# Effect of carvacrol on mature biofilms and bacterial quorum sensing

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July 2012

Extended research internship as part of Master veterinary medicine

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IRAS-VPH report number: 2012.276

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## Abstract

The biofilm offers a protective environment for bacteria to grow in and survive under diverse conditions. This means that the bacteria will be protected against environmental factors such as pH changes, UV radiation and osmotic shock but as well against antimicrobial agents and the immunerespons of a host.[1,8] Because of this protective layer of the biofilm it is very hard to remove them but possible anti-biofilm strategies are to prevent the bacteria to colonize a surface, the inhibition of signal molecules used for communication and breaking down the biofilm matrix.[3,4] The communication process used by the bacteria in a biofilm is called quorum sensing and has shown to be necessary for mature biofilm formation, most biofilms will end at the first stages without quorum sensing.[5,6]Using quorum sensing inhibitors might be making the biofilms more susceptible to antimicrobial treatments and the host immune system.[22]

A natural phytochemical with broad antimicrobial properties is carvacrol, a component of oregano, thyme and marjoram. It is used as a flavouring compound in for example, candy and baked goods.[7]

The aim of this project is to determine the effect of carvacrol on in vitro mature biofilms of *Staphylococcus aureus*, *Salmonella enterica* serovar Typhimurium and *Pseudomonas aeruginosa*. And to assess if carvacrol has a quorum sensing inhibitor activity, for which *Chromobacterium violaceum* will be used.

The biofilms formed by *Staphylococcus aureus* showed a significant reduction after adding carvacrol of concentrations up to 4mM. For the in vitro biofilms of *Salmonella enterica* serovar Typhimurium and *Pseudomonas aeruginosa* there was no significant effect of carvacrol up to 8mM. Between concentrations of 0.2mM and 0.4mM carvacrol reduces the production of violacein, induced by quorum sensing, of *Chromobacterium violaceum* without a significant decrease of viable bacteria being present.

## Introduction

Individual bacteria can move freely in a liquid environment: swimming or as plankton. They can also adhere to a surface close to each other and in this state they will start forming a biofilm.[1,2] A biofilm is defined as 'an uninterrupted multilayer of bacterial cells attached to the surface and to each other and embedded into a biopolymer matrix'.[1] Biofilms are more antimicrobial resistant then free living bacteria and this will be explained in the rest of the introduction.[3,4] To be able to form a mature biofilm, communication is necessary between the bacteria that are part of this process and is called quorum sensing. This will also be explained more detailed in the introduction but inhibiting the quorum sensing might weaken the biofilm and make it more susceptible for antimicrobial therapy or the host immune system.[5,6] Carvacrol is a phytochemical which is present in oregano, thyme and marjoram.[7] In this research project carvacrol will be used as an antimicrobial agent and the effect on in vitro formed biofilms will be assessed as well as a possible anti-quorum sensing activity.

### Biofilms

A biofilm can be formed on abiotic, air-water interfaces and minerals, and biotic surfaces such as animals or other microbes. In humans biofilms will be formed wherever there is colonization by bacteria for example on skin, intestine but also on heart valves and central venous catheters. This ability to form biofilms is a source of problems in the medical field as well as in economic areas.[1] Single and mixed species biofilms exist and offers a protective environment to grow in, survival in diverse conditions and the opportunity to disperse and colonize new surfaces/niches. Dispersal is possible because biofilms are dynamic.[8]

#### *Biofilm matrix*

A biofilm consists of 10% bacteria and 90% matrix. The matrix is produced by the bacteria and consists of extracellular polymeric substances (EPS) which main components are exopolysaccharides, proteins (pili, fimbriae, lectins and autotransporters), eDNA and lipids.[1,2] The exopolysaccharides are a major constituent of the matrix and without most bacteria will not be able to form a multilayer biofilm. eDNA also has an important role in the structure and stability of the matrix. The origin of the eDNA is believed to be genomic DNA that is released after cell lysis of bacteria present in the biofilm. Matrix components can also consist of minerals, surfactants, membrane vesicles and glycolipids.[1,2,9] The matrix provides stability and possibility for small molecules to enter or leave the matrix and has an important protective role. This protection is mainly against harmful environmental factors such as pH changes, UV radiation, dehydration and osmotic shock but as well to protect against antimicrobial agents and the immunerespons of a host.[1,5,9]

#### *Antimicrobial resistance*

There are some mechanism which make biofilms more resistant to antimicrobial agents then free living bacteria. As already mentioned the matrix provides a protective barrier for the bacteria living in the biofilm. This barrier might bind some agents so they will not diffuse to all the bacteria. In some parts of the biofilm bacteria are present in a different metabolic state than planktonic cells are because of lack of nutrients, aerobe and anaerobe parts. Because of this the bacteria exist in several stages: aerobe, fermentative, dormant and dead. The action of most antimicrobials is to disrupt a microbial process for which some cellular activity will be necessary.

Resistance may also occur because of resistant phenotypes in the biofilm existing as subpopulations as plasmids are exchanged easily between bacteria that are close to each other with the plasmids possible containing genes for antimicrobial resistance.[3,4,8,10] Because of these mechanisms it might prove very difficult to remove or inactivate a biofilm and antimicrobial therapy usually only will stop the symptoms caused by the bacteria released by the biofilm. Possible strategies are: preventing bacteria to adhere to and/or colonize a surface, inhibition of signal molecules that regulate formation and existing biofilms and breaking down the biofilm matrix.[3,4,11]

*Biofilm infections*

Biofilms play an important role in hospital-acquired infections especially those related to implanted medical devices such as intravascular- or urinary catheters, orthopaedic prostheses, cardiac pacemakers and endotracheal tubes (*figure 1*). Next to increased resistance against antimicrobial agents, as mentioned before, there also is resistance to the immune system of the host and an estimate of 80% of all the infections are biofilm related by the National Institutes of Health (United States).[10] Because of the recalcitrance of biofilm related infections the best 'treatment' option usually is to remove the infected device where possible. Another possibility would be coating of medical devices with antimicrobial agents to prevent colonization of the device. Problem with the most used medical devices for this approach would be possible development of resistance because of long lasting low released concentration.[1,3,8,10] Most of the nosocomial infections are caused by *Staphylococcus epidermidis* and *Staphylococcus aureus*. Staphylococci have a very good ability to attach and colonize plastic surfaces, and they play a very important role in medical device related infections, were *Pseudomonas aeruginosa* is a very important causative bacteria too.[8,10,12]

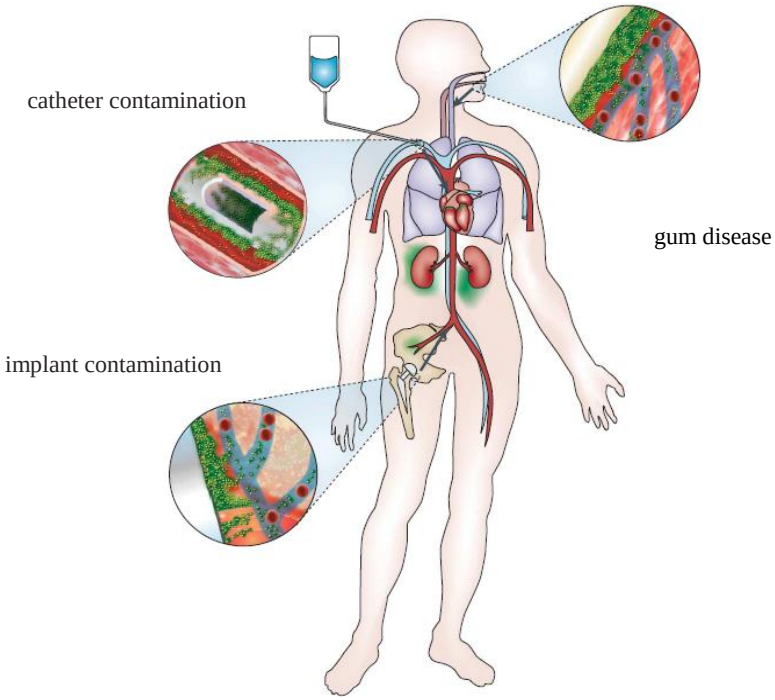


***Fig. 1*** **Showing three possible entry points in the body for bacteria able to form a biofilm: gum disease, catheter contamination and implant contamination. The arrows show how the biofilm (green) could spread through the body.** [8]

*Staphylococcus aureus*
*Staphylococci* are gram-positive bacteria that are facultative anaerobes. They can be found as commensal on mucous membranes, skin and intestines of humans and animals. They can also be found in the nose and 20% of the human population are nasal carriers. A lot of infections are opportunistic and can be seen in patients with trauma, metabolic or endocrine disturbances or immunosuppresion. In these situations *Staphylococcus aureus* can cause infections as skin infections, food poisoning or endocarditis. *Staphylococcus aureus* is also relatively stable in the environment and should be seen as an potential environmental contaminant which can cause infections.[6,13]

*Salmonella enterica* serovar Typhimurium
*Salmonella* is a gram-negative bacterium that are frequently found as an intestinal pathogen of both humans and animals. The clinical manifestation of salmonellosis in animals can be septicaemia and/or enteritis the acute, subacute and chronic form. In humans it is mostly seen as gastroenteritis (food poisoning), septicaemia or bacteraemia. After the clinical signs have subsided an asymptomatic carrier state is not an exception again both in animals and in humans. Infection mostly occurs by ingestion of food (and water) which has been contaminated. An infection or epidemic usually can be traced back to food products containing meat, milk, eggs or poultry. Cases by direct contact with an infected human or animal are rare.[14]

*Pseudomonas aeruginosa*
*Pseudomonas* is a gram-negative aerobe bacterium which can be found worldwide in soil, water and on plants. It is also present on mucous membranes and skin. Because *Pseudomonas aeruginosa* possesses flagella and pili it can adhere to injured cells which means that for an infection to take place the host defence should be weakened. It can cause opportunistic infections in wounds, patients with immunodeficiency, cystic fibrosis or young patients. It will rarely infect a healthy person or animal.[9,13,14]

**Biofilm communication/signalling**
Bacteria secrete and detect signal molecules by way of communication. When the concentration of these molecules reaches a certain threshold value the expression of specific genes will alter. Because a certain number (quorum) of bacteria is required to achieve the threshold concentration, this communication process is often referred to as quorum sensing. Gram-negative bacteria use acylated homoserine lactones (AHLs) and gram-positive bacteria oligo-peptides as autoinducers. This way bacteria in the biofilm will alter compared to the free living ones and will be better at resisting different circumstances (phenotypical tolerance) and they will be able to react as a community.[5,6] It is also suggested that before the quorum sensing system starts there is signalling between the bacteria by direct contact during the attachment phase of the biofilm through adhesion forces. This signalling is for short distances and the quorum sensing is for communication over longer distances after the biofilm has more matured.[15]

*Membrane vesicles*

Membrane vesicles are 'complex and chemically heterogenous bilayered structures derived from the outer membrane of a wide variety of gram-negative bacteria'.[16] Bacteria, in biofilms and planktonic ones, can release membrane vesicles which can act as virulence factors or lysis of cells in the biofilm. The membrane vesicles only occur in gram-negative bacteria and not in gram-positive ones. Membrane vesicles released from the walls of the bacteria can stay inside the biofilm and be a component of the biofilm matrix or be released into the surrounding environment. Pieces of the cell wall are entrapped in the vesicles so they might contain virulence factors. Lysis of cells can also occur as a function of the vesicles but the lysed cells must be under conditions of low nutrition and poor growth. These lysed cells will be used as nutrients and growth factors for the remaining bacteria. The membrane vesicles can contain DNA and enzymes which they can transfer to other gram-negative bacteria possibly passing on virulence and antibiotic resistance factors. The antimicrobial resistance can also be carried out by the vesicles trough binding the agents before they even come into contact with the biofilm. More specific for *Pseudomonas aeruginosa* were some strains can release membrane vesicles with β-lactamase which then can degrade β-lactam antibiotics.[1,2,16,17]

*Quorum sensing*

For a molecule to be seen as a quorum sensing signal it must meet some criteria, the amount of molecules should be dependent on the bacterial density, it should be recognized by the bacteria and affect gene transcription.[18] The quorum sensing communication occurs both within bacterial species and between for a mixed species biofilm. As mentioned above gram-negative and gram-positive use different molecules for their quorum sensing communication. The main goal is the same, to coordinate gene expression and react as a community. The interspecies communication is mediated by autoinducer AI-2 and the gene responsible for the production of this autoinducer is present in both gram-negative and gram-positive bacteria. Quorum sensing has also been shown to be necessary for proper biofilm formation; the biofilms of most bacteria will not mature and end at the first stages without quorum sensing.[6]

In gram-positive bacteria a quorum sensing inhibitor protein has been effective in the treatment and prevention of biofilm infections on intravascular and urinary catheters and orthopaedic implants.[3] For Staphylococci the factors used for initial colonization are thought to be repressed after this first stage by the quorum sensing system and therefore, inhibition of quorum sensing in *S. aureus* is most effective in acute stages of infection. For *S. aureus* the effect of quorum sensing on gene transcription, positive or negative, seems to depend on growing conditions and this way having an influence on the development of the biofilm.[12,19,20,21] The quorum sensing inhibitors might have a role in anti-biofilm treatment making the biofilms more susceptible to the host immune system and the antimicrobial treatment.[22]

*Chromobacterium violaceum*

This bacterium is a motile gram-negative, facultative anaerobe which can be present in soil and water especially in regions with a subtropical or tropical climate. Septicaemic infections can occur in humans, dogs, pigs and sheep, mostly as an opportunistic infection. These infections are seen accordingly to the geographical occurrence of the bacterium in subtropical and tropical climate regions.

In vitro the bacterium can be recognized by the production of a non-diffusible violet pigment.[13,14]

**Biofilm life cycle**

The formation of a biofilm takes place in four steps. Step one and two are loose attachment to the surface, followed by actual adhesion. In Staphylococci, eDNA, resulting from bacterial lysis, is used for the adherence to a surface and the first steps of formation.[10] After this motility (surface projections, flagella, pili) on the surface takes place followed by formation and maturation. Dispersal of the biofilm can occur later (*figure 2*). The structure of biofilms is influenced by changes in nutrient conditions, hydrodynamic conditions, intercellular communication and can be mushroom shaped or flat.[1,2,3,8]

*Biofilm formation*

The maturation phase of biofilms consists of two parts, first the intercellular aggregation and second the biofilm structuring where the mushroom-shaped towers arise from. The channels in between are filled with fluid which seem to exist to get nutrients into the deeper layers of the biofilm and allows for dispersion of cells. The channels which are formed by controlled cell death is part of the biofilm development. The remaining part of the microcolony where the lysed cells are dispersed from stays intact.[12,23,24]

*Biofilm dispersal*

Biofilm dispersal can either be considered passive or active. Dispersal can be seen as passive after biofilm removal strategies (for example to control contamination or infection of indwelling medical devices or water disposal system) but can also be an active mechanism activated by the biofilm itself. Active dispersal seems to be induced to create opportunities to colonize new surfaces. Wild type biofilms release a small amount of cells under good conditions continuously and after longer periods of growth major dispersion will occur.[8,25,26]

*Mechanisms / compounds dispersal*

Factors that can start the dispersal are mechanical forces, a stop in production of matrix materials and/or other detachment factors either external or coming from the biofilm itself. For the dispersal there are three different modes. First the swarming dispersal where microcolonies are releasing individual cells. Second the clumping dispersal where shedding of cells takes place in aggregates as emboli and third the surface dispersal where structures of the biofilm will be moving across a surface.[8,12] There are several environmental, inter- and intracellular signals that can initiate and regulate the dispersal, some are changes in nutrients, autoinducers, phage-mediated cell lysis, rhamnolipids (extracellular surfactants) or cell division at the outside surface of the biofilm.[25]

*Advantages / disadvantages bacteria & medical*

The dispersal from a biofilm is an important route for pathogenic bacteria to spread from the environment to a host, between hosts and within a host. The spread from the environment to a host can be achieved through for example hands of healthcare staff and (respiratory) therapy equipment.[25]

During clumping dispersal the biofilm cells that are shed are still surrounded by matrix and maintain the same antimicrobial resistance as in the still attached part of the biofilm (*figure 2*). This way there will be efficient colonization and spreading of the infection to other parts and tissues of the host. For the surface dispersal strategy this applies as well and presents a challenge compared to swarming dispersal because of the higher resistance to antimicrobials.[8] The dispersal of biofilms as a target to fight the infection is an interesting approach as loss of the biofilm will expose the individual bacterial cells to antimicrobials and the immune system of the host.[3]

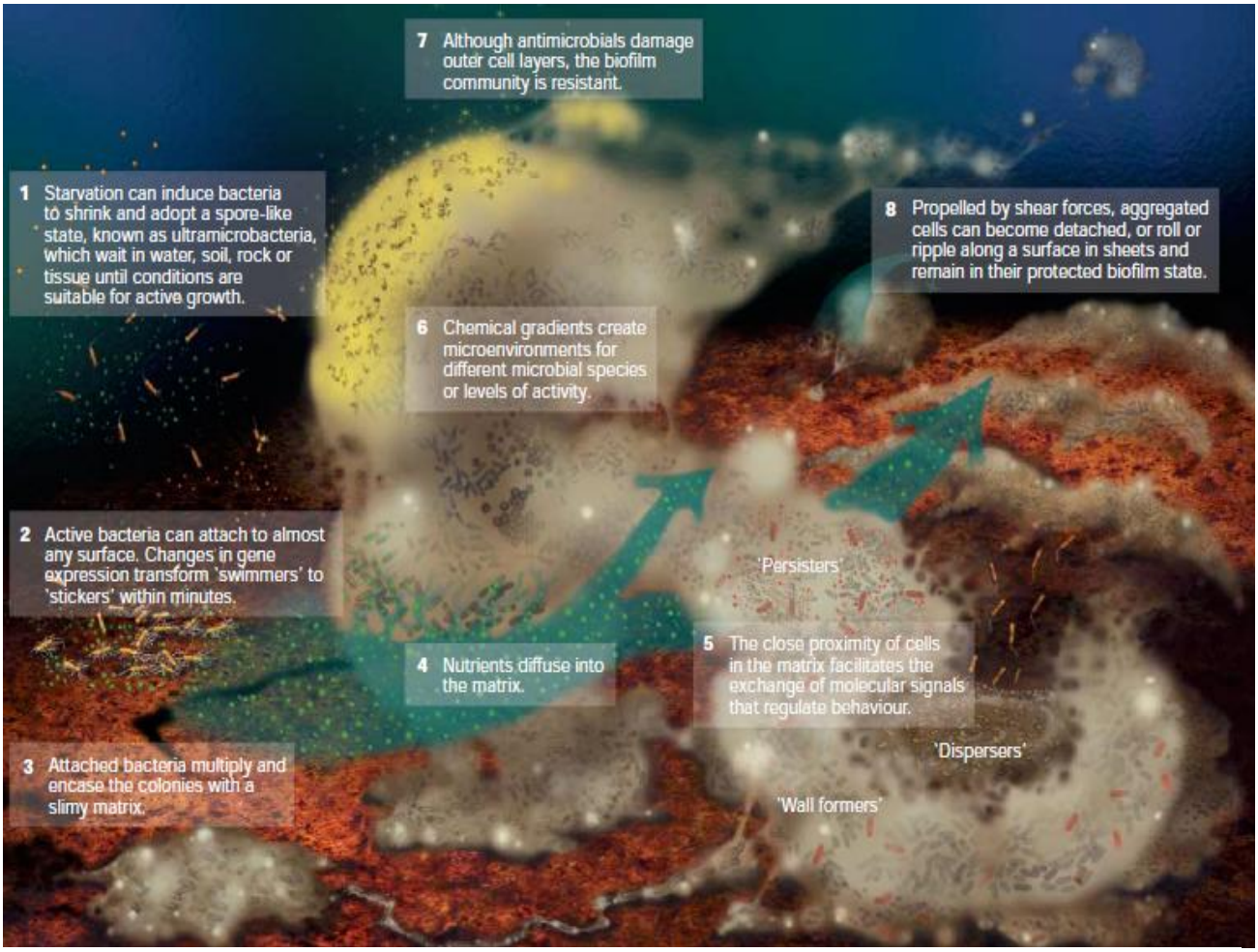


*Fig. 2* Biofilm development and dynamic behaviors.[8]

## Carvacrol

Carvacrol is a natural phytochemical and a component of oregano, thyme and marjoram. It is used as a flavouring compound in for example, candy and baked goods.[7] The antimicrobial activity of carvacrol is seen as broad-spectrum and it is effective against fungi, yeast and bacteria, it was also able to inactivate dried biofilms on stainless steel.[7] The effect of carvacrol is biocidal, it can destabilize the cytoplasmic membrane and act as a proton exchanger (*figure 3*). It has been shown that the hydroxyl group of carvacrol is important for the antimicrobial activity.[27] This way the (pH) gradient across the cytoplasmic membrane will chance which will lead to depletion of ATP, a chance of the osmotic gradient and cell death.[27]

Carvacrol has very strong aromatic properties which is why it has limited possibilities for the application in food preservation.[7]

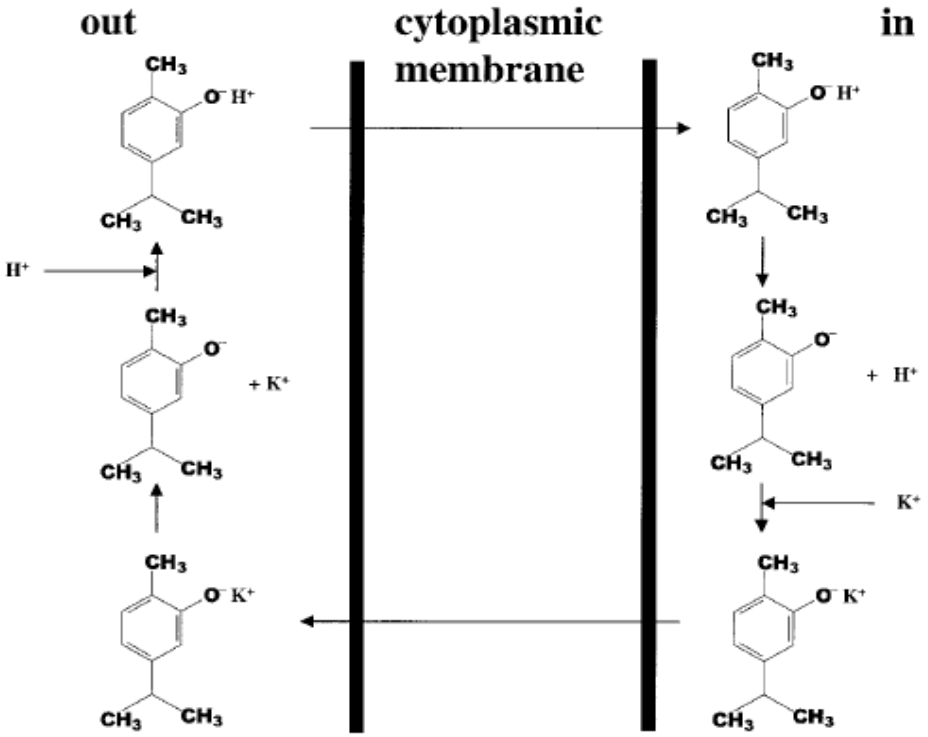


*Figure 3* hypothesized activity of carvacrol.[27] Carvacrol passes the cytoplasmic membrane where it releases a proton after which it returns with a cation (here potassium) through the cytoplasmic membrane. After releasing the cation carvacrol can take up a proton and is able to pass the cytoplasmic membrane again.[27]

**Aim of the project**

The aim of this project was to determine in which degree and which concentrations carvacrol exhibits an effect on mature monospecies biofilms and if there is a difference in effect of the treatment depending on the specific bacteria that formed the biofilm. Furthermore this project aimed to assess whether carvacrol possesses a quorum sensing inhibitor activity.

## Materials and methods

### Biofilm destruction assay

This assay was carried out to determine if carvacrol can remove the biofilms of *Staphylococcus aureus* 0074, *Salmonella* enterica serovar Typhimurium DT104 and *Pseudomonas aeruginosa* and if there is a concentration dependent effect of carvacrol. For this purpose biofilms were formed after which removal was attempted by adding different concentrations carvacrol. The description of the biofilm destruction assay was carried out for each of the bacteria with some specific additions per bacteria. The additions are described after the general assay.

*Biofilm preparation*

An overnight culture of bacteria incubated at 37ºC was prepared. The bacterial suspension was diluted with the growth medium (broth) to an OD of 0.01 at a wavelength of 590nm.

To determine the number of bacteria in the suspension used, decimal dilutions were made with phosphate buffered saline (PBS, 0.01M) and plated out on Trypton Soya Agar (TSA, Oxiod CM129 3% + 1.5% agar) plates incubated overnight at 37ºC and the colony forming units (cfu) were counted the next day.

The bacterial biofilms were grown in a 96-wells plate (flat bottom medium binding, Greiner bio-one 655101). Of the bacterial suspension 100µl was added to the wells in the 96-wells plate. There was also only Trypton Soya Broth-Glucose (TSBG, Oxoid CM 129 3% + glucose 0.25%) added to the wells serving as a blank. All the remaining wells were filled with PBS to prevent the contents of the wells with bacterial suspension and TSBG from drying out. The 96-wells plates were incubated during 24 hours. The bacteria in the 96-wells plate were removed and the wells were washed twice with PBS to remove any loose bacteria. Formed biofilms were stained by adding crystal violet (CV) to the wells.

*Biofilm removal*

The bacteria were removed from the 96-wells plate after the 24 hour incubation period. To the wells was added aqua dest as a blank and (negative) control. Ethanol (96%) dissolved in aqua dest was added as a control. From a 1M stock solution, concentrations carvacrol (98%, Aldrich) of 2, 4, 6 and 8mM were added as well. The 96-wells plate then was incubated for 24 hours at 20ºC without shaking.

*Biofilm staining*

The wells were emptied and washed twice with PBS to remove loose bacteria. Staining of the biofilms was done by adding CV to the wells for a defined period, then washed three times with PBS. The bound CV was solubilized in 33% acetic acid and the OD was read on a plate reader at a wavelength of 590nm. The mean of the blank column was subtracted from columns with the treatments and 24 hour biofilm.

The assay was performed three times for *S. aureus* and *S.* Typhimurium and twice for *P. aeruginosa*.

***Biofilm destruction assay additions per bacterium***

*S. aureus* 0074

The overnight culture of *S. aureus* was prepared in TSBG. TSBG was also used to dilute the bacterial suspension to an OD of 0.01. The two 96-wells plates were

incubated the first 24 hours at 37ºC on a plate shaker at 600rpm. Biofilms were stained by adding 100µl of 0.4% CV Boom (12191, colour index: 42555) for 15 minutes to the wells.

*S. enterica* serovar Typhimurium DT104
The growth medium used for the *S.* Typhimurium overnight culture was Trypton Soya Broth (TSB, Oxoid CM 129 3%) and for forming the biofilms 1/20 diluted TSB.
The first 24 hour incubation period at 25ºC was performed without shaking.
The staining of the biofilms was done by adding 100µl methanol for 15 minutes after washing twice with PBS. Then 0.25% CV (Klinipath, 1170, colour index: 42555) was added for 10 minutes.

*P. aeruginosa*
The *P. aeruginosa* assay was carried out the same way as the assay used for *S.* Typhimurium except that 0.25% CV (Klinipath) was added for 5 minutes.

**Quorum sensing inhibitor activity assay**
The bacterium used for this assay was *Chromobacterium violaceum* ATCC 12472. This bacterium produces a violet pigment when the bacteria are signalling through quorum sensing. As the quorum sensing decreases the production of the violet pigment will decrease as well and can be used this way to determine the amount of quorum sensing.
An overnight culture of *C. violaceum* was made in Luria Bertani (LB, trypton1%, yeast 0.5%, NaCl 0.5%) broth at 26ºC and was diluted until an OD of 0.01 was reached (590nm).

To determine the number of bacteria present in the suspension used, decimal dilutions were made in PBS and plated out on LB agar plates and incubated 48 hours at 26ºC. After the incubation period the colony forming units (cfu) were counted.

A range of carvacrol concentrations in LB was set up as follows. 1M stock solution of carvacrol was made by adding 157µl carvacrol to 843µl ethanol. Stock solution was added to 10ml portions of LB broth to achieve concentrations of 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.8mM. All bottles were inoculated with 10µl of *C. violaceum* and incubated for 72 hours at 26ºC.

*Bacteria count*
The number of surviving bacteria in each concentration of carvacrol was determined by performing serial decimal dilutions from the bacterial suspension of all eight bottles and plating out on LB agar plates. After incubation for 48 hours at 26ºC the cfu were counted and used to calculate the cfu per ml of the bacterial suspensions.

*Violacein measurement*
Of every bacterial suspension 4ml was centrifuged for 3 minutes at 13.000*g* and the supernatant was removed. The pellet was resuspended in 200µl LB broth and 200µl 10% SDS (1g SDS in 10ml aqua dest) was added and left for 5 minutes at room temperature. A blank sample was included as well. After the 5 minutes waiting time 900µl n-BuOH ($H_2O$ saturated) was added and then centrifuged for 5 minutes at 13.000*g*. The supernatants were transferred to a 96-wells plate and read on a plate

reader (Versa max tunable microplate reader) at a wavelength of 590nm and the averages corrected for the average of the blank samples.

The entire assay was performed three times for *C. violaceum*.

**Statistical analysis**

The data were compared with a one way ANOVA carried out in Excel (2003). The treatments consisting of aqua dest and each concentration carvacrol were compared to the 24 hour biofilm and differences are statistically significant if $p < 0.05$. In case of $p < 0.05$ for the ANOVA a Dunnett‘s two-sided post hoc test was used to determine which treatment was significantly different in SPSS 16.

## Results

### Biofilm destruction assay

*S. aureus* 0074

The biofilm destruction assay was carried out three times for *S. aureus*. The results are shown as percentages and as absolute OD values in *figures 4* and *5*.

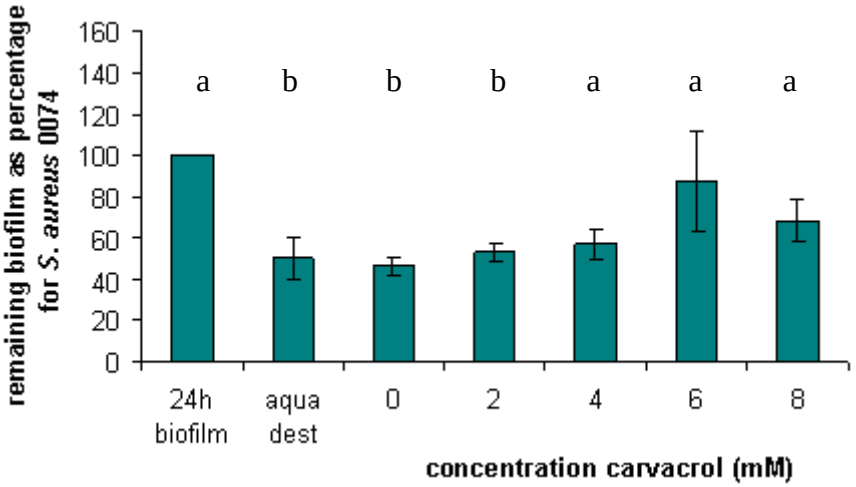


***Fig. 4*** **Average of three biofilm destruction assays for *S.* aureus 0074. The biofilms are expressed as a percentage of the 24 hour biofilm with a ± SEM. Significant differences compared to the 24h biofilm are indicated with different letters ($p < 0.05$).**

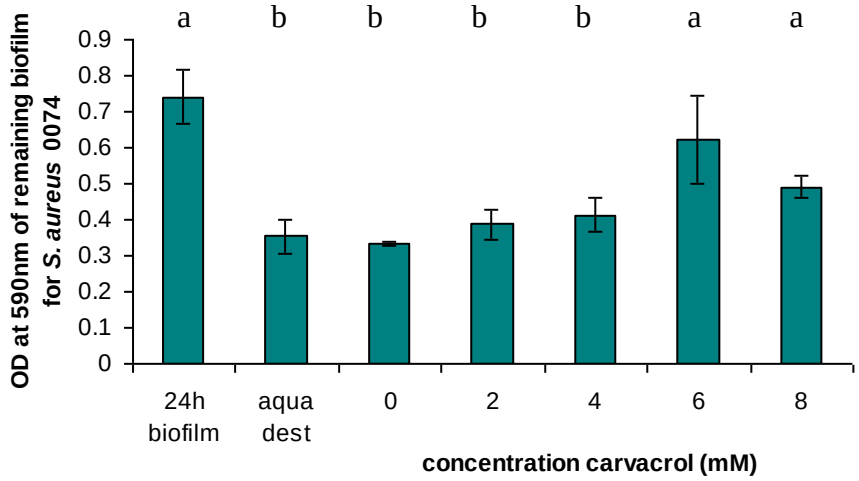


***Fig. 5*** **Average of three biofilm destruction assays for *S.* aureus 0074. The OD at a wavelength of 590nm is shown as the amount of biofilm with ± SEM. Significant differences compared to the 24h biofilm are indicated with different letters ($p < 0.05$).**

The results for *S. aureus* in *figures 4* and *5* both show a decrease in the amount of biofilm for all treatments compared to the 24 hour biofilm. The biofilm removal effect is higher for the treatment with aqua dest, 0mM, 2mM and 4mM carvacrol than for the 6 and 8mM carvacrol.

For both the results as percentages and as absolute OD values $p < 0.05$ indicating a significant difference in the effect of biofilm destruction for the used treatments compared to the 24 hour biofilm. For the biofilms as percentages there is a significant effect for the aqua dest, 0mM and 2mM carvacrol. The results as OD values have a significant effect for the treatments with aqua dest, 0mM, 2mM and 4mM carvacrol ($p < 0.05$).

*S. Typhimurium DT104*

The averages of the results of the three *S.* Typhimurium DT104 biofilm destruction assays are shown in percentages and as absolute OD values (*figures 6* and *7*).

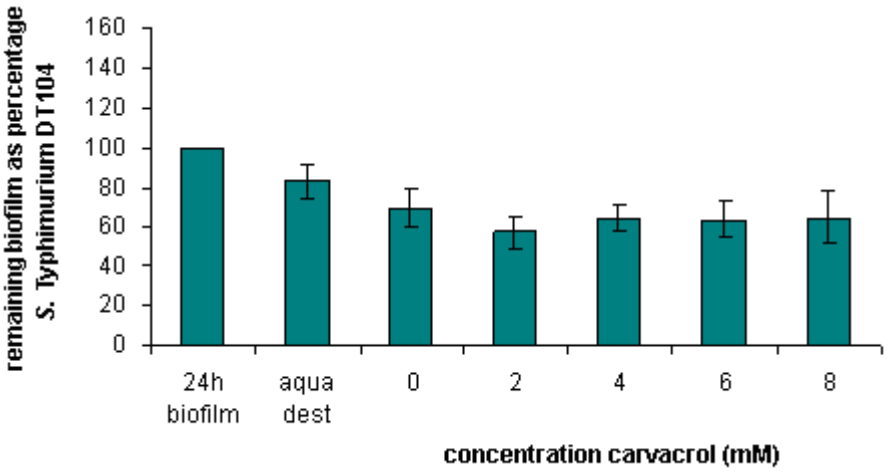


***Fig. 6*** **Average of three biofilm destruction assays for *S.* Typhimurium DT104. The biofilms are expressed as a percentage of the 24 hour biofilm with a ± SEM.**

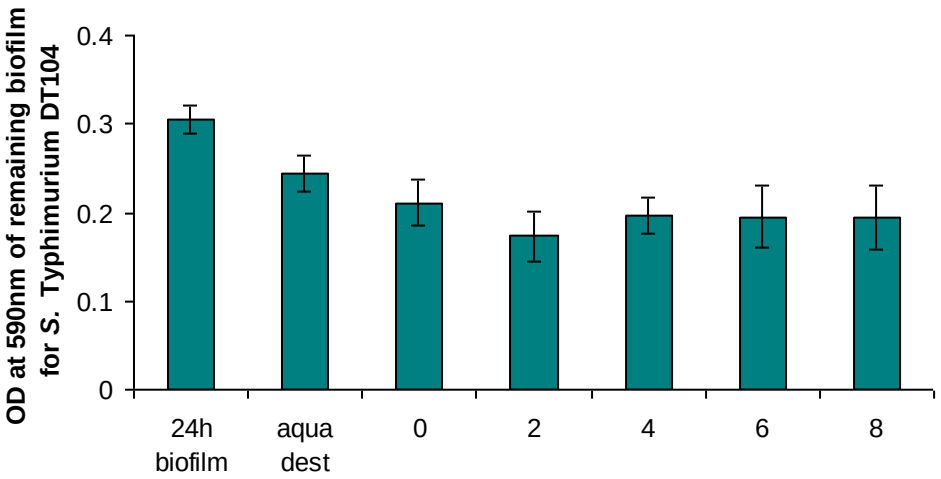


***Fig. 7*** **Average of three biofilm destruction assays for *S.* Typhimurium DT104. The OD at a wavelength of 590nm is shown as the amount of biofilm with ± SEM.**

In the results for the biofilms as percentages as well as for the OD values (*figures 6* and *7*) the same trend is occurring. Compared to the 24 hour biofilm the treatment with aqua dest, 0mM and 2mM carvacrol show a very slight reduction in biofilm, which is not significant ( $p > 0.05$ ).

*P. aeruginosa*

The biofilm destruction assay was carried out two times for *P. aeruginosa*. The results are shown in *figures 8* and *9* as percentages and OD values respectively.

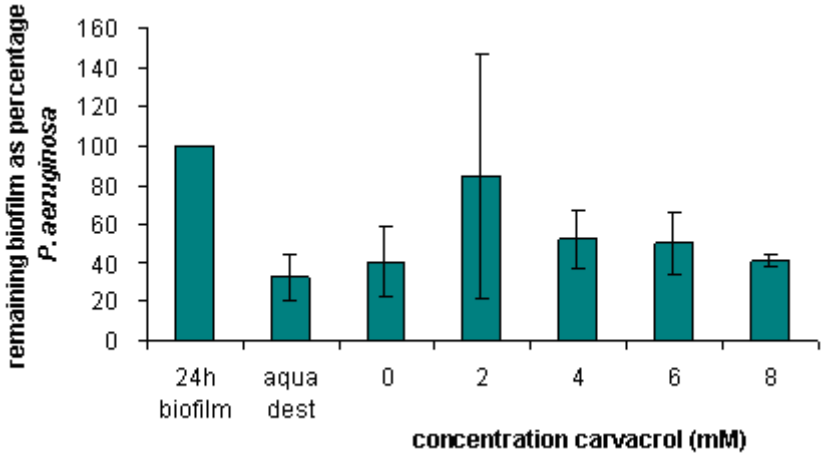


***Fig. 8*** **Average of two biofilm destruction assays for *P. aeruginosa*. The biofilms are expressed as a percentage of the 24 hour biofilm with a ± SEM.**

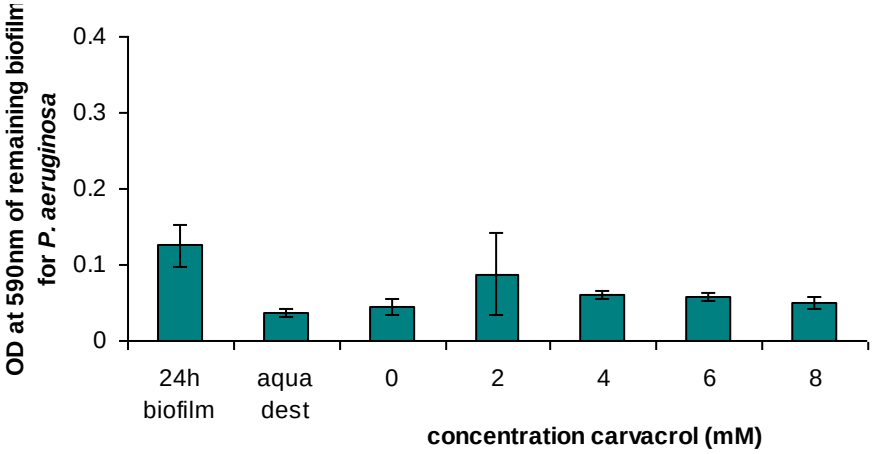


***Fig. 9*** **Average of two biofilm destruction assays for *P. aeruginosa*. The OD at a wavelength of 590nm is shown as the amount of biofilm with ± SEM.**

The results show a decrease in biofilm for all the treatments. There are some differences in effect on biofilm destruction with 2mM carvacrol being the least effective.

For the results as percentages and as OD values $p > 0.05$ meaning there are no significant differences in the biofilm destruction ability of the treatments.

## Quorum sensing inhibitor activity assay

*C. violaceum* ATCC 12472

The quorum sensing inhibitor activity assay was carried out three times for *C. violaceum*. The results are shown in *figures 10* and *11* as percentages and absolute values for the OD of the violacein protein and as absolute values for the number of bacteria present (log cfu/ml).

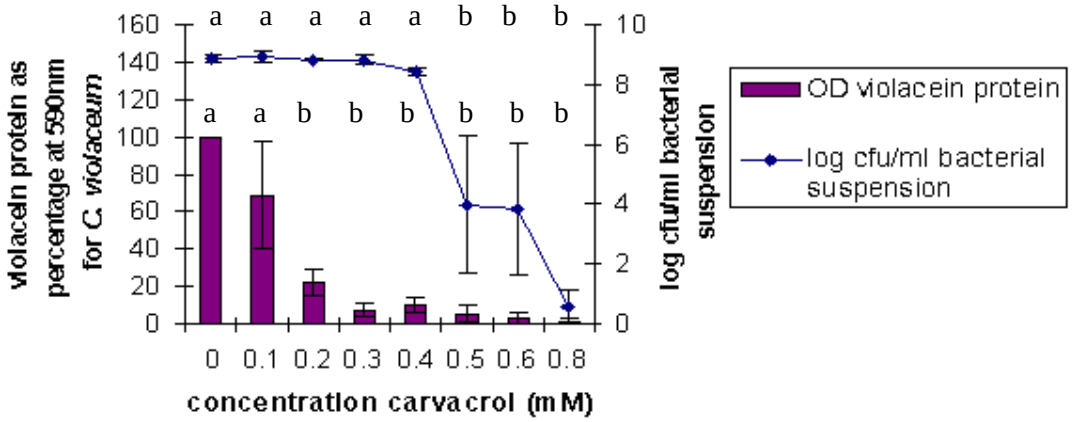


***Fig. 10*** **Average of three quorum sensing inhibitor activity assays for *C. violaceum*. The amounts of produced violacein protein per concentration carvacrol are expressed as a percentage of the 0mM concentration and the log of the cfu per ml of the bacterial suspension is shown as a line. Both the data have a ± SEM. Significant differences are indicated by different letters ($p < 0.05$)**

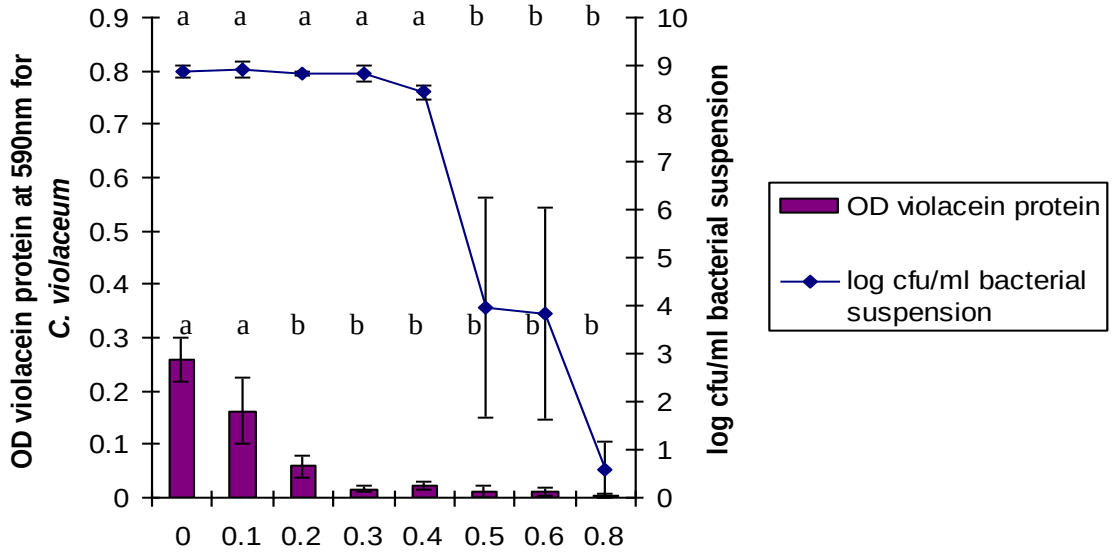


***Fig. 11*** **Average of three quorum sensing inhibitor activity assays for *C. violaceum*. The OD at a wavelength of 590nm is shown as the amount of violacein protein with ± SEM (bars) and the log of the cfu per ml of the bacterial suspension is shown as the line with ± SEM. Significant differences are indicated by different letters ($p < 0.05$)**

Violacein production for both the absolute values and as a percentage shows a decrease as the concentration of carvacrol rises. Violacein production is not signicanlty changed at 0.1 mM carvacrol and is significantly reduced from 0.2 mM carvacrol upwards ($p < 0.05$). The number of bacteria does not decrease up to 0.4mM carvacrol and decreases significantly from 0.5mM carvacrol concentration ($p < 0.05$).

## Discussion

### Biofilm destruction assays

The absolute values for the biofilms of *P. aeruginosa* are low compared to the biofilms formed by *S. aureus* and *S.* Typhimurium. The reason for the low absolute biofilm values should be examined by performing more assays with this bacterium. Due to time constraints the biofilm destruction assay for *P. aeruginosa* was only performed twice compared to three times for the other two bacteria. To be able to make a good comparison the assay should be repeated for Pseudomonas.

For the *S. aureus* biofilm assays the results showed a significant decrease of a 24 hours biofilm for the absolute values for the concentrations carvacrol of 0, 2 and 4mM and as percentages for the 0 and 2mM carvacrol. Adding aqua dest for 24 hours also showed a significant decrease in formed biofilm. The higher concentrations carvacrol of 6 and 8mM might have killed all the bacteria entrapping them in the formed biofilm. With the 0, 2 and 4mM concentrations the bacteria might still be alive but experience such a decrease in environmental conditions that they started shedding from the biofilm. Before the biofilms are stained in the assay the wells are washed to remove all the loose bacteria. To determine whether there is a difference in composition and ratio live/dead bacteria in the biofilms for the different concentrations carvacrol a live/dead stain could be carried out.

Adding the carvacrol concentrations to biofilms of *P. aeruginosa* and *S.* Typhimurium did not show a significant difference or decrease in the amount of biofilm. As discussed above for *P. aeruginosa* there might not have been enough biofilm present to be able to detect a significant difference and it might be useful here as well to determine the number of actual live bacteria present in the biofilm after the treatments with carvacrol. The absolute values for the biofilms formed by *S.* Typhimurium are high enough to be able to detect a significant decrease but here a live/dead stain could also help determine if the cells in the biofilm are really not affected by the carvacrol or they did (partly) get killed and were therefore not shed by the biofilm.

The results for *S. aureus* are obtained under in vitro conditions and if there is an actual effect in vivo needs to be examined. Consideration should also be given to the fact that in vivo most biofilms are not monospecies and the effect on a mixed species biofilm might be different also the environmental conditions that will differ in vivo and one bacterium might make different types of a biofilm under different conditions. The carvacrol itself probably will not remove an entire biofilm but might be able to give antibiotics more effect on the bacteria in the weakened biofilm or destroy the bacteria that are loosened from it. The loosened bacteria might also present danger to the host as they can lead to embolism or potentially spread the infection so it becomes systemic.

### Quorum sensing inhibitor activity assay

The 0.2, 0.3 and 0.4mM carvacrol seem to have an effect that is quorum sensing inhibiting as for these concentrations there is no significant decrease in the number of bacteria but there is a decrease in violacein production which cannot have been caused by fewer bacteria present.
If the quorum sensing inhibiting effect can be extrapolated to other bacteria that make use of the same quorum sensing system as *Chromobacterium violaceum* ATCC 12472

does need to be examined, but that carvacrol does inhibit quorum sensing in this bacterium is shown. *C. violaceum* uses autoinducer AI-2 for quorum sensing and this autoinducer is used in gram-negative and gram-positive bacteria meaning that carvacrol possibly can inhibit quorum sensing in different bacteria.[6,28]
Quorum sensing is used to form a biofilm in the initial stages but also plays a role in the mature biofilm and in the expression of virulence factors in both gram-negative and gram-positive bacteria. The assay was carried out on bacteria growing in broth and not growing in a biofilm. There might also be a difference of the quorum sensing inhibition by carvacrol for the biofilm mode of growth.

In case of the formation of a biofilm the quorum sensing inhibiting properties might be useful to combine with antibiotic therapy as antibiotics can sometimes induce biofilm formation in some pathogens and might be prevented by combining the treatments.[22] But here as well the results of these assays were obtained in vitro an it might be very difficult to predict how the quorum sensing inhibiting properties of carvacrol will be in vivo as the quorum sensing pathways used by pathogens are very complex. In vivo experiments also need to be carried out as to determine the actual biological activity/availability in the host(tissue). Because of the complexity of the quorum sensing pathways it also should be considered what other effects inhibition can have during the formation and maturation of a biofilm. For example for *S. aureus* the quorum sensing system is repressed in a mature biofilm and is active during dispersal so inhibition might not have any effect on a mature biofilm but only in the first stages of development, making it more difficult to treat and if used for this type of bacterium to be sure that the infection is in an early state.[19] Furthermore the effect of quorum sensing in a biofilm seems to be highly dependent on the environmental conditions making it even more difficult to predict the effect of it's inhibition under different circumstances and in a host.

The inhibition of quorum sensing might be a good target as the bacteria themselves don't experience any loss of growth due to the inhibition so it is believed that there won't be any fast development of resistance against quorum sensing inhibiting compounds.[10] In combination with antibiotic treatment it might prevent an active infection going into biofilm state in a host.

## Conclusion

Carvacrol did not have a significant effect on in vitro mature biofilms formed by *Salmonella enterica* serovar Typhimurium DT104 and *Pseudomonas aeruginosa* for concentrations up to 8mM. For the mature biofilm formed by *S*taphylococcus *aureus* 0074 there was a significant reduction of biofilm for concentrations carvacrol up to 4mM. These results were obtained from assays with monospecies biofilms.

At concentrations higher than 0.2mM carvacrol reduces the production of violacein of *Chromobacterium violaceum* ATCC 12472 and inhibits violacein production induced by quorum sensing in this bacterium. For concentrations carvacrol between 0.2 and 0.4mM the number of viable bacteria present was not significantly decreased indicating that the decrease in violacein production is not caused by fewer bacteria being present.

Carvacrol shows promise in the area of quorum sensing inhibition. Whether the results of the in vitro tests can be extrapolated to in vivo must be researched further and as well if the quorum sensing inhibition action of carvacrol also has an effect on bacteria other than *Chromobacterium violaceum* ATCC 12472 which make use of the same quorum sensing system.

## Acknowledgements

I want to thank my supervisors, Sara Burt and Edwin Veldhuizen, for their time and dedication during the project and for all their help and patience during the finishing stages of this research project.

Another special thanks is for Betty who was willing to give her time and help which was tremendous in figuring out the best protocol for some of the assays that where performed. Without her help this would not have been such a success.

Also I want to thank all the members of the microbiology lab for their help and assistance and willingness to answer all my questions where needed and making me familiar with the lab, especially Ali and Angèle.
And all the members of the IRAS-VPH division for their helpfulness.

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