Oral Biofilm as a Reservoir for Antimicrobials
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Chapter 2

Acute and substantive action of antimicrobial toothpastes and mouthrinses on oral biofilm *in vitro*

Marieke P.T. Otten, Henk J. Busscher, Henny C. van der Mei, Chris G. van Hoogmoed, Frank Abbas


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Abstract

The aim of this study was to compare acute action by killing or disrupting oral biofilms by antimicrobial toothpastes and mouthrinses *in vitro* and to investigate substantive action by absorption of antimicrobials in a biofilm. Biofilms were grown from freshly collected human saliva in 96-wells microtiter-plates. After removal of saliva, wells were washed with sterile water (control), or exposed to dilution series of mouthrinses (Corsodyl, Listerine, Meridol, Crest Pro Health) or toothpaste-slurries (Prodent Coolmint, Colgate Total, Zendium Classic, Crest Pro Health, Oral B Pro Expert, Crest Cavity Protection). Acute action was concluded from reduced continued (16 h) growth of treated biofilms with respect to the control. Substantive action was studied by exposing dead biofilms to mouthrinses or toothpaste-slurry. Substantive action through absorption and subsequent release of antimicrobials from biofilm was concluded from reduced growth on top of the treated biofilms. All formulations showed acute action in the highest concentrations studied. Further dilution yielded loss of efficacy, or even stimulation of biofilm growth. Antimicrobial absorption in and release of antimicrobials from dead biofilms in effective concentrations was demonstrated for three selected antimicrobial products, meaning that antimicrobials remain bio-available for substantive action on new biofilms.
Introduction

All surfaces exposed to the human oral cavity are covered within seconds with a layer of adsorbed salivary proteins (“pellicle”) to which a large variety of different oral microbial strains and species can adhere and grow to form a biofilm (“plaque”). Under appropriate conditions, the number of pathogenic bacteria in an oral biofilm will rise and as a consequence diseases, like caries and periodontitis can develop. Removal of oral biofilms by toothbrushing is the most effective way to prevent caries and periodontal diseases, but complete removal of oral biofilm by brushing is impossible for most people. Especially interdental areas, fissures and gingival pockets are little accessible to the bristle ends of a toothbrush and constitute places where plaque is easily left behind, and may also be difficult to remove by other mechanical means. Antimicrobials are added to toothpastes and mouthrinses in order to assist in achieving oral health through killing of oral biofilm organisms. A wide range of different antimicrobials can be added to toothpastes and mouthrinses, including enzymes, metal ions, stannous fluoride, chlorhexidine and triclosan. Chlorhexidine is generally considered to be the most effective oral antimicrobial, and it is often used as a positive control in studies.

Chemical control of oral biofilms by antimicrobials can occur by “acute” or “substantive” action by killing or disrupting the biofilm. Acute action takes place immediately after the use of an oral antimicrobial, leaving behind a dead or partly dead or disrupted biofilm. Substantivity is defined by the Oxford Dictionary of Dentistry as a characteristic of an antimicrobial product whereby it remains active in the oral cavity for a longer period. Recently, we suggested on the basis of a clinical study, that also plaque left behind after brushing, which is inevitably the case in most people, can absorb antimicrobials to yield substantive killing of new plaque. In this study we hypothesize that oral antimicrobials can absorb in
biofilms to inhibit further growth of and kill new organisms, a process we refer to as substantive action.

Consequently, the aim of this study is not only to measure the acute action by killing or disrupting oral biofilms achieved by a single exposure of antimicrobial mouthrinses and toothpastes, but also to investigate whether antimicrobials, absorbed in dead biofilms, have the ability to exert prolonged action on organisms growing from fresh saliva on top of the dead biofilm.

**Materials and methods**

**Saliva, toothpastes and mouthrinses**

In order to study acute and substantive action, stimulated human saliva of five volunteers was collected by chewing Parafilm and pooled in accordance with the guidelines set out by the Medical Ethical Committee at UMCG, Groningen, The Netherlands. The saliva was sonicated twice for 10 s at 30 W (Vibra Cell model 375, Sonics and Materials Inc., Danbury, CT, USA) in order to break bacterial chains and clumps.

For acute action, four different mouthrinses and six different toothpastes were commercially obtained, as listed in Table 1, together with their main active ingredients. Toothpastes were diluted in demineralized water to a 25% (w/w) slurry, which was centrifuged at 10,000g for 5 min at 10°C (Beckman J2-MC Centrifuge, Fullerton, CA, USA) in order to remove particulate matter. Subsequently, the supernatant of the toothpaste was further diluted to 12.5%, 2.5% and 0.25% (w/w) with demineralized water. Mouthrinses were used full-strength or diluted to 50%, 10% and 1% (v/v) in demineralized water. For the demonstration of substantive action through antimicrobial absorption in biofilms, Corsodyl and Crest Pro Health mouthrinse (100% (v/v)) and Crest Pro Health toothpaste-slurry (25% (w/w)) were used.
Table 1. Toothpastes and mouthrinses used in this study, together with their main active components and manufacturer.

<table>
<thead>
<tr>
<th>Mouthrinse</th>
<th>Main active components</th>
<th>Manufacturer</th>
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<tbody>
<tr>
<td>Corsodyl®</td>
<td>Chlorhexidine digluconate 0.2%</td>
<td>GlaxoSmithKline, Middlesex, UK</td>
</tr>
<tr>
<td>Listerine®</td>
<td>Alcohol, Phenols and Essential oils</td>
<td>Pfizer Consumer Healthcare, Morris Plains, NJ, USA</td>
</tr>
<tr>
<td>Meridol®</td>
<td>Amine fluoride, Stannous Fluoride</td>
<td>GABA Group, Basel, Switzerland</td>
</tr>
<tr>
<td>Crest Pro Health® mouthrinse</td>
<td>Cetylpyridinium chloride</td>
<td>Procter &amp; Gamble, Cincinnati, USA</td>
</tr>
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<table>
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<tr>
<th>Toothpaste</th>
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<tbody>
<tr>
<td>Prodent Coolmint®</td>
<td>Sodium Fluoride, Sodium Lauryl Sulphate (SLS)</td>
<td>Sara Lee Household &amp; Bodycare, Exton, USA.</td>
</tr>
<tr>
<td>Crest Cavity Protection®</td>
<td>Sodium Fluoride, SLS</td>
<td>Procter &amp; Gamble, Cincinnati, USA</td>
</tr>
<tr>
<td>Colgate Total®</td>
<td>Triclosan, Polyvinyl methylether maleic acid, Sodium Fluoride, SLS</td>
<td>Colgate-Palmolive Company, Piscataway, USA</td>
</tr>
<tr>
<td>Zendium Classic®</td>
<td>Sodium Fluoride, Colostrum, Lactoperoxidase, Lysozyme Glucose oxidase, Amyloglucosidase</td>
<td>Sara Lee Household &amp; Bodycare, Exton, USA</td>
</tr>
<tr>
<td>Crest Pro Health® toothpaste</td>
<td>Stannous Fluoride, Sodium Hexametaphosphate, SLS</td>
<td>Procter &amp; Gamble, Cincinnati, USA</td>
</tr>
<tr>
<td>Oral B Pro Expert®</td>
<td>Sodium Fluoride, Stannous Fluoride Sodium Hexametaphosphate, SLS</td>
<td>Procter &amp; Gamble UK, Weybridge, UK</td>
</tr>
</tbody>
</table>
Chapter 2

**Acute and substantive action of antimicrobial toothpastes and mouthrinses**

Acute action on microorganisms in 4 h old, initial oral biofilms by mouthrinses and toothpaste slurries was evaluated in sterile 96-wells microtiter plate (Greiner Bio-one, Cellstar, F-bottom, Alphen a/d Rijn, The Netherlands). First, a 4 h old biofilm of initially adhering micro-organisms was grown. To this end, wells were filled with 175 µl freshly collected human saliva and incubated for 4 h at 37°C, with shaking at 150 rpm (Incubator Shaker, Innova 4000, New Brunswick Scientific, Edison, NJ, USA). After 4 h, the liquid was aspirated from each well and the wells were rinsed 3 times with 200 µl sterile water. Subsequently, the biofilms were exposed to 175 µl mouthrinse or toothpaste slurry for 30 s or 2 min, respectively, after which the wells were washed 3 times with 200 µl sterile water. Control biofilms were exposed to sterile water. After these exposures, 175 µl sterile tryptic soya broth (TSB) was added to each well to allow continued growth of the exposed biofilms for 16 h, at 37°C and shaking at 150 rpm.

Substantive action by absorbed antimicrobial mouthrinse and toothpaste ingredients was evaluated in sterile 96-wells microtiter plate from biofilm growth on dead biofilms after absorption of antimicrobials. Evaluations were done after oral antimicrobial absorption in initial, 4 h old and thicker, 24 h old matured biofilms. First 4 h old biofilms were prepared, as described above, whereas 24 h old biofilms were made by removal of saliva after 4 h incubation, followed by incubation in 175 µl TSB for 20 h at 37°C and shaking at 150 rpm. After 4 h or 24 h, the liquid was aspirated from each well, wells were rinsed 3 times with 200 µl sterile water, and biofilm organisms were killed by exposure to 70% (v/v) ethanol for 3 min. Biofilms were killed in order to study the mechanism of prolonged or substantive action of antimicrobials released from a biofilm, without interference of bacterial growth. Complete killing was verified by attempting to continue growth of the biofilm in sterile TSB (175 µl) during 16 h. Crystal violet staining and measurement of OD₅₇₅ (see below) demonstrated that 70% (v/v) ethanol had
fully impeded further growth. After killing, the dead biofilms were exposed to Corsodyl, Crest Pro Health mouthrinse (both 100% (v/v)) or Crest Pro Health toothpaste-slurry (25% (w/w), as described for acute action to facilitate antimicrobial absorption. After washing, 175 µl freshly collected saliva, was added to each well in order to grow a biofilm on the dead biofilm. Each experiment included six replicate wells and was performed three times with separately collected saliva.

**Evaluation of biofilm growth**

In order to quantitatively evaluate the amount of biofilm growth, wells with biofilm were rinsed three times with 200 µl phosphate buffer (PBS) and 175 µl of a 2.3% (w/v) crystal violet solution (CV, Crystal Violet Solution, Sigma-Aldrich Inc., St Louis, MO, USA) was added to each well. After 30 min at room temperature, excess of CV solution was removed by washing the plates four times with 200 µl ultrapure water. Finally, bound CV was released by adding 225 µl ethanol/acetone (80/20% (v/v)) to each well and the OD signaled was measured using a microtiter plate reader (FLUOstar OPTIMA, BMG labtech, Offenburg, Germany).

Acute action was concluded from a reduction in the amount of biofilm after growth of the exposed biofilms (OD$_{exposed}$) with respect to the control (water treatment; OD$_{control}$), while substantive action was concluded from a reduction in the amount of biofilm grown on a dead biofilm (OD$_{exposed}$) with respect to a water control. Accordingly, for acute and substantive action, the percentage reduction in biofilm growth was concluded using the following equation

\[
%\text{reduction} = \left( \frac{OD_{control} - OD_{exposed}}{OD_{control}} \right) \times 100\%
\]

(1)
All OD values were corrected for possible adsorption of toothpaste slurries, mouthrinses and CV at the walls of the wells, while for substantive action OD values were also corrected for CV absorption in the dead biofilm.

**Statistical analysis**

All reductions in biofilm growth after exposure to (diluted) mouthrinses or toothpaste slurries were compared to the control, i.e. water exposure using the unpaired Students *t*-test, ANOVA and LSD post hoc test. For statistical analysis SPSS 16.0 software for Windows (SPSS Inc., Chicago, IL, USA) was used. P-values less than 0.05 were considered to be statistically significant.

**Results**

**Acute action of antimicrobial toothpastes and mouthrinses**

All undiluted mouthrinses and 25% (w/w) toothpaste slurries reduced the continued growth of an exposed biofilm significantly (p < 0.05), compared to the control which is indicative of acute action by killing or disrupting (see Figures 1 and 2). Corsodyl was effective even at a 1% dilution (see Figure 1), whereas Listerine and Crest Pro Health rinse lost efficacy at a concentration of 1% (v/v). Meridol showed loss of efficacy at a dilution of 50% and even stimulated biofilm growth when used in 10% and 1% (v/v) dilutions (p < 0.05).

Toothpaste slurries of Prodent Coolmint were the only ones yielding significant reductions in continued growth of exposed biofilms over the entire dilution range (see Figure 2), whereas most toothpaste slurries lost efficacy at 0.25% (w/w) dilution. Note that Crest Pro Health paste and Crest Cavity Protection stimulated growth at their highest dilutions (only statistically significant for Crest Cavity Protection at p < 0.05). Zendium Classic already lost efficacy and stimulated growth at 12.5% (w/w) dilution, although growth stimulation was not statistically significant.
Figure 1. Percentage reduction in continued growth of an initially adhering, 4 h old biofilm, after 30 s exposure with different dilutions of mouthrinses as compared to a control, i.e. exposure to sterile water. Note that negative reductions denote increased growth with respect to the control. *A significant (P < 0.05) reduction compared with the control. The SE values were calculated from the results obtained from 18 experiments.
Figure 2. Percentage reduction in continued growth of an initially adhering, 4 h old biofilm, after 2 min exposure with different dilutions of toothpaste slurries as compared to a control, i.e. exposure to sterile water. Note that negative reductions denote increased growth with respect to the control. *A significant (P < 0.05) reduction compared with the control. The SE values were calculated from the results obtained from 18 experiments.
Substantive action through absorption in and release from biofilms

Biofilm growth on dead biofilms after absorption of mouthrinse or toothpaste components was significantly ($p < 0.05$) reduced for all three antimicrobial products, demonstrating absorption and release of bio-available antimicrobials in effective concentrations (see Fig. 3). Growth reduction after absorption of Corsodyl was less than achieved by Crest Pro Health rinse or Crest Pro Health toothpaste slurry, although this effect was only significant for the rinse. The reductions by absorption and release of components in 24 h old dead biofilms were very similar to the 4 h old, initial biofilms.

**Figure 3.** Percentage reduction in biofilm growth on dead, 4 h and 24 h old biofilms after absorption of antimicrobial components from mouthrinses (30 s absorption) or a toothpaste slurry (2 min absorption) compared to a control, i.e. exposure to sterile water. The asterisk (*) indicates a significant difference with respect to the control, while the hash (#) indicates significant difference with Corsodyl. The SE values were calculated from the results obtained from 18 experiments.
Discussion

Conceivably, antimicrobial agents can influence oral biofilm formation in different ways, e.g. by preventing bacterial adhesion to surfaces, affecting bacterial viability or by disrupting an existing biofilm. Here we studied acute action on organisms in oral biofilms and substantive action through absorption and release of antimicrobials in and from dead biofilms. All antimicrobial products included showed acute action when applied full-strength (mouthrinses) or in 25% (w/w) toothpaste slurries. Moreover, antimicrobial absorption in and release of bio-available antimicrobials from dead biofilm in effective concentrations was demonstrated to contribute to substantive action for three selected antimicrobial products, viz. Corsodyl, Crest Pro Health mouthrinse and Crest Pro Health toothpaste.

The use of dilutions series of mouthrinses and toothpaste slurries to assess the acute action efficacy of these oral antimicrobial products is new. Mouthrinses are always used full-strength and clinically only undergo minor dilution in saliva. For toothpastes, 25% (w/w) slurries are the standard for in vitro evaluations, as based on the average amount of toothpaste used during brushing, and the total amount of fluid in the oral cavity. Yet, there are large individual variations and during brushing and further dilution will occur due to salivation and swallowing. Moreover, most people add water to the toothbrush. Therewith the decrease in growth reduction of exposed biofilms upon dilution is indicative for the antimicrobial efficacy of the product in clinical use. Most antimicrobial products evaluated for their acute action remain effective up to substantial dilutions, which is in line with clinical observation on their plaque control efficacy.

In Corsodyl, chlorhexidine is the component responsible for clinical antimicrobial efficacy, while for Listerine, Meridol and Crest Pro Health rinse the responsible components are essential oils, the combination of stannous- and amine-fluoride, and bio-available cetylpyridinium chloride, respectively. In most toothpaste
formulations, fluoride and sodium lauryl sulphate (SLS) contribute to a certain degree of acute action, as found in this study for Prodent Coolmint and Crest Cavity Protection. Toothpastes with antimicrobial claims include Colgate Total, Crest Pro Health and Oral B Pro Expert\cite{12,13} and indeed these toothpaste slurries cause acute action up to substantial dilutions. Note that Oral B Pro Expert differs only from Crest Pro Health with respect to an increased fluoride level (1450 ppm instead of 1100 ppm in Crest Pro Health). The higher fluoride concentration, also present in Prodent Coolmint, is probably the reason why these products still show growth reduction at the highest dilution. Zendium Classic, based on colostrums and enzymes enhancing the host defense system, loses antimicrobial efficacy already at a dilution of 12.5\%. Also in clinical studies\cite{14}, it was shown that the antimicrobial effect of enzymes-containing toothpastes was minor.

In general, metal ions act as essential co-factors assisting enzymes to work as a catalyst for metabolic reactions in cells\cite{15}, but they are toxic to cells and bacteria in high concentrations\cite{16}. In this respect, it is interesting to note that two of the products based on stannous (Meridol mouthrinse and Crest Pro Health toothpaste) show growth stimulation at the highest dilution, i.e. the lowest stannous concentration, as has also been shown by Aranha \textit{et al.}\cite{17}. However, the stannous-containing formulation complemented with a high concentration of NaF (Oral B Pro Expert) does not show growth stimulation, which confirms that fluoride too can exert antimicrobial effects\cite{4}.

In addition to their acute action efficacy, components from the antimicrobial mouthrinses Crest Pro Health and Corsodyl and the toothpaste Crest Pro Health demonstrate absorbance in dead biofilms and remain bio-available for subsequent release and substantive action on biofilm organisms growing on the exposed biofilm. Hitherto, the substantivity of oral antimicrobial products has been evaluated predominantly \textit{in vivo}\cite{6,10} and suggested to be due to adsorption of antimicrobials to the abundantly available soft tissue surfaces in the oral cavity.
from which they are slowly released to yield substantivity\textsuperscript{3,5}. This \textit{in vitro} study confirms our clinical findings that plaque can act as a reservoir for oral antimicrobials\textsuperscript{8} and identifies the absorptive capacity of oral biofilm as a factor, contributing to the prolonged activity of antimicrobial health care products. Substantivity through absorption in oral biofilms can only be effective if the absorbed antimicrobials remain bio-available, i.e. can be released. In this respect it is interesting that substantivity of the chlorhexidine-containing rinse through absorption in and release from oral biofilm is smaller than of the other products, possibly because this large cation had become irreversibly trapped in the biofilm, consisting of negatively charged bacteria\textsuperscript{18}. We could not establish here with adequate statistical significance that thicker, 24 h old biofilms had a greater absorption capacity than 4 h old biofilms.

In conclusion, antimicrobial toothpastes and mouthrinses are able to acutely act on biofilm organisms \textit{in vitro}, even when they are considerably diluted, as occurring in clinical situations. Furthermore, antimicrobials from mouthrinses and toothpaste slurries may remain bio-available in a dead biofilm, resulting in prolonged killing of new biofilm, therewith providing evidence in support of a new mechanism of substantive action. This is of clinical importance, because patients usually are not capable of complete removal of all oral biofilm by tooth cleaning. The new mechanism outlined enables them to benefit from active toothpaste and mouthrinse, absorbed in biofilm left after cleaning.

\textbf{Conflict of interest}

The authors declare that there are no conflicts of interest in this study.
Reference List


