Does bacterial communication play a role for the effect of triclosan, Corsodyl and Listerine on biofilm formation and growth of *Streptococcus mutans*?

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Introduction

Biofilm and biofilm formation

Bacteria colonize biological and inert surfaces in the form of matrix-encapsulated communities referred to as biofilms (1). These microbial biofilms are a highly distinct form of microbial life compared with the planktonic, or freely floating, form of microbial life that has been exhaustively studied for the last century (2). Bacterial biofilms account for the majority of chronic diseases, including gingivitis, endocarditis and nosocomial infections (1). Microbial biofilms are involved in approximately 65 % of human bacterial infections and up to 60 % of hospital acquired infections are caused by biofilms that contaminate implants and catheters (3). Oral diseases, such as dental caries and periodontal disease, should be considered as consequences of ecologically driven imbalances of oral microbial biofilms. Control of oral biofilms is fundamental to the maintenance of oral health and to the prevention of dental caries, gingivitis, and periodontitis (4).

The biofilm mode of growth seems to be advantageous for microorganisms. The biofilms consist of microorganisms enveloped in extracellular polymeric substances, organized in three-dimensional structures, with networks of intervening water channels and multiple layers of cells (5). The matrix that holds the biofilm together is a mixture of polysaccharides, proteins, and DNA secreted by the cells (4).

The process of biofilm formation is thought to be well regulated. The process starts with the adhesion of planktonic microorganisms to a surface, followed by colonization and co-adhesion, growth and maturation, and finally detachment of some microorganisms (4).

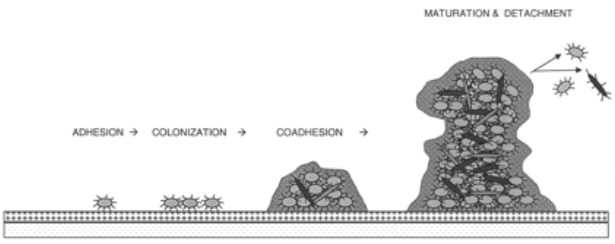


Figure 1. Biofilm formation (modified from article 4)

At the first stage, the microorganisms are reversibly attached, and not yet committed to the biofilm differentiation process. To develop a mature biofilm, the adhesion must be irreversible and the microorganisms must grow in population. There are three mechanisms that may lead to colonization and co-adhesion:

1) Redistribution of attached cells by surface motility
2) Division of attached cells
3) Recruitment of planktonic cells to the developing biofilm

The biofilm formation is a dynamic process, thus attachment, co-adhesion and growth may occur at overlapping times (6). Nutrient contents of the growth medium, such as glucose, serum, availability of iron and CO_2, osmolarity, pH, and temperature, influence biofilm production among different bacteria (7). The maturation of the biofilm results in a complex architecture with channels and pores (6). The bacteria use these channels and pores to transport nutrients, waste products and many signal molecules (8).

When organized in biofilms, the microorganisms are less susceptible to anti-microbials and more resistant to immune defense mechanisms. The concentration of an agent which kills planktonic microorganisms might have to be increased by 10-1000 times to have the same efficacy on microorganisms in a biofilm (9).

Apart from chlorhexidine and fluorides, only a few of the existing oral prophylactic agents have significant effects. One probable explanation for this low efficacy is the fact that the microorganisms involved organize into complex biofilm communities with features that differ from those of planktonic cells (4). The construction of the biofilm prevents diffusion of active molecules into the depth of the biofilm, or the molecules get inactivated on the surface of the biofilm. Another explanation for this resistance is the bacterial growth rate in the biofilm. A bacterial culture that has a fast growth will be more sensitive to antimicrobial agents. Bacteria grow more slowly when they grow in a biofilm, and therefore they are less sensitive (8).

Bacteria in biofilms colonize a wide variety of medical devices, such as catheters, artificial cardiac pacemakers, prosthetic heart valves and orthopaedic appliances, and are associated with several human diseases, such as native valve endocarditis, burn wound infections, chronic otitis media with effusion and cystic fibrosis (7). Bacteria in a biofilm are more highly resistant to antibiotics than planktonically growing bacteria, thus the potential impact of biofilm formation could be significant (7).

Communication within a biofilm

Bacterial microorganisms exist in large cooperative populations by employing communication systems necessary for their virulence and survival. Quorum sensing is one such system that enables bacteria to coordinate their gene regulation and trigger collective population behaviors (1). This system is one type of cell to cell communication in which bacteria control their gene expression in response to cell density (10), and thereby a density-dependent regulation of gene expression by small signaling molecules, autoinducers (AI). Through this quorum sensing system, bacteria are able to monitor their population by releasing AI- signals and consequently responding to a specific threshold accumulation of AI signals (1). Many bacteria have several quorum sensing systems (11).

The biochemical synthesis of AI-2 involves enzymatic steps starting from S-adenosylmethionine (SAM), particularly that catalyzed by LuxS, which produces AI-2 as a side product in addition to the primary role of this enzyme in the activated methyl cycle metabolism (11).

AI-2 is a collective term to describe cyclic derivatives of 4,5-dihydroxy-2,3-pentanedione (DPD), a highly reactive metabolic by-product of the activated methyl cycle. The AI-2 signal and its enzymatic synthase LuxS are broadly encountered in gram-positive and gram-negative bacteria, suggesting that AI-2 is an inter-and intraspecies communication signal. This communication system has been shown to play a role in the vital functions, including virulence and biofilm formation, of several bacteria (1). The LuxS coding gene is conserved in a majority of sequenced Gram-positive and Gram-negative microbial genomes, suggesting that AI-2 may function as a universal language for interspecies communication (6).

Prevention of biofilm infections

The recent emergence and spread of multi-resistant micro-organisms and refractory biofilm-induced infections have prompted an intense search for novel antibiotics that inhibit pathogenic microorganisms through novel targets (4). Infections once being easy to cure with antimicrobials are now becoming difficult, and sometimes even impossible to treat due to multidrug resistance. The antimicrobial used today were developed and generally tested against planktonic microorganisms in the laboratory, disregarding the increased resistance against antimicrobials in microorganisms living in biofilms.

It is therefore necessary to look for new approaches to combat biofilm infections (6).

There is growing interest throughout the oral health care profession in therapeutic agents that complement and enhance the mechanical removal of biofilms in the oral cavity (12). Antiseptic mouthrinses solutions are used in many clinical situations for different prophylactic and therapeutic purposes.

Triclosan

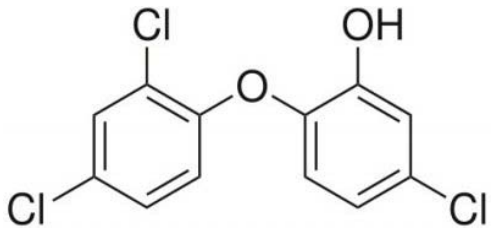


Figure 2.The molecule structure of triclosan

Triclosan (*2, 4, 4'-trichloro-2'-hydroxydiphenyl ether*), also known as Irgasan, is a non-ionic, lipid-soluble biphenol with both hydrophilic and hydrophobic properties. It has a broad spectrum of antibacterial activity and it has activity against many Gram-positive bacteria, some Gram- negative bacteria, fungi and viruses (13).

Because of its high anti-microbial effectiveness, the use of triclosan has increased dramatically in recent decades in the US and Europe, and it is incorporated in a range of personal hygiene items such as hand soaps, deodorant soaps, shampoos and medical devices such as sutures and plastics (14). Triclosan has also been used in dermatological products such as skin cream thus providing protection of the skin (15). In addition, this material has also been incorporated in many oral hygiene products such as mouthrinses and toothpastes. It has been shown to be effective in the inhibition of plaque, calculus, caries and gingivitis (16).

Early in the 1960s, triclosan was synthesized and developed by Ciba Geigy Co. (Basel, Switzerland) and in a review done by Bhargava and Leonard (17), this material is described as an odorless crystalline powder with a molecular weight of 289,5. Furthermore, they write that triclosan resolves poorly in water but is moderately soluble in alkaline, and dissolves easily in most non-polar organic solvents.

In terms of activity against microorganisms, triclosan is concentration dependent. Low concentrations of triclosan interfere with bacterial nutrient uptake (18, 15), whereas high concentrations induce leakage of intracellular components. The mechanism of triclosan’s antiseptic action is by acting on the microbial cell membranes. It intercalates into bacterial cell membranes (14) and disrupts the cytoplasmic membrane, RNA, lipid and protein synthesis (19, 20) and thereby resulting in inhibition of the microorganism or killing via cell lysis.

Triclosan also shows to have an anti-inflammatory action, due to inhibition of enzymes of the arachidon metabolism, which plays a key role in the development of inflammation of the gingiva (21). It was shown that by brushing teeth with triclosan-containing dentifrice, it could reduce the subgingival microbiota both in quantity and qualitaty, and in some cases it could also retard the progression of periodontitis (16).

Corsodyl

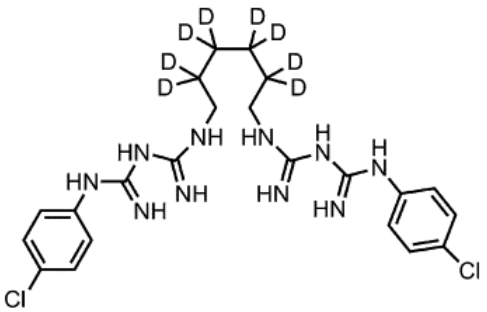


Figure 3. The molecule of chlorhexidine

The active agent in Corsodyl mouthrinse is chlorhexidine, which has a wide spectrum of antibacterial activity, and has been used in health care settings for several decades (22). This agent has particularly good activity against gram-positive bacteria, but also certain fungi and viruses (23). It does not affect mycobacterium or bacterial spores due to the inability of the antiseptic to reach target sites within the cell (24). Petti et al. have reported that chlorhexidine shows also to have caries-preventive effect, as well as remineralising properties and has few toxic effects (25). Chlorhexidine is a cationic bis-biguanide which is available as the acetate (diacetate), hydrochloride and gluconate salts (24). As a cationic agent, chlorhexidine becomes incompatible with anionic surfactants and the efficacy of this agent can be affected by the presence of a sodium lauryl sulphate (SLS)-containing dentifrice (26).

Corsodyl has few side effects (25). Transient taste disturbances are common, and some have discoloration of teeth and tongue after 1-2 weeks of use because of its affinity for dietary compounds. In some cases, staining of the teeth is severe, and removal requires a professional prophylaxis. Burning sensation and desquamation of mucosa occur sometimes. Allergic reactions have been reported occasionally (23).

The antibacterial activity of chlorhexidine is believed to be concentration dependent (27). At low dosages, the integrity of the bacterial cell membrane is altered and thereby resulting in a reversible leakage of bacterial low-molecular-weight components (28). At higher doses, this agent has a bactericidal effect via membrane disruption and cell lysis due to a disturbance of the bacterial metabolism (29, 30). Exposure to chlorhexidine causes membrane damage and leakage of cytoplasmic cell components (31).

Listerine

Listerine is a mouthrinse which consists of different phenolic compounds such as eucalyptol, methyl salicylate, menthol and thymol. Due to their low toxicity and high antibacterial activity (32), these essential oils have been incorporated into the mouthrinse. Listerine exhibits its antibacterial activities by these four active agents. In addition, it also contains, among other substances, alcohol (22 %) and fluoride (0,05 %).

This product has been shown to inhibit or reduce plaque accumulation and the severity of gingivitis (32). The effect of Listerine on gingivitis may partly be because of the anti-inflammatory properties of the essential oils incorporated in the rinse (33). Essential oils are aromatic oily liquids obtained from plant material (leaves, bark, herbs and wood) (34) and are regarded as natural alternatives to chemical preservatives.

The antibacterial activity of the phenolic compounds against bacterial cells is described to be relatively complex. It also involves denaturation of proteins along with cell lysis resulting in the leakage of cellular substances (32) such as ions, ATP and nucleic acid. The effect of Listerine is therefore ascribed to its bactericidal

properties (33). Each of the phenolic compounds has some form of activity against bacteria and fungi.

Eucalyptol is one of the most important and most widely planted genera and includes more than 700 species (35). Eucalyptol is a rich source of bioactive compounds and has significant biological activities, including antibacterial, antimalarial, anti-inflammatory and antioxidant properties (35, 36).

The main use of the molecule *methyl salicylate* is in the flavour industry to create several aromas, such as strawberry, banana, mint, peach, tomato, raspberry and cherry (37). Methyl salicylate can either be obtained by synthesis or from two natural sources: essential oil of wintergreen and sweet birch bark (37). This molecule has also shown to possess some antibacterial activity against several bacteria (38) and thereby give a reduction in bacterial growth.

Thymol is one of the major compounds in oregano essential oil (34) and is responsible for its activity against microorganisms. Thymol interacts with the lipid bilayer of cytoplasmic membranes causing loss of integrity and leakage of intracellular components (34).

Menthol is a naturally occurring alcohol of plant origin, which gives plants of the Mentha species their distinctive smell and flavours (39) and has mainly been used for medical purposes. The ability of menthol to act as penetration enhancer by disrupting the lipid bilayer (39), it shows to be active against a variety of microorganisms (40).

Hypothesis and aim of our study

The major aim of the study was to investigate the effect of triclosan, Corsodyl and Listerine on the planktonic growth and the biofilm formation. We also looked at the efficacy of selected mouthrinses on established biofilm. To test the hypothesis, that AI-2 communication is involved in biofilm formation and sensitivity to triclosan, Corsodyl and Listerine in *Streptococcus mutans* - *S.mutans* UA159 WT (wild-type) and UA159 MT (Mutant) – the bacteria created with inactivated AI-2 signal molecule, were assayed for their ability to initiate biofilm formation in the prescense of an antimicrobial agent. If communication via AI-2 is important for biofilm formation and antimicrobial tolerance, one would expect a decrease in planktonic growth and biofilm formation in UA159 MT (Mutant) with inactivated AI-2 signal molecule, compared to the wild type.

S. mutans has been considered as the main pathogen associated with dental caries. It induces mineral loss due to its strong adhesion to the tooth surface and to the acid production resulting from fermentable carbohydrates, which keeps local pH low (41).

The results of this study will tell us something about the value of the communication with the signal molecule, AI-2, as an universal language for interspecies communication.

The present study should be viewed as a pilot study. In this sense the aim is to discover tendencies that need further and more thorough investigations.

Materials and methods

S. mutans UA 159 WT (Wild type) and *S. mutans* UA 159 AI-2 negative (Mutant) were assayed for their ability to initiate biofilm formation in the presence of selected antimicrobial agents. This would allow comparison of the antibiofilm efficacy and the importance of AI-2 communication.
S. mutans wild type and mutant were prepared by growing the streptococci at 37^{o}C in 5 % CO_2 in air for two over-nights in TSB (Tryptic Soya Broth) medium.

For the first over-night culture, bacterial colonies from TSB agar plates were transferred to 5 ml TSB-medium. The bacterial suspensions were incubated at 37 ^{o}C for 18 hours to avoid the bacterial cells to die.
For the second overnight, 10 µl from the first over-night culture were transferred to a new 5 ml TSB medium and these bacterial suspensions were again incubated at 37 ^{o}C.

<u>Project 1 Inhibition of biofilm formation and planktonic growth</u>
The antimicrobial agents used were triclosan, Listerine and Corsodyl.
The optical density (OD) from the second over-night culture was adjusted with TSB to OD=1.0 at 600 nm. Then the pre-grown cell suspension was diluted 1:500 in TSB medium.

75 µl of TSB medium was added in the individual wells of 96 well polystyrene microtiter plates from 2A-H to 10 A-H. We used three plates for the antimicrobial agent, respectively.
Triclosan was diluted 1:100 in TSB medium and Listerine and

Figure 4. Antimicrobial agents and laboratory equipment

Corsodyl were diluted 1: 250 in TSB medium, respectively, before adding 200 µl of the agent into 1A-H. We created a dilution series by transferring 125 µl from column 1 to column 2 etc., and even wells in column 10 for each row A-H.

Then we removed 125 µl from column 10. It is important to point out that it was used three plates for the wild-type and three plates for the mutant. The growth of biofilms was initiated by adding 75 µl of diluted bacteria suspension in all of wells from 1A-H to 10 A-H and 150 µl in column 11. In column 12, we added 150 µl of TSB medium. Column 11 was positive control, and column 12 was negative control (no bacteria).

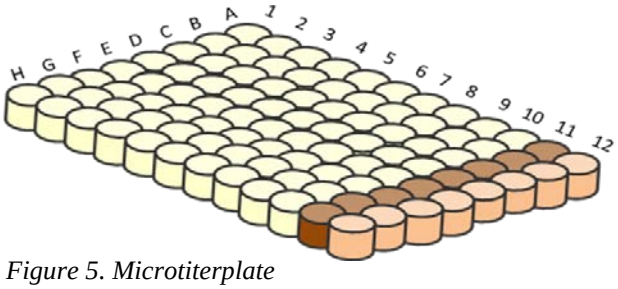


Figure 5. Microtiterplate

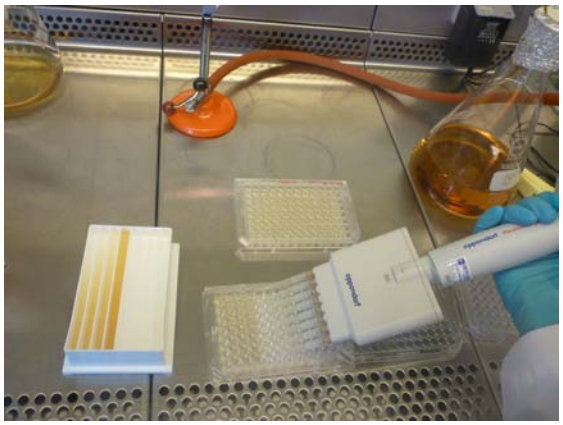


Figure 6. Adding TSB medium to microtiter plates.

After this 18 hour incubation period, the liquid medium was transferred to new 96 well polystyrene microtiter plates and quantified by measuring the absorbance of the planktonic growth at 600 nm with a microplate reader. The biofilm plates were rinsed twice with sterile distilled water (dH_2O). The plates were then air-dried and stained with 0.1 % safranin for 10 minutes. After being stained, the plates were rinsed with dH_2O to remove excess dye. The plates were then air-dried once again. The biofilms were quantified by measuring the absorbance of the staining of biofilms at 530nm. This optical density is most accurately measured when the dye is evenly distributed in the wells. This was obtained by adding 150 µl of 30 % acetic acid in each well, releasing the dye from the stained biofilms into a homogenous solution. 100 µl of this solution was transferred from each well into a second microtiter plate, from which the optical density was measured.

The experiment was run in 3 parallels and was repeated three times.

Project 2 Effect on established biofilm

The antimicrobial agents used were Listerine and Corsodyl.
The optical density (OD) from the second over-night culture was adjusted with TSB to OD=1.0 at 600 nm. Then the pre-grown cell suspension was diluted 1:100 in TSB medium.
The growth of biofilms was initiated by adding 150 µl of diluted bacteria suspension in all of wells from 1A-H to 11 A-H. In column 12, we added 150 µl of TSB medium. Column 11 was positive control, and column 12 was negative control (no bacteria). The microtiter plate was incubated at 37 ^{o}C with 5% CO_2 for 18 hours. We used three plates for the wild-type and three plates for the mutant.

After this 18 hour incubation period, the liquid medium was transferred to new 96 well polystyrene microtiter plates and quantified by measuring the absorbance of the planktonic growth at 600nm with a microplate reader. The biofilm plates were rinsed twice with sterile PBS (phosphate buffered saline). We used three plates for the wild-type and three plates for the mutant

New 96 well polystyrene microtiter plates were used to create the dilution series of the antimicrobial agents. 75 µl of PBS was added in the individual wells from 2A-H to 10 A-H. 200 µl of the agent was then added to 1A-H and 125 µl transferred from column 1 to column 2 etc., and even wells in column 10 for each row A-H. Then we removed 125 µl from column 10. Finally, we added 75 µl PBS from 1A-H to 10 A-H. Here, we used the antimicrobial agent directly in the individual wells 1A-H without diluting it in TSB medium. From this plate, we transferred 125 µl with dilutions to the corresponding wells on the biofilm plates. After the transfer, the plates were set to slight shaking for 2 hours.

The biofilm plates were rinsed twice with PBS. The plates were then air-dried and stained with 0.1 % safranin for 10 minutes. After being stained, the plates were rinsed with dH_2O to remove excess dye. The plates were then air-dried once again. The biofilms were quantified by measuring the

absorbance of the staining of biofilms at 530nm.

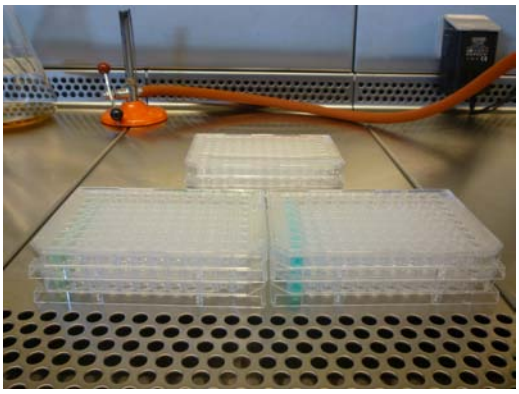


Figure 7. Microtiterplates with antimicrobial agents.

The experiment was run in 3 parallels and was repeated three times.

These two projects were performed to investigate the efficacy of different antimicrobial agents on biofilm formation and on established biofilm.

Results

The antibacterial activity of triclosan, Corsodyl and Listerine was evaluated using *S. mutans* UA159 WT (wild-type) and UA159 MT (Mutant). All bacterial strains were obtained from the department of Oral Biology, Faculty of Dentistry.

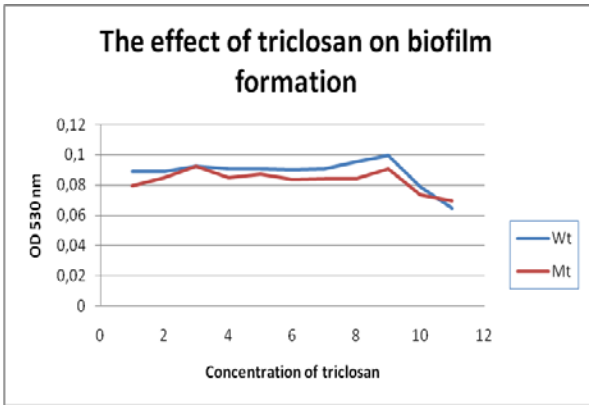
<u>Project 1</u>

Test results for triclosan

From the graph we can see that both the biofilm formation and the planktonic growth of the bacteria are affected by the antimicrobial agent, triclosan. With low concentrations of this agent, we can observe that the inhibitory effects are low, both on the planktonic growth and the biofilm formation. Eventually when we increase the concentration of the agent, we see that the density of the bacteria decreases. This means that the effect of triclosan on both the planktonic growth and biofilm formation only is essential in higher concentrations. If we compare the effects of triclosan on *S.mutans* UA159 WT and UA159 MT (AI-2 negative), we see no significant difference.

Column	Concentration (µM)
1	0
2	0,509
3	0,815
4	1,304
5	2,086
6	3,338
7	5,341
8	8,544
9	13,672
10	21,875
11	35

Table 1. Concentration of triclosan in each collumn on the microtiterplate used. Each collumn is represented on the x-axis on the graphs below.



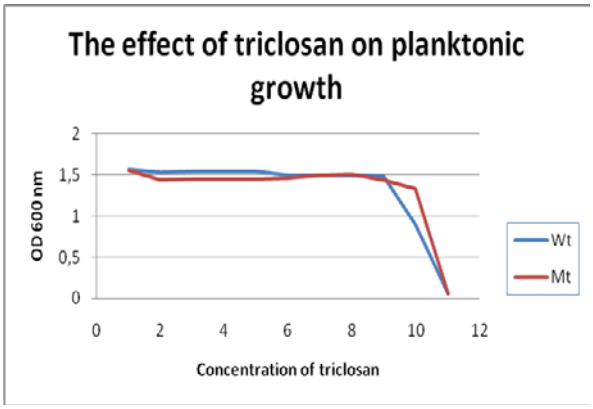


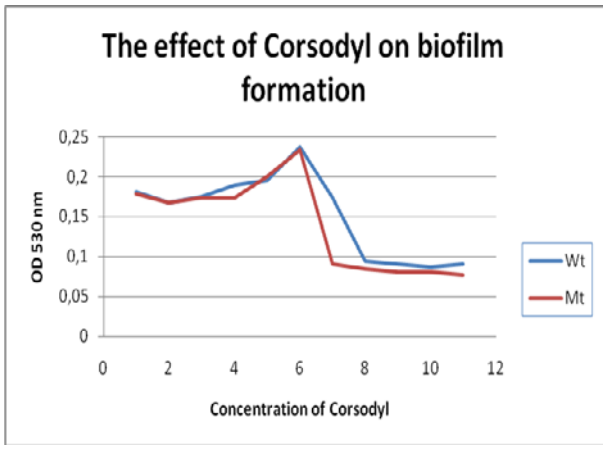
Figure. 8 The effect of triclosan on biofilm and planktonic growth of S. mutans *UA159 WT and UA159 MT.*

Test results for Corsodyl

From the graphs below, we can see that Corsodyl has an inhibitory effect on both the planktonic growth and the biofilm formation. Also here, we observe that in low concentrations, the antimicrobial agent does not have an inhibitory effect on neither the planktonic growth nor the biofilm formation. But with higher concentration we see a clearly inhibitory effect. When looking at the graph for planktonic growth we can observe that the bacterial density is powerfully decreased at higher concentrations of this agent. When we look at the test results for *S. mutans* UA159 WT and UA159 MT, we see no clear differences.

Column	Concentration (µM)
1	0
2	0,128
3	0,205
4	0,328
5	0,525
6	0,839
7	1,343
8	2,148
9	3,438
10	5,5
11	8,8

Table 2. Concentration of Corsodyl in each collumn on the microtiterplate used. Each collumn is represented on the x-axis on the graphs below.



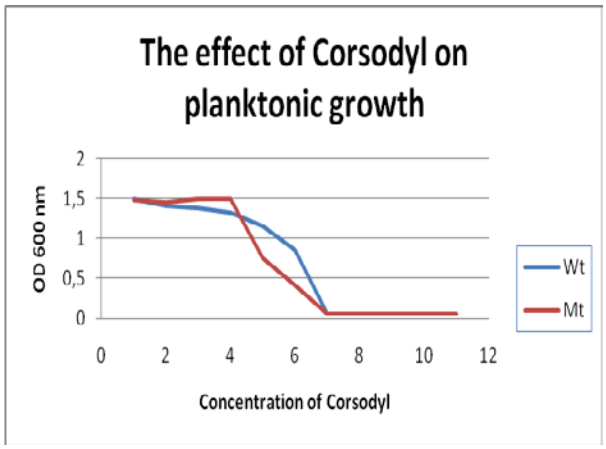


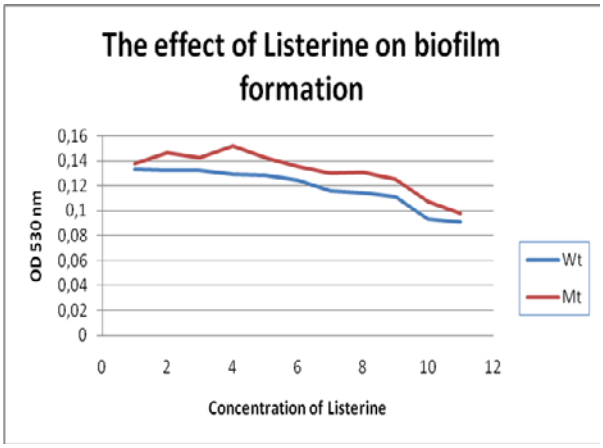
Figure. 9 The effect of Corsodyl on biofilm and planktonic growth av S. mutans *UA159 WT and UA159 MT.*

Test results for Listerine

From the graphs below we can see that Listerine only has an inhibitory effect on the biofilm formation, and not on the planktonic growth. The bacterial density in the planktonic growth stays approximately the same, despite the increase in concentration of antimicrobial agent. Also here we got the same test results as for triclosan and Corsodyl when we compared the results for *S. mutans* UA159 WT and UA159 MT. We see from the graphs that there were no clear differences between these genotypes of *S. mutans* UA159.

Column	Concentration (%)
1	0
2	0,0058
3	0,0093
4	0,0149
5	0,0238
6	0,0381
7	0,061
8	0,0977
9	0,1563
10	0,25
11	0,4

Table 3. Concentration of Listerine in each collumn on the microtiterplate used. Each collumn is represented on the x-axis on the graphs below.



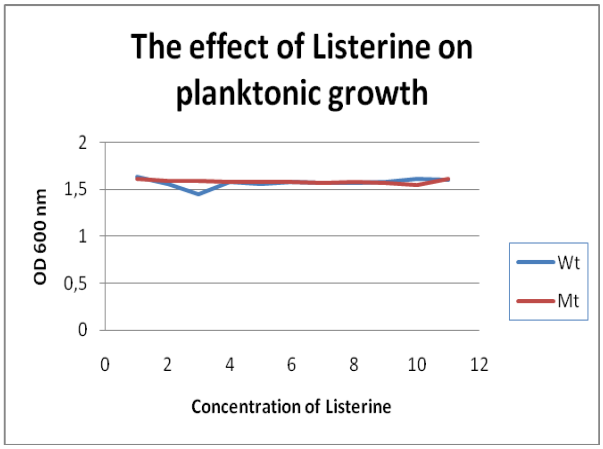


Figure. 10 The effect of Listerine on biofilm and planktonic growth av S. mutans *UA159 WT and UA159 MT.*

Project 2

Here we investigated the antibacterial agent's efficacy on established biofilm. These results show that in this experiment, with the concentrations used, we couldn't find any effect of antimicrobials on established biofilm. We see from the graphs that there were no clear differences between these types of bacteria.

Column	Concentration (µM)
1	0
2	0,128
3	0,205
4	0,328
5	0,525
6	0,839
7	1,343
8	2,148
9	3,438
10	5,5
11	8,8

Table 4. Concentration of Corsodyl in each collumn on the microtiterplate used. Each collumn is represented on the x-axis on the graphs below.

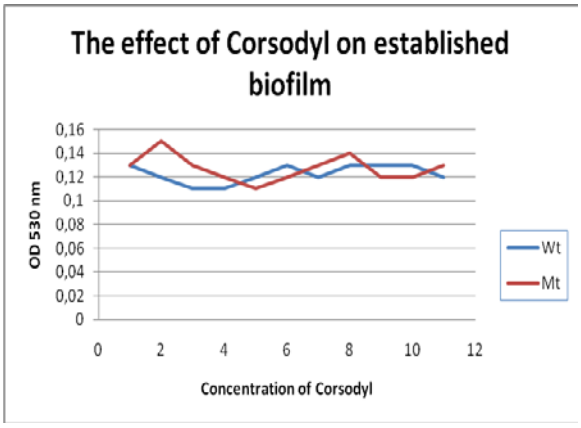


Figure 11. Effect of Corsodyl on established biofilm of S. mutans *UA159 WT and UA159 MT.*

Column	Concentration (%)
1	0
2	0,0058
3	0,0093
4	0,0149
5	0,0238
6	0,0381
7	0,061
8	0,0977
9	0,1563
10	0,25
11	0,4

Table 5. Concentration of Listerine in each collumn on the microtiterplate used. Each collumn is represented on the x-axis on the graphs below.

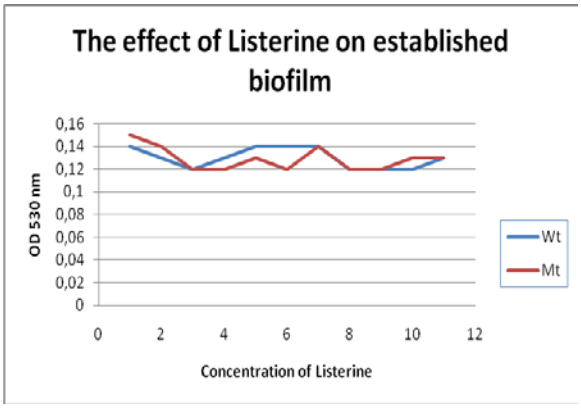


Figure 12. Effect of Listerine on established biofilm of S. mutans *UA159 WT and UA159 MT.*

Discussion

Regarding our hypothesis, we found that the antimicrobial agents used in our experiment (project 1) were effective in inhibiting biofilm formation and planktonic growth of *S. mutans* UA159 WT (wild-type) and UA159 MT (Mutant). We observed that streptococcal biofilm formation was reduced during growth in the presence of these antimicrobials. But we only found an inhibitory effect from triclosan and Corsodyl, but not for Listerine. Taking a look again at figure 10, Listerine only has an inhibitory effect on the biofilm formation, and not on the planktonic growth. The planktonic growth seems to remain stable, despite the increase in concentration of the antimicrobial agent. These findings could be due to a systematic error arising when depositing antimicrobial (Listerine) in the wells with the aid of a pipette. On the other hand, triclosan and Corsodyl showed remarkable bactericidal effects. This can be explained by the fact that the rate of a reaction involving an antimicrobial agent depends on the concentration of the agent at the active site (42). It is reasonable to expect that the concentration at the active site will depend on the bulk concentration of the antimicrobial agent in the medium surrounding the microorganisms. One of the reasons why Listerine did not show effect could be caused by precipitation or too low concentrations of active agent in the medium.

There was a slight tendency, but no clear differences between the two bacteria used, *S. mutans* UA159 WT (wild-type) and UA159 MT (Mutant) – the bacteria created without the AI-2 signal molecule. This was in contrast to what we had expected. We believe that the result from our study is not enough to conclude whether the AI-2 signal molecule is crucial for biofilmformation or not. To confirm the hypothesis need therefore further investigation.

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