Efficacy and mechanisms of non-antibacterial, chemical plaque control by dentifrices—An in vitro study

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1. Introduction

Dental plaque is not a random film of microbial and macromolecular origin but has been described as an ordered structure, adhering in a spatially organized manner to the pellicle, which is in turn adsorbed to the tooth surface. Dental plaque is not only spatially organized, but also its development in time occurs in an ordered fashion. Primary colonizers...
Actinomyces and various streptococcal strains are recognized among the initial colonizers, and have the important role to connect the entire plaque mass growing on top of them to the tooth surface. To emphasize this important role of the initial colonizers, we have proposed to call the layer of initially adhering organisms the “linking film part of the biofilm”.

Palmer et al. recently demonstrated the role of co-adhesion in the spatiotemporal development and prevalence of mixed-species of streptococci and actinomyces in vivo, in early dental plaque using confocal laser scanning microscopy on enamel chips, affixed with stents in the human oral cavity.

Plaque control is the key to the prevention of most oral diseases, most notably caries and periodontal disease and is also a target for reducing stain and calculus formation. Unfortunately, even the use of powered toothbrushes in combination with a conventional 0.2% sodium fluoride containing dentifrice is not enough to remove all plaque especially not from gingival margins, interproximal spaces and fissures. Consequently, additional means of plaque control are required. Chemotherapeutics added to dentifrices include chemical chelants which may affect pellicle surface physico-chemistry, detergent systems which may disperse or detach plaque and antimicrobial ingredients to decrease pathogenicity of adherent plaque. Fig. 1 summarizes the different working mechanisms for chemical plaque control. As can be seen, both bactericidal and non-bactericidal (e.g. cleansing or anti-adhesion) mechanisms may contribute individually or in combination in assisting the control of dental plaque formation and retention. Oral hygiene measures are, in a first instance, aimed at plaque removal but a clear secondary aim is to prevent or delay de novo adhesion of organisms stimulating maturation of the plaque or to decrease the pathogenicity of residual plaque.

With respect to cleansing actions, studies have been conducted evaluating mouthrinses pre-brushing rinses and individual oral detergents for their ability to stimulate detachment of plaque organisms from surfaces, but very few subsequently look at de novo adhesion which is important to provide sustained antiplaque actions by these mechanisms. In an in vitro adhesion assay involving single strain experiments, it was demonstrated that formulations with hexametaphosphate had good cleaning capacities for detachment, while transiently yielding more hydrophilic tooth surfaces in vivo than other commercial dentifrice formulations.

Recently, we have introduced pairs of co-adhering oral bacteria as a more relevant in vitro model for initial plaque formation to surfaces than single strain models. In a study on mechanistically stimulated bacterial detachment from pellicle surfaces, co-adhering actinomyces and streptococci were stimulated to detach by various modes of brushing in the absence of dentifrice components. The different modes of brushing not only removed the (co-)adhering organisms in different numbers, but also de novo adhesion of streptococci depended on the mode of brushing. Sonic brushing removed nearly all (co-)adhering streptococci and actinomyces from pellicle surfaces, but re-deposition of a co-adhering streptococcal strain was more extensive than of a non-co-adhering streptococcal strain. This was taken as an indication that not only actinomyces left adhering on the pellicle surface can act as preferential sites for re-deposition of co-adhering streptococcal strains, but moreover suggests that fimbrial material left on the pellicle surface after brushing may attract co-adhering streptococci. Such important conclusions could obviously not have been drawn from single strain experiments.

Fig. 1 – Schematic presentation of the different mechanisms of action of active ingredients in dentifrice formulations for chemical plaque control (adapted from Busscher et al.).
The purpose of this research was to examine the in vitro ability of commercial dentifrice supernates with different chemotherapeutic components added, to stimulate detachment of co-adhering actinomycies and streptococci from pellicle surfaces and to discourage de novo streptococcal deposition. Dentifrices chosen for comparison included antibacterial formulations (containing triclosan and stannous fluoride) and tartar control and whitening formulations (containing pyrophosphate and sodium hexametaphosphate), as well as a regular dentifrice containing fluoride and sodium laurel sulphate.

2. Materials and methods

2.1. Bacterial strains, culture conditions and harvesting

A co-adhering pair of initial colonizers of pellicle surfaces in the oral cavity has been selected for this study, that has previously been extensively studied with regard to co-aggregation, and in oral cleansing models. Streptococcus gordonii J22 was cultured in Todd Hewitt Broth (OXOID, Basingstoke, Great Britain) at 37 °C in ambient air. Actinomyces naeslundii T14V-J1 was cultured in Schaedler’s broth supplemented with 0.01 g/l hemin in an anaerobic cabinet (Concept 400, Ruskinn Technology Ltd., West Yorkshire, UK) in an atmosphere of 10% H2, 85% N2 and 5% CO2 at 37 °C. Strains were pre-cultured on blood agar plates from which single colonies were taken as an inoculum for an overnight batch culture. This culture was used to inoculate a second culture which was grown for 16 h, harvested by centrifugation for 5 min at 6500 × g and washed twice with adhesion buffer (2 mmol/l potassium phosphate, 50 mmol/l potassium chloride and 1 mmol/l calcium chloride at pH 6.8). To break up bacterial aggregates, bacteria were sonicated intermittently while cooling in an ice/water bath for 35 s at 30 W (Vibra Cell model 375; Sonics and Materials, Danbury, CT, USA). These procedures were found not to cause cell lysis in any strain nor did the supernatant of pelleted sonicated cells cause co-aggregation of the partner cells. Actinomyces were suspended in adhesion buffer to a concentration of 1 × 10^8 bacteria/ml. Streptococci were suspended in adhesion buffer (2 mmol/l potassium phosphate, 50 mmol/l potassium phosphate and 1 mmol/l calcium chloride) and washed twice with adhesion buffer supplemented with 1.5 mg/ml lyophilized human whole saliva to a concentration of 3 × 10^8 bacteria/ml.

2.2. Saliva collection and preparation

Human whole saliva from 20 healthy volunteers of both sexes was collected into ice-cooled beakers after stimulation by chewing Parafilm and pooled, centrifuged, dialyzed and lyophilized for storage. For experiments, lyophilized saliva was dissolved at a concentration of 1.5 mg/ml in adhesion buffer. All volunteers gave their informed consent to saliva donation, in accordance with the rules set out by the Ethics Committee at the University Medical Center Groningen.

2.3. Dentifrice formulations

The composition of dentificries used are shown in Table 1 and described below:

- Crest Regular Dentifrice (CR): A standard 0.243% sodium fluoride dentifrice containing sodium laurel sulphate. This dentifrice does not contain added tartar control ingredients or antimicrobial ingredients.
- Crest Tartar Control Dentifrice (CTC): This dentifrice also contains 0.243% sodium fluoride and sodium laurel sulphate. The dentifrice contains a high concentration (about 50,000 ppm neat) of soluble pyrophosphate derived from various sodium salts as an antitartar control. This dentifrice does not contain added antimicrobial ingredients.
- Crest Gum Care Dentifrice (CGC): This dentifrice contains 0.454% stannous fluoride and sodium laurel sulphate. The stannous fluoride provides both caries and antimicrobial effects and the formulation is aimed to prevent gingivitis.
- Colgate Total Dentifrice (CoT): This dentifrice also contains 0.243% sodium fluoride and sodium laurel sulphate, next to polyvinyl methylfhe maleic acid (PVM/MA, “Gantrez”) as an antitartar, antimicrobial delivery and retention ingredient. This dentifrice contains triclosan antimicrobial for the provision of antigingivitis benefits.
- Crest Dual Action Whitening Dentifrice (CDAW): This dentifrice also contains 0.243% sodium fluoride and sodium laurel sulphate, but also a high concentration of sodium hexametaphosphate (>50,000 ppm as polymeric hexametaphosphate) as an antitartar and whitening ingredient. This dentifrice does not contain added antimicrobial ingredients.

2.4. Parallel plate flow chamber, image analysis and bacterial deposition protocol

Bacterial adhesion was observed on the bottom glass plate of a parallel plate flow chamber (17.5 cm × 1.7 cm) with a channel height of 0.075 cm. The glass was cleaned by sonication in a 2% surfactant RBS 35 (Fluka Chemie, Buchs, Switzerland),

| Table 1 – Dentifrices and their main detergents and other antimicrobial ingredients, as included in this study |
|---------------------------------------------------------------|------------------------------------------|------------------------------------------|
| Dentifrice          | Ingredients                  | Manufacturer                |
| Crest® Regular (CR) | NaF                        | Procter & Gamble, Cincinnati, OH, USA |
| Crest® Tartar Control (CTC) | NaF, pyrophosphate          | Procter & Gamble, Cincinnati, OH, USA |
| Crest® Gum Care (CGC) | SnF2                      | Procter & Gamble, Cincinnati, OH, USA |
| Colgate® Total (CoT) | Polyvinyl methylfhe maleic acid, triclosan, NaF | Colgate-Palmolive Co., New York, NY, USA |
| Crest® Dual Action Whitening (CDAW) | Hexametaphosphate, NaF | Procter & Gamble, Cincinnati, OH, USA |

Note that all formulations contain sodium laurel sulphate (SLS).
followed by alternately rinsing with, tap water, methanol and
demineralised water. The glass plate was coated with saliva
for 16 h at room temperature in order to create a salivary
pellicle. Observation were carried out with CCD-MXR camera
(High Technology, Eindhoven, The Netherlands) mounted on a
phase contrast microscope (Olympus BH-2) equipped with a
40× ultra long working distance objective (Olympus ULWD-CD
Plan 40 PL). The camera was connected to an image analyzer
(TEA, Difa, Breda, The Netherlands). Live images were stored
on disk for enumeration.

Before each experiment, all tubes and the flow chamber
were filled with buffer, and 10 min perfusion with buffer was
applied to remove remnants of saliva from the flow chamber.
Subsequently, the actinomyces suspension was flowed first
through the system until a surface coverage of $1 \times 10^6$ bac-
teria/cm$^2$ was reached as enumerated by the image
analysis system. Thereafter, flow was switched again to
buffer to remove unattached bacteria from the flow chamber
and the tubes for 15 min. (This step was separately shown not
to remove any attached actinomyces.) Co-adhesion was
initiated by switching the flow to the streptococcal suspension
in saliva for 2 h. Solutions were circulated through the
system by means of hydrostatic pressure at a wall shear rate
of $10 \text{s}^{-1}$ which corresponds to physiological conditions of low
shear$^{16}$ and yields a laminar flow (Reynolds number 0.6).
Hereafter, flow was again switched to buffer to remove
unattached bacteria from the flow chamber and tubes
for 15 min.

Prior to perfusing the flow chamber with dentifrice super-
nates, five images were taken from different areas on the
substratum surface after which the flow chamber was
perfused with 4.6 ml of a 25 wt% slurry of dentifrice super-
nates in water. Supernates of the dentifrices listed inTable 1
together with their main active components were obtained by
centrifugation (10,000 $\times$ g) for 5 min at 10 $^\circ$C. In all detachment
experiments, only dentifrice supernates were used in order to
study exclusively the chemical action of the dentifrices, while
furthermore deposition of abrasion particles will interfere
with the image analysis.

Again, five images from different areas on the pellicles
surfaces were taken, the flow chamber was flushed with
120 ml of adhesion buffer to remove remnants of detergents or
antimicrobials and filled again with a fresh streptococcal
suspension in saliva and re-deposition was initiated for
another 2 h.

![Fig. 2 – The percentage distribution of aggregate sizes on
salivary pellicle surfaces prior to exposure to dentifrice
supernates, involving Actinomyces naeslundii T14V-J1 and
Streptococcus oralis J22.](image)

Analysis of the images included determination of the
number of adhering organisms per cm$^2$ and the percentage
distribution of the organisms in aggregates of different size.
All data presented represent averages of triplicate runs with
separately cultured bacteria.

3. Results

Prior to exposure to dentifrice supernates, on average
$3.5 \pm 0.6 \times 10^6$ bacteria were adhering per cm$^2$ pellicle surface
(mean ± S.D. of 15 different experiments with separately
cultured bacteria), of which $1 \times 10^6$ cm$^2$ were actinomyces.
Streptococci adhered to the pellicle as single organisms, but
also co-adhered with already adhering actinomyces. Quantita-
tive analysis of the aggregate size distribution prior to
exposure to dentifrice supernates is given in Fig. 2 and shows
that 25% of the adhering S. oralis J22 and A. naeslundii T14V-J1
adhere in the form of single cells. About 42% of them form
large aggregates on the pellicle surface, containing 10 or more
bacteria.

The percentage detachment as well as the absolute number
of organisms remaining, immediately after exposure to
dentifrice supernates (i.e. before application of the rinsing
buffer) are summarized in Table 2. The percentage removal
varies per dentifrice and detachment is almost absent for CGC.
Also for CDAW detachment is initially low (30%), but for this

<table>
<thead>
<tr>
<th>Dentifrice</th>
<th>Before dentifrice supernate ($\times 10^6$ cm$^{-2}$)</th>
<th>After dentifrice supernate ($\times 10^6$ cm$^{-2}$)</th>
<th>% detachment</th>
<th>After de novo deposition ($\times 10^6$ cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR</td>
<td>2.7 $\pm$ 0.3</td>
<td>1.6 $\pm$ 0.2 a</td>
<td>43 $\pm$ 4 a</td>
<td>2.0 $\pm$ 0.3 a</td>
</tr>
<tr>
<td>CTC</td>
<td>3.7 $\pm$ 0.3</td>
<td>1.9 $\pm$ 0.3 a</td>
<td>49 $\pm$ 10 a</td>
<td>2.6 $\pm$ 0.9 a</td>
</tr>
<tr>
<td>CGC</td>
<td>3.6 $\pm$ 0.3</td>
<td>3.6 $\pm$ 0.4 b,c</td>
<td>2 $\pm$ 1 b</td>
<td>3.9 $\pm$ 0.1 b</td>
</tr>
<tr>
<td>CoT</td>
<td>3.4 $\pm$ 0.6</td>
<td>1.7 $\pm$ 0.1 a</td>
<td>47 $\pm$ 6 a</td>
<td>Could not be determined</td>
</tr>
<tr>
<td>CDAW</td>
<td>4.0 $\pm$ 0.8</td>
<td>2.9 $\pm$ 0.9 c</td>
<td>30 $\pm$ 10 c</td>
<td>1.1 $\pm$ 0.5 c</td>
</tr>
</tbody>
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$a \neq b \neq c$ at $p < 0.001$ (paired Student’s t-test).
dentifrice detachment continued during re-deposition, as can be seen from the numbers in Table 2 as well. For the other three dentifrices based on NaF and sodium lauryl sulphate (SLS), detachment is not significantly different and amounts between 43 and 49%.

Fig. 3A and B presents images of (co-)adhering organisms after exposure to dentifrice supernates (Fig. 3A: CoT and Fig. 3B: CDAW) and followed by rinsing with buffer. Whereas the supernate containing hexametaphosphate in addition to SLS (Fig. 3B), leaves predominantly single adhering organisms on the pellicle, the triclosan/Gantrez containing supernate (Fig. 3A), which also possesses SLS, causes massive re-deposition of large aggregates, despite the fact neither the supernate nor the rinsing buffer contained any suspended organisms. These large aggregates likely originate from bacteria detached from other parts of the flow chamber forming aggregates in suspension and depositing on the pellicle surface. As can be seen by comparison of Fig. 3B and C, detachment after exposure to hexametaphosphate in CDAW continues despite the fact that in this phase of the experiment the flow chamber was perfused with streptococci again.

After re-deposition, adhering streptococci have a tendency to form aggregates again with remaining actinomyces. The percentage of organisms involved in large aggregates, comprising more than 10 organisms, is given in Fig. 4 for the different dentifrices. The re-deposition of streptococci after rinsing with CoT could not be determined due to the presence of massive aggregates prior to re-deposition (see above). (Note, that in the absence of significant detachment caused by CGC, we do not consider the data in Table 2 and Fig. 4 as being representative for de novo deposition, but rather for the stationary end-phase as already achieved in the initial deposition phase of the experiment.) Of the remaining formulations, the tartar control dentifrice CTC yielded the largest re-deposition of streptococci (Table 2), in combination with a high percentage of bacteria adhering in large aggregates. An opposite behaviour was observed for CDAW, for which a continued detachment was observed after perfusing the flow chamber with a dentifrice supernate, even during subsequent flow with a streptococcal suspension (see also Table 2). In addition, CDAW yielded the smallest percentage of bacteria which adhered in large aggregates.
Consequently, instead of de novo adhesion, we observed continued detachment combined with a continuous decrease in the number of organisms adhering in large aggregates (see also Fig. 3C).

4. Discussion

In this study, we investigated the detachment and re-deposition of a pair of co-adhering initial colonizers of dental hard surfaces in the oral cavity and effects of treatment with supernates of commercial dentifrice formulations. Effects observed will be discussed in relation with the active ingredients included in a specific dentifrice. This approach is valid, because in dentifrice manufacturing the base components remain largely the same, as otherwise manufacturing would become impossible and too costly. The use of two strains in studying bacterial detachment by dentifrices in vitro is new and should be more realistic for the clinical situation. Differences in chemical plaque control through different components of dentifrices were not only revealed by their differential capacity to detach adhering organisms from a pellicle surface, but moreover by different chemical effects on the pellicle surface and the surfaces of remaining bacteria. This caused large bacterial aggregates in de novo deposition of streptococci after some dentifrices. In de novo deposition after CR and CDAW (NaF and hexametaphosphate containing dentifrice, respectively) only small percentages of organisms adhered in large aggregates. Streptococci are abundantly available in saliva and it is interesting to observe that, immediately after chemically stimulated detachment, flowing with a streptococcal suspension may cause the formation of co-adhering aggregates on the pellicle surfaces. It has been suggested3 and recently clinically confirmed 5 that these aggregates constitute micro-environments for certain organisms, such as anaerobes, to grow under optimal conditions as the on-set of de novo plaque.

Previously, we11 compared detachment of S. oralis J22 and A. naeslundii T14V-J1 by dentifrice supernates in single strain experiments. As in the present co-adhesion detachment model, CGC containing SnF2 and SLS did not stimulate significant bacterial detachment, which was attributed to charge neutralization by Sn2+ of pellicle and bacterial cell surface components impeding detachment. Alternatively, it is of great interest to note that the results of this study show CR, CTC and CoT are all more effective in detaching co-adhering pairs than in stimulating detachment of the individually adhering strains making up this pair. Conversely, addition of hexametaphosphate to a NaF and SLS containing dentifrice as in CDAW, yielded reduced detachment (30%) of co-adhering streptococci and actinomyces as compared with the 70% detachment on S. oralis J22 in a single strain experiment. However, detachment of the co-adhering pair was more extensive than of actinomyces in a single strain experiment (10% detachment observed). Co-adhesion therewith is a definitive factor in chemical plaque control, both with respect to detachment as well as with respect to de novo deposition of bacteria.

Rose et al.17 have proposed a model for the uptake and subsequent release of calcium and fluoride in plaque. Fluoride was suggested to break calcium binding between co-adhering pairs and subsequently adsorb under optimal pH conditions (5.0–5.5), to calcium, bound to bacterial cell surfaces. Calcium binding sites would then be blocked for de novo co-adhesion. In an earlier study, involving detachment of co-adhering bacterial pairs from pellicle surfaces by different modes of brushing, we could not confirm that fluoride performed such a role in breaking Ca-bonds between actinomyces and streptococci during brushing in the presence of NaF (see Fig. 5). This study demonstrates, however, that the dentifrices containing NaF and SLS (CR, CTC, CoT and CDAW), all stimulate bacterial detachment from pellicle surfaces. However, immediately after detachment by CoT large aggregates of detached pairs deposited on the pellicile. Although we disagreed with the suggestion by Rose et al.17 that NaF on its own can break bi-dentate calcium bridges between co-adhering bacteria, this study shows that NaF in combination with SLS has that ability. Hypothetically, fluoride breaks bi-dentate calcium-bonds, but leaves other bonds between co-adhering organisms intact, that are readily disrupted by SLS. Furthermore, in line with the suggestions of Rose et al.17 fluoride is likely to block calcium binding sites left on the bacterial cell surfaces (see also Fig. 5), as few large aggregates were found in de novo streptococcal deposition after CR and CDAW (see Fig. 4). Pyrophosphate, as

Fig. 5 – Proposed scheme for the interference of NaF in combination with SLS on bi-dentate calcium bridges in the co-adhesion between actinomyces and streptococci, including the role of pyrophosphate.
in CTC, evidently intervenes in the fluoride blocking of calcium binding sites, possibly because pyrophosphate can serve as a bi-dentate bridge between calcium bound on the bacterial cell surfaces. As a result, pyrophosphate enables de novo co-adhesion and the development of large aggregates.

In clinical studies, the dentifrices studied here produce pronounced and varied actions. For example, the stannous fluoride dentifrice is known to be effective in the control of caries and gingivitis.21–23 Similarly, CoT, the triclosan/“Gan-trez” dentifrice is proven effective for inhibition of gingivitis and dental calculus formation.21–23 Both CTC (pyrophosphate) and CDAW (hexametaphosphate) dentifrices have no proven antibacterial actions in reducing plaque or gingivitis and yet both are highly effective in the control of dental calculus formation and in the control of extrinsic stain on teeth.24–29

The clinical effects of ‘hygiene’ and topically applied dentifrices and mouthrinses are complex. Nevertheless, the cost and complexity of clinical studies recommend the development of laboratory methods effective in predicting clinical actions and assisting in the design of therapeutic improvements. It is likely that cleansing, conditioning and antibacterial mechanisms of topically applied formulations all play an integrated role in influencing the quantity, tenacity and virulence of developed intraoral biofilms. The study of effects on adhesion, co-adhesion and detachment mechanisms may provide useful insights in the development of more effective and better tolerated chemotherapeutic dentifrices to maintain oral health.

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References


