An active attachment biofilm model to develop anti-caries strategies
Exterkate, R.A.M.

Citation for published version (APA):
Exterkate, R. A. M. (2014). An active attachment biofilm model to develop anti-caries strategies
Chapter 6

Summary and Future Perspectives.
There is a need to develop new preventive agents that target the oral microflora. For this adequate testing models are required that allow for the screening of these agents.

This thesis describes a number of experiments that were performed to develop, test and utilize a biofilm model suitable for this purpose. The prerequisites for such a model were: (i) biofilm formation should be reproducible and based on the active attachment of bacteria to a substratum (ii) besides single species biofilms it should allow the screening of complex polymicrobial biofilms, (iii) it should be possible to use various types of substrata and (iv) it should be possible to compare various antimicrobial compounds, concentrations and treatment times in a single experiment.

Based on the available models in combination with these prerequisites, a new active attachment model was developed. This model consisted of a custom designed stainless steel lid with 24 clamps that fitted on top of standard 24-well plates (Greiner, The Netherlands). The clamps allowed for the use of various substrata to grow biofilms on. The advantage of the model was that all biofilms, even though grown in separate wells, could be ‘handled’ at the same time. The fact that biofilm substrata were attached to the lid, allowed for a high degree of control with respect to exposure to nutrients and/or agents for treatment. The 24 biofilms grown independently in one experiment allowed for a range of concentrations or agents to be tested in a single run. For the testing of different exposure times multiple models should be run in parallel.

In chapter 2 the suitability of the model was evaluated using both single species \textit{S. mutans} and saliva derived polymicrobial biofilms. The output parameters of the model were total CFU count, and lactic acid production. First, the reproducibility of the model in terms of CFU counts of the biofilms that were formed from overnight cultures of \textit{S. mutans} C180-2, freshly collected saliva and frozen saliva was determined. Subsequently, the effect of biofilm type (single species biofilm or polymicrobial biofilm) and biofilm age on the treatment efficacy of a range of concentrations of aminefluoride (Olaflur, Gaba, Switzerland) was measured. Overall this data showed that the model is adequately sensitive to allow dose-response
relations to be assessed, both in terms of CFU counts and in lactic acid production. Polymicrobial biofilms proved less sensitive to aminefluoride with respect to cell viability, but aminefluoride significantly inhibited the metabolic activity of both single species *S. mutans* C180-2 and polymicrobial biofilms. This study showed that single species biofilms lack the complexity necessary to allow for a proper evaluation of caries preventive compounds. Using polymicrobial biofilm models proved to be a more appropriate approach. Moreover, the efficacy of a potential antimicrobial agent needs to be judged on the reduction of acid formation (i.e. cariogenic potential) as well as on the bacterial viability.

In chapter 3 the effect of adding arginine to the growth medium on biofilm metabolism was studied. Arginine is a substrate for alkaline production by bacteria. In the past, studies on arginine effects on alkaline production have focused on single species, saliva sediment or plaque. Studying arginine effects in polymicrobial biofilm models could be an approach to bridge the gap between data obtained from single species experiments and *ex vivo* or *in vivo* studies. Alkaline production was found to be reduced by the presence of sucrose, a low buffer capacity and a low pH (≤ pH 4.5). Alkaline production increased with increasing biofilm age, irrespective the presence or absence of sucrose. In fact, the inhibiting effect of sucrose decreased with increasing biofilm age. Studying the effects of specific additives on the metabolism of polymicrobial biofilms might help to extend our knowledge beyond the data obtained from well-controlled single species experiments.

In the studies described in chapters 2 and 3 polymicrobial biofilms were formed but their composition was not determined. As novel developed agents are not necessarily aimed at eradicating oral bacteria but should be aimed at limiting plaque growth or even at selectively suppressing specific species, it was deemed important to determine the composition of the biofilms formed. The composition of polymicrobial biofilms is difficult to be determined by culturing techniques. Recently, culture independent DNA-based technologies have become available and these are more appropriate than traditional culturing techniques. But at the same time DNA-based technologies do not allow for the discrimination between live and dead cells, which is a major drawback. Killing efficacy of antimicrobial treatments might be underestimated as the DNA of non-vital cells is
A treatment with propidium monoazide (PMA) has been proposed to overcome this problem. For this, cells are incubated with PMA allowing PMA to penetrate cells with compromised membranes and bind to their DNA. Photolysis of PMA using bright visible light forms a covalent link to DNA. As a result this DNA cannot be amplified by PCR. Any remaining unbound PMA is simultaneously inactivated by reacting with water. In chapter 4 this approach was tested on saliva derived polymicrobial biofilms. The effect of PMA on viable cells was evaluated by treating the saliva with PMA prior to biofilm formation. Biofilms were formed with both untreated and PMA-treated saliva. After 48 h the formed biofilms were treated once with a 0.2% chlorhexidine rinse (0.2% CHX, Meridol perio, GABA, Switzerland). After the treatment lactic acid production by the biofilms was determined.

Subsequently, the biofilms were harvested and left untreated or treated with PMA. Exposure to 0.2% CHX reduced the viability and metabolic activity of 48 h biofilms. The shift in the bacterial composition of the biofilms observed after the CHX exposure was enhanced after a post-rinse treatment with PMA. The effect of PMA on the bacterial composition of water treated biofilms was small. When saliva was treated with PMA, i.e. prior to the biofilm formation, viability was reduced and the bacterial composition of both saliva and saliva-derived biofilms was shifted. It was concluded that a PMA treatment after an anti-microbial treatment improved the detection of shifts in \textit{in vitro} polymicrobial biofilms after an antimicrobial treatment. But at the same time the study showed that PMA influenced the ability of cells to grow. Therefore, PMA should be used bearing these limitations in mind. The results obtained in chapter 4 lead to the question whether shifts in bacterial composition after short-term use of antimicrobials \textit{in vivo} would also be masked by the presence of non-vital bacteria. Therefore in chapter 5 a study is described which evaluated the use of PMA in clinical studies involving a mild antimicrobial treatment. For this, six subjects were sampled for buccal plaque, tongue scrapings (with a microbrush) and saliva, twice before a two-week period of oral-rinse use and twice at the end of that period. Subjects were asked to brush with a regular toothpaste (Prodent CoolMint, The Netherlands) and to use an oral rinse twice daily (Meridol, GABA, Switzerland). Subjects refrained from oral hygiene, except for the oral rinse, in the 24 h before the sampling. At each time point two buccal plaque samples, two tongue scrapings and
one saliva sample (that was subsequently split into two samples) were collected. One
sample of each of these sampling sites was treated with PMA and the other one was
left untreated. In total 24 samples per subject were collected. Data showed a clear
shift in the bacterial composition of tongue and saliva samples after a 2-weeks use of
the oral rinse. Buccal plaque samples did not show an effect of the oral rinse. Only in
2 out of the 6 subjects there seemed to be an effect on the bacterial composition.
PMA treatment enhanced the observed effects only for the saliva samples. The
measured composition of both buccal plaque and tongue samples was not altered by
the PMA treatment. It was concluded that the necessity to use a PMA treatment to
block the DNA from non-vital cells in clinical studies aimed at measuring
compositional shifts after the use of a mouthwash is limited to salivary samples.

Overall the data showed that the new biofilm model allows for the screening of
potential antimicrobial compounds. The sensitivity of the model was high enough to
show dose response relations both in terms of CFU counts and metabolic activity
with a selected anti-microbial. Moreover the model could show the effect of
compounds added to the growth medium on important metabolic output
parameters such as pH and ammonium production. Shifts in the bacterial
composition of the polymicrobial biofilms could be monitored. Adding a PMA
treatment step enhanced the observed effects. The concept of adding a PMA
treatment step when measuring shifts in the composition of polymicrobial samples
was also tested on clinical samples. Apparently a prolonged exposure to oral rinses
restricted the need for the PMA treatment to show changes in bacterial composition
of buccal plaque and tongue samples, whereas it still proved to be necessary when
analyzing changes in saliva.

**Future research**
The experiments described in this thesis were limited to cariogenic biofilms. The
biofilms grown in the respective studies were all grown in the presence of sucrose,
resulting in biofilms dominated by Streptococci and Veillonella species. A possible
target for future studies would be to grow biofilms that more closely reflect the
original inoculum. The inoculum in the studies described in this thesis was diluted
saliva, but studies that use dispersed plaque or even paperpoint samplings from subgingival plaque as inoculum also seem feasible. Growing biofilms that reflect the inoculum requires growth conditions that allow for different types of biofilms to be formed. Studies with different growth media and refreshment schedules are needed in order to increase the applicability of the model.

Ideally, future studies would allow the model to be used for biofilms reflecting different types of pathology. This would allow for the differentiation in the efficacy of agents based upon the composition of the biofilms.

In the past couple of years the model has been utilized in PhD studies of fellow researchers within our research group. The model has also been used in research in collaborating labs outside of ACTA.

When the model is developed further, allowing for different types of biofilms to be formed, it will be feasible to resolve some of the issues mentioned above.