found to be have antimicrobial activity in this study target the bacterial membrane and therefore have a similar working mechanism as the earlier tested alkyl gallates and their derivatives (Savietto et al., 2018; Silva et al., 2013). The exact working mechanism of the alkyloxy benzene-1,2-diols will have to be further investigated. Since the earlier tested alkyl gallates and their derivates show anti-Ftsz activity on top of membrane disruption (Król et al., 2015; Nazaré et al., 2018; Savietto et al., 2018), it would be interesting to see whether the alkyloxy benzene-1,2-diols have an effect on the activity of Ftsz and cell division overall.

Since this study indicates that the ethers target the bacterial membrane, it is of interest to consider the differences in membrane composition between the bacterial species where they are effective against and the ones they do not show any antimicrobial activity for.

The ethers containing 6, 7 and 8 carbons in their alkyloxy tail have the highest activity against *B. subtilis*. This is in line with earlier found results; Nazaré et all found the highest antimicrobial activity against *X. citri* for the alkyl dihydroxybenzoates with a carbon tail of around 7 carbons (Nazaré et al., 2018), and the alkoxy benzene-1,2-diols with a tail length of 5, 6, 7 or 8 carbons showed the highest activity against *X. citri* (Cavalca, unpublished data). With around 7 carbons in the tail, there is likely the best balance between hydrophilic and lipophilic properties. A study investigating the design of antimicrobial agents by Nihei et al. shows that an increased tail length often increases antimicrobial activity, but decreases the solubility of the compounds in water, which can lead to the formation of micelles. Micelles are not able to pass the bacterial membrane, which would explain why further increasing the carbon tail length leads to a lower antimicrobial activity (Nihei, Nihei, & Kubo, 2003).

It can also be said that both the two hydroxyl groups and the carbon tail are essential for the antimicrobial activity of the alkyloxy benzenediols in this study. BTO as well as 7cp did not show any antimicrobial activity on *B. subtilis*. BTO has the 2 hydroxyl groups but misses the carbon tail present in the alkyloxy group, while protected 7C has this tail but lacks the hydroxyl groups. Nihei et al. also found that having at least 2 hydroxyl groups was essential for antimicrobial activity to be present in their tested compounds, which are 3,4-dihydroxybenzoates and 3,4 dihydroxyphenyls (Nihei et al., 2003). However, a side-wise comparison like in this study has not been done yet for the antimicrobial agents effective against *X. citri*.

When looking at longer growth curves of *B. subtilis*, I saw that some bacteria treated with compound started to grow after several hours. One of the possible causes for this, is that the compounds could get degraded, or perhaps aggregate and come out of solution, over time. Preliminary results suggest that the compounds get degraded or come out of solution in LB medium (Cavalca, unpublished data). LB has a neutral pH, while the medium used by our research group to growth *X. citri* is acidic. When testing the antimicrobial activity of our compounds against *X. citri*, no indications for compound degradation were found. This suggests that the compounds are stable in acidic medium, but not in neutral medium.

Part of this study was the search for a good investigation method to establish the values for the minimal bacteriostatic concentration. Several problems came to light; bacteria treated with compound would start to grow after a time period way longer than the one of 4 hours that was used and the REMA has proven not to be a good measure for bacterial growth. Changing to a time period of 24 hours and leaving out REMA as a method to establish the MIC solved these problems partly. By using a time period of 24 hours, I am also more in line with other studies, since the MIC is most often defined as the minimal inhibitory concentration of a compound needed to inhibit bacterial growth for a period of 24 hours. This makes comparison of the ethers in this studies to other antimicrobial compounds easier. Despite this, it is still important to consider the limitations of the current method. The effect of compound degradation is also bigger when using a longer time frame, something that should be considered as well.

In this study the activity of the alkyloxy benzene-1,2-diols was tested against two bacterial species, *B. subtilis* and *E. coli.* It would be of interest to test the antimicrobial activity against other bacterial species as well. First on the list is *Lactococcus lactis;* this bacterium beneficial for us humans, and is yet another Gram-positive model organism. It would also be interesting to test the activity of the compounds against species that are pathogenic for humans. Like stated in the introduction, it is important to know the effect against other bacterial species when wanting to implement one or several of the compounds in nature.

My and many researchers objective is to find an alternative method to tackle citrus canker, that is less toxic than the ones currently used. Toxicity is therefore an important factor to measure. The compounds used in this study were sent to collaborators regarding toxicity. The ethers do not show to be toxic themselves, however, the production process of the alkyloxy benzene-1,2-diols out of BTO has shown to be toxic. The ethers are more stable than the earlier tested esters and they can be derived from agricultural waste. Therefore, it is worthy to investigate the production process in order to obtain a less toxic route of synthesis.

5. Bibliography

Cavalca, L. B., unpublished data

Dewdney, M. M., Roberts, P. D., Graham, J. H., Chung, K. R., & Zekri, M. (2013). Homeowner Fact Sheet : Citrus Canker. Retrieved April 9, 2020, from https://edis.ifas.ufl.edu/pp116

Król, E., de Sousa Borges, A., da Silva, I., Polaquini, C. R., Regasini, L. O., Ferreira, H., & Scheffers, D. J. (2015). Antibacterial activity of alkyl gallates is a combination of direct targeting of FtsZ and permeabilization of bacterial membranes. *Frontiers in Microbiology*, *6*(APR). https://doi.org/10.3389/fmicb.2015.00390

Molecular Probes. (2004). LIVE/DEAD® BacLight™ Bacterial Viability Kits ™.

Nazaré, A. C., Polaquini, C. R., Cavalca, L. B., Anselmo, D. B., Saiki, M. de F. C., Monteiro, D. A., … Regasini, L. O. (2018). Design of antibacterial agents: Alkyl dihydroxybenzoates against xanthomonas citri subsp. citri. *International Journal of Molecular Sciences*, *19*(10). https://doi.org/10.3390/ijms19103050

Nihei, K. I., Nihei, A., & Kubo, I. (2003). Rational design of antimicrobial agents: Antifungal activity of alk(en)yl dihydroxybenzoates and dihydroxyphenyl alkanoates. *Bioorganic and Medicinal Chemistry Letters*, *13*(22), 3993–3996. https://doi.org/10.1016/j.bmcl.2003.08.057

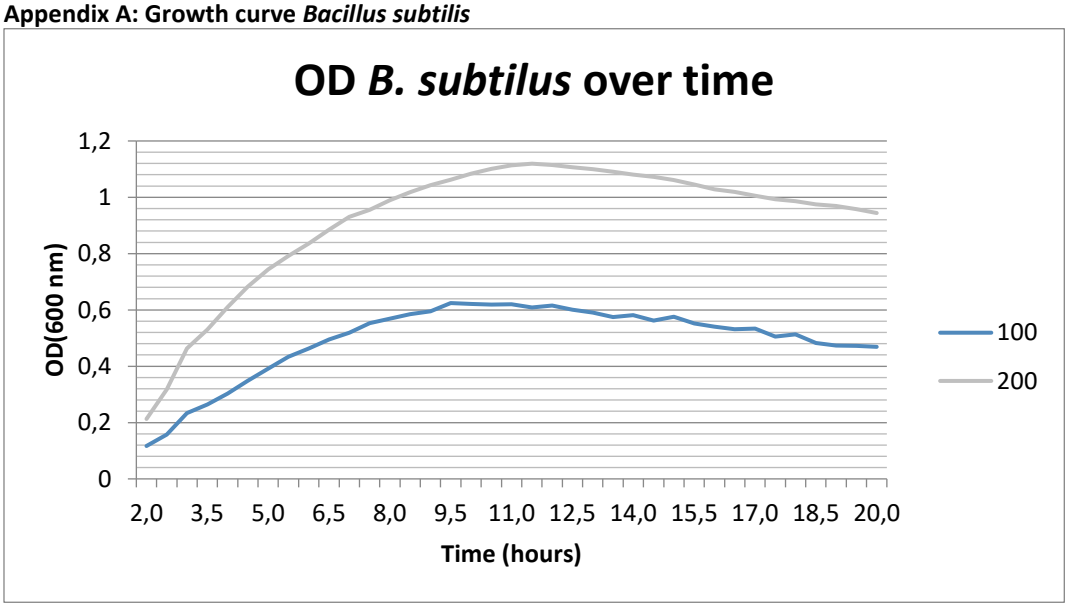
Nikon. (n.d.). Nikon Imaging Software.

Savietto, A., Polaquini, C. R., Kopacz, M., Scheffers, D. J., Marques, B. C., Regasini, L. O., & Ferreira, H. (2018). Antibacterial activity of monoacetylated alkyl gallates against Xanthomonas citri subsp. citri. *Archives of Microbiology*, *200*(6), 929–937. https://doi.org/10.1007/s00203-018-1502-6

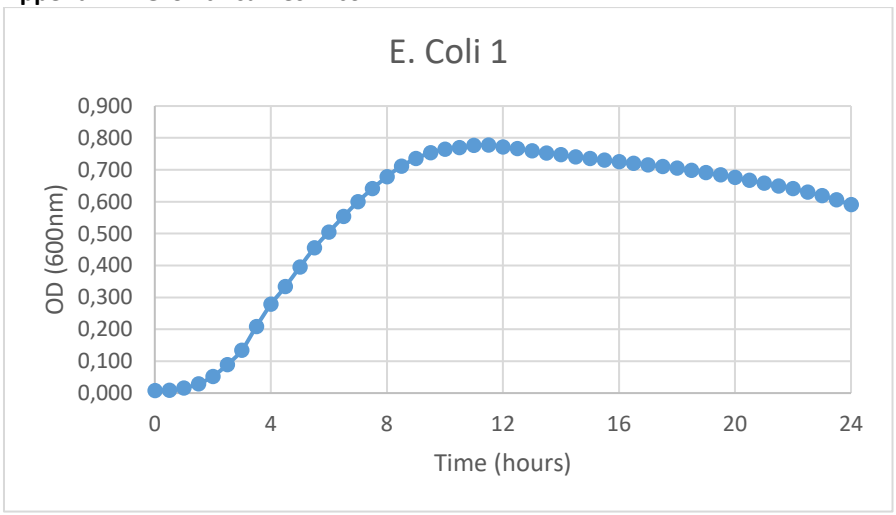
Silva, I. C., Regasini, L. O., Petrãnio, M. S., Silva, D. H. S., Bolzani, V. S., Belasque, J., … Ferreira, H. (2013). Antibacterial activity of alkyl gallates against Xanthomonas citri subsp. citri. *Journal of Bacteriology*, *195*(1), 85–94. https://doi.org/10.1128/JB.01442-12

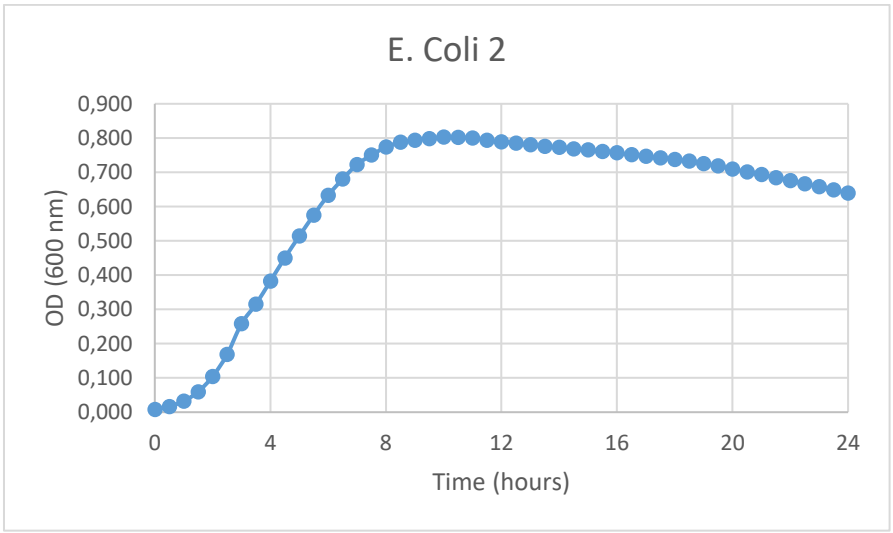
6. Appendices

Appendix A: Growth curve *Bacillus subtilis*

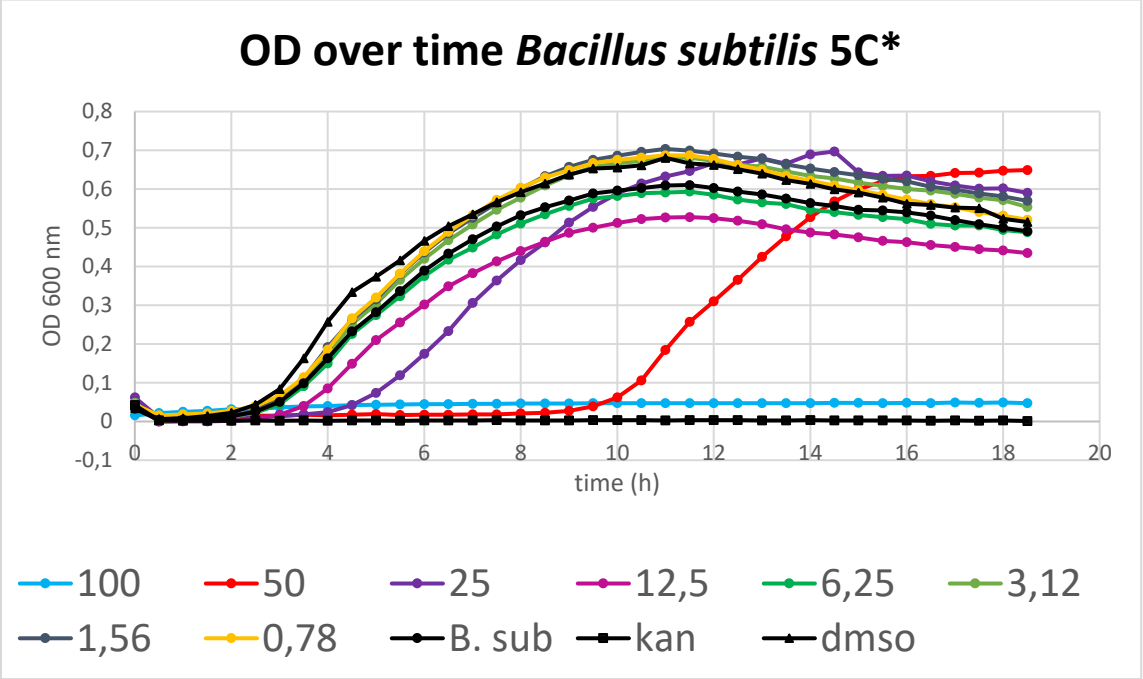


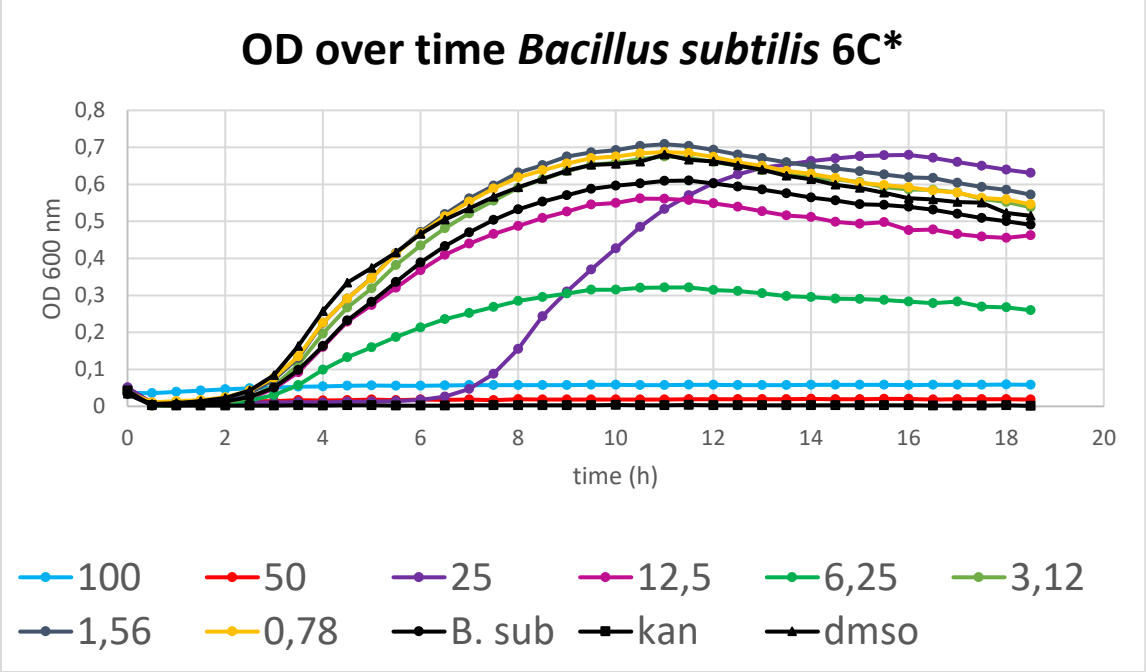
Appendix B: Growth curves *E. coli*

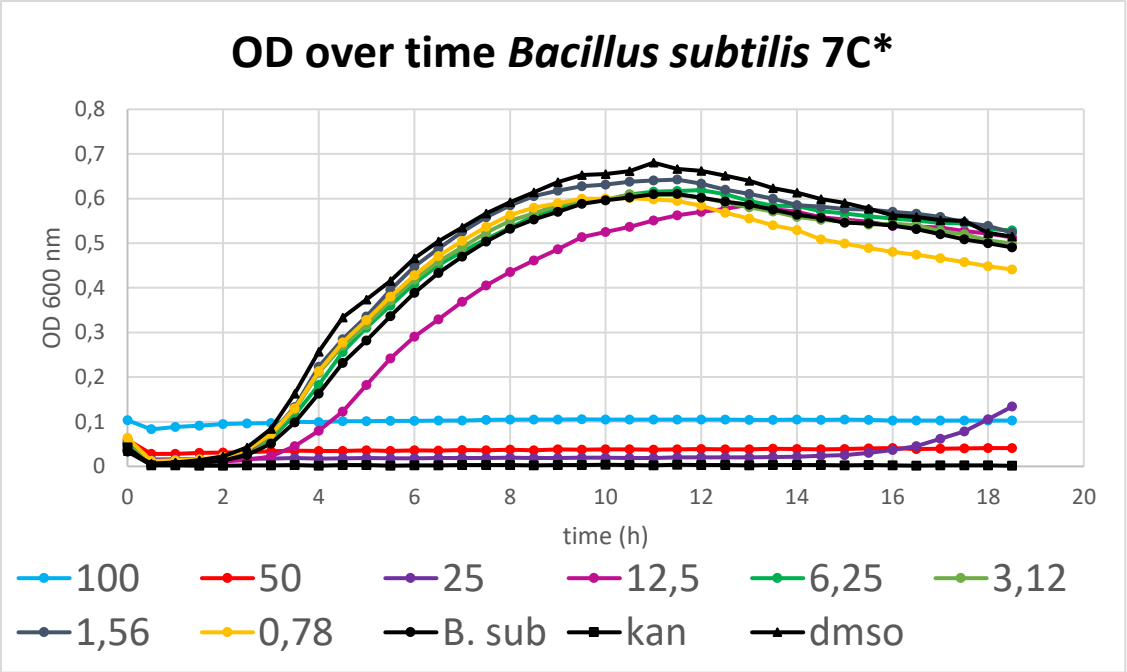




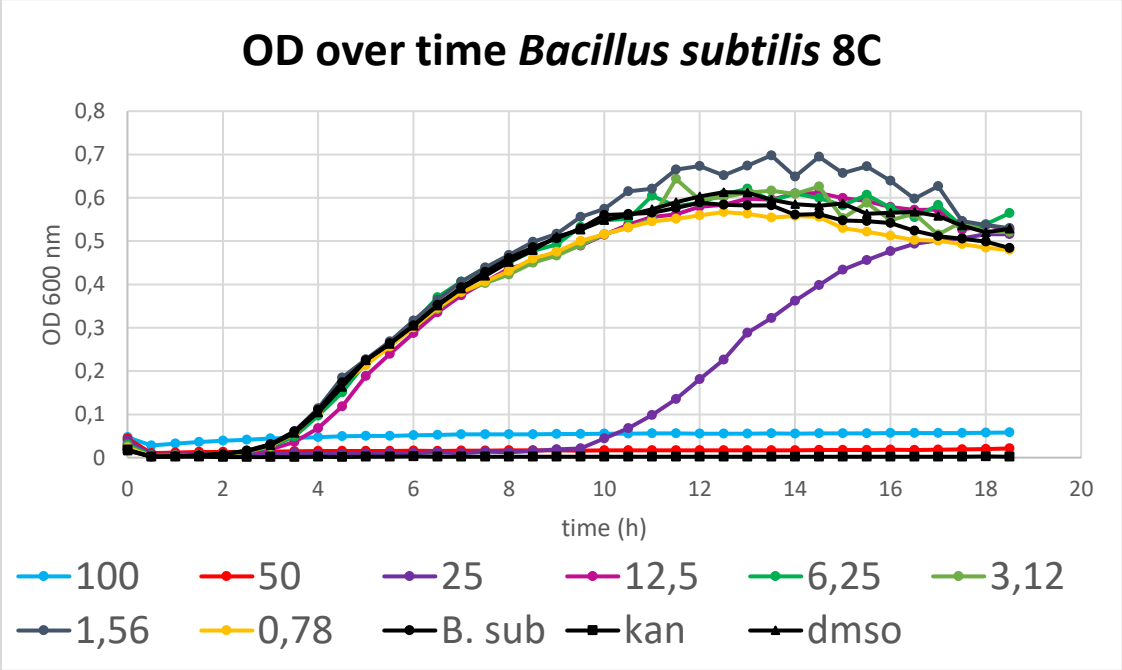
Appendix C: Growth curves *B. subtilis after treatment with compound*

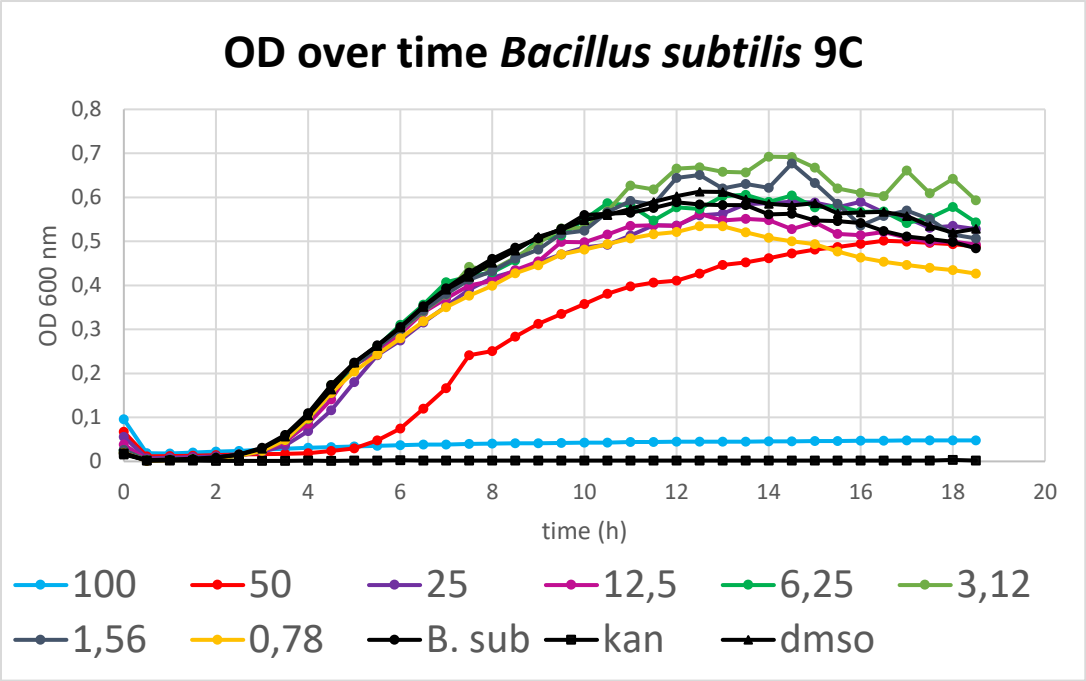






*Instead of chemical control only blank subtracted




OD over time Bacillus subtilis 9C
OD 600 nm
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
0
2
4
6
8
10
12
14
16
18
20
time (h)
100
50
25
12,5
6,25
3,12
1,56
0,78
B. sub
kan
dmso