Chapter 6

Synergy of brushing mode and antibacterial use on \textit{in vivo} biofilm formation


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ABSTRACT

Objectives: Orthodontic, multi-strand retention-wires are used as a generalized model for oral retention sites to investigate whether biofilm left-behind after powered toothbrushing in-vivo enabled better penetration of antibacterials as compared with manual brushing.

Methods: 2-cm multi-strand, stainless-steel retention-wires were placed in brackets bonded bilaterally in the upper arches of 10-volunteers. Volunteers used NaF-sodium-lauryl-sulphate-containing toothpaste and antibacterial, triclosan-containing toothpaste supplemented or not with an essential-oils containing mouthrinse. Opposite sides of the dentition including the retention-wires, were brushed manually or with a powered toothbrush. Health-care-regimens were maintained for 1-week, after which wires were removed and oral biofilm was collected.

Results: When powered toothbrushing was applied, slightly less bacteria were collected than after manual brushing, regardless whether an antibacterial-regimen was used or not. Powered-toothbrushing combined with antibacterial-regimens yielded lower biofilm viability than manual brushing, indicating better antibacterial penetration into biofilm left-behind after powered brushing. Major shifts in biofilm composition, with a decrease in prevalence of both cariogenic species and periodontopathogens, were induced after powered brushing using an antibacterial-regimen.

Conclusion: Oral biofilm left-behind after powered brushing in-vivo enabled better penetration of antibacterials than after manual brushing.

Clinical significance: Mechanical removal of oral biofilm is important for prevention of dental pathologies, but biofilm is always left-behind, such as in fissures, buccal pits, interproximal areas and gingival margins and around orthodontic appliances. Use of antibacterial toothpastes or mouthrinses can contribute to removal or killing of biofilm bacteria, but biofilm structure hampers antibacterial penetration. A synergy between brushing mode and antibacterial-regimen applied exists with clinically demonstrable effects.
INTRODUCTION

Amount, viability and composition of oral biofilm play a major role in the development of oral pathologies, such as caries, gingivitis and periodontitis. Prevention of biofilm-related oral pathologies can be achieved either by mechanical or chemical removal of biofilm, changing its composition or preventing its formation. Mechanical biofilm removal by powered toothbrushing has been demonstrated to be superior to manual brushing. However, complete biofilm removal can never be achieved and after a single self-performed brushing, the amount of oral biofilm can only be reduced by 50-60%, leaving biofilm behind at locations out of reach for mechanical removal such as fissures, buccal pits, posterior interproximal areas and gingival margins, where oral pathologies mostly develop. In orthodontic patients, the number of locations out of reach of mechanical removal is even higher, making orthodontic patients more prone to oral pathologies than non-orthodontic patients.

The use of antibacterial containing toothpastes or mouthrinses can be a valuable addendum to mechanical biofilm control in order to reduce the viability of biofilm left-behind after brushing. However, the general structure and composition of oral biofilm hampers penetration of oral antibacterials through the depth of an entire biofilm. Oral biofilm consists of a large variety of adhering bacteria embedded in an extracellular-polymeric-matrix that acts both as a glue for bacteria as well as a barrier against penetration of antibacterials. Powered toothbrushing of in vitro oral biofilm has been demonstrated to impact the structure of biofilm left-behind to create a more open structure, more amenable to antibacterial penetration, especially when the bristles of the brush have not been able to touch the biofilm and remove it. This more open structure is caused by a high energy transfer from a powered toothbrush into the biofilm through strong fluid flows, air bubble inclusion and acoustic waves. Accordingly it has been demonstrated in vitro that due to this more ‘fluffed-up’, open biofilm structure chlorhexidine and cetylpyridinium-chloride penetrate and kill bacteria to a greater depth into biofilm left-behind after powered brushing. Also, once oral antibacterials have penetrated the biofilm, the biofilm left-behind might act as a reservoir for the oral antibacterial agent ensuring a prolonged action of the agent. The impact of these in vitro findings for the clinical situation has never been demonstrated and could only be speculated upon, however.

In order to determine whether the improved penetration of antibacterial agents into biofilm left-behind after powered brushing as observed in vitro, also yields clinical benefits, we here aim to compare biofilm formation and composition in vivo on orthodontic, multi-strand retention wires after manual versus powered toothbrushing using a control, NaF- sodium lauryl sulphate containing toothpaste or an antibacterial, triclosan-containing toothpaste supplemented or not with the use of an essential-oils containing mouthrinse. Orthodontic,
multi-strand retention wires are known to be difficult to clean\textsuperscript{16,17} and were employed as a generalized model for oral retention sites. Different regimens of oral health care were maintained for 1-week in a group of volunteers, equipped with multi-strand, stainless steel retention wires, after which oral biofilm left-behind after different modes of brushing was evaluated.

**MATERIALS & METHODS**

*Retention wires, volunteers, inclusion criteria and oral hygiene regimens*

In this study, biofilm growth was evaluated on multi-strand, stainless steel retention wires (Quadcat\textsuperscript{®}, PG Supply, Inc., Avon, USA), serving as a model for oral sites that are difficult to reach with a toothbrush. In addition, retention wires are easily removable for evaluation of biofilm formed. Brackets (SPEED System Orthodontics, Cambridge, Canada) were bonded to the buccal side of the first molar and the second premolar bilaterally in the upper arch of 10 healthy volunteers (5 male, 5 female) in agreement with the rules set out by the Ethics Committee at the University Medical Centre Groningen (letter June 23\textsuperscript{rd}, 2011). Volunteers were included in the study, provided that they had a healthy and complete dentition, no bleeding upon probing and did not use any medication. All volunteers granted a written informed consent. Wires with a length of 2 cm were placed between the brackets. The wires were sterilized in 70\% ethanol before use and stayed in situ for one week during which the volunteers were instructed to brush twice a day for 2 min with a manual toothbrush (Lactona iQ X-Soft, Lactona Europe B.V., Bergen op Zoom, The Netherlands) on one side of the dentition or with a powered toothbrush (Sonicare DiamondClean\textsuperscript{®}, Philips Nederland B.V., Eindhoven, The Netherlands) on the other side. Volunteers were furthermore instructed to use a NaF-sodium lauryl sulphate (NaF-SLS) containing toothpaste without antibacterial claims (Prodent Softmint\textsuperscript{®}, Sara Lee Household & Bodycare, Exton, USA), or a triclosan-containing toothpaste (Colgate Total\textsuperscript{®}, Colgate-Palmolive Company, Piscataway, USA) with antibacterial claims. In addition, the use of the triclosan containing toothpaste was supplemented with the use of an essential-oils containing mouthrinse (Cool Mint Listerine\textsuperscript{®}, Pfizer Consumer Healthcare, Morris Plains, NJ, USA). The order in which the regimens were applied in the different volunteers was determined at random. In between regimens and before the start of the experiment, a washout period of 6 weeks was applied during which only the NaF-SLS containing toothpaste was allowed to be used. The duration of the washout period was based on the results of a pilot study that indicated that the composition of the oral biofilm returned to base line values within 5 weeks after use of an antibacterial toothpaste.

Regimens were maintained for 1 week, after which wires were removed and oral biofilm was collected from the wires and the buccal enamel surfaces surrounding the brackets using a
cotton swab. Wires were removed in the morning after breakfast and regular brushing by the volunteers. Wires and enamel biofilms collected were stored in an Eppendorf tube containing 1.0 mL filter sterile reduced transport fluid (RTF)\textsuperscript{18} for transportation from the orthodontic clinic to the laboratory.

Upon arrival in the laboratory, retention wires with adhering biofilm and biofilm collected from enamel surfaces were separately sonicated three times for 10 s with 30 s intervals in Eppendorf tubes containing 1.0 mL RTF on ice chilled water, to disperse bacteria. Part of the bacterial dispersions were stored at -80°C until use for PCR- Denaturing Gradient Gel Electrophoresis (DGGE), while another part was used to determine bacterial number and viability. For enumeration of the numbers of adhering bacteria, bacteria were enumerated in a Bürker-Türk counting chamber, while the percentage viability of the biofilms was evaluated after live/dead staining (BacLight\textsuperscript{TM}, Invitrogen, Breda, The Netherlands) of the dispersed biofilms. Live/dead stain was prepared by adding 3 μL of SYTO\textsuperscript{9}/propidium iodide (1:3) to 1 mL of sterile, demineralised water. Fifteen μL of the stain was added to 10 μL of the undiluted bacterial dispersion. After 15 min incubation in the dark, the number of live and dead bacteria were counted using a fluorescence microscope (Leica DM4000B, Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) and expressed as a percentage viability. Note that strictly speaking, live/dead staining is not a measure of microbial killing but of membrane damage.\textsuperscript{19,20} The membrane of live bacteria is permeable to SYTO9, staining both live and dead organisms and yielding green fluorescence. Propidium-iodide can only enter through damaged membranes, where it replaces SYTO9, yielding red fluorescence of dead or damaged cells.

**DGGE analysis of in vivo biofilms**

After all dispersed biofilms were collected, PCR-DGGE was carried out in order to compare their bacterial composition, as described previously.\textsuperscript{17} Briefly, for extraction of DNA, frozen bacterial dispersions were thawed, centrifuged for 5 min at 13,000 g, washed and vortexed with 200 μL TE-buffer (10 mM Tris-HCl, 1 mM EDTA pH 7.4) and again centrifuged. After DNA extraction, PCR was performed on 100 ng DNA with a T-gradient thermocycler for PCR amplifications. PCR products were analyzed by electrophoresis on a 2.0% agarose gel containing 0.5 μg/mL ethidium bromide. DGGE of PCR products generated with the F357-GC/R-518 primer set was performed, as described by Muyzer et al.\textsuperscript{21} The PCR products were applied on 0.08 g/mL polyacrylamide gel in 0.5 X TAE buffer (20 mM Tris acetate, 10 mM sodium acetate, 0.5 mM EDTA, pH 8.3). The denaturing gradient consisted of 30 to 80% denaturant (100% denaturant equals 7 M urea and 37% formamide). A 10 mL stacking gel without denaturant was added on top. Electrophoresis was performed overnight at 120 V and 60°C. Gels were stained with silver nitrate.\textsuperscript{22} Each DGGE gel was normalized according
to a marker consisting of 7 reference species comprising common bacterial species associated with oral health and disease. The reference strains included Streptococcus oralis ATCC 35037, Streptococcus mitis ATCC 9811, Streptococcus sanguinis ATCC 10556, Streptococcus salivarius HB, Actinomyces naeslundii ATCC 51655, Lactobacillus sp., Streptococcus sobrinus ATCC 33478, Streptococcus mutans ATCC 10449, Porphyromonas gingivalis ATCC 33277 and Prevotella intermedia ATCC 49046.

Statistical analysis
Data were analyzed with the Statistical Package for Social Sciences (Version 16.0, SPSS Inc., Chicago, IL, USA). A one-way analysis of variance (ANOVA) was used to compare the number of bacteria and their percentage viability. A Bonferroni test was used for post-hoc multiple comparisons. Statistical significance was set at \( p < 0.05 \).

DGGE gel images were converted and transferred into a microbial database with GelCompar II, version 6.1 (Applied Maths N.V, Sint-Martens-Latem, Belgium). Similarities in bacterial composition of the different biofilms were analysed using a band based similarity coefficient and a non-weighted pair group method with arithmetic averages was used to generate dendrograms indicating similarities in composition.

RESULTS
When powered toothbrushing was applied, slightly less bacteria were collected from retention wires than after manual brushing, while enamel surfaces harvested insufficient amounts of biofilm for enumeration, providing a validation for the use of orthodontic, multi-strand retention wires as a model for oral retention sites. Within the regimens involving manual brushing, there were no significant differences (\( p < 0.05 \)) in the numbers of bacteria collected from retention wires after use of a NaF-SLS-containing toothpaste and the use of an antibacterial, triclosan-containing toothpaste, regardless of supplementation with an essential-oils containing mouthrinse (Table 1). When powered toothbrushing was applied however, significantly less bacteria (\( p < 0.01 \)) were collected when using the antibacterial, triclosan-containing toothpaste whether or not supplemented with an essential-oils containing mouthrinse, than when using the NaF-SLS-toothpaste.

More strikingly, viability of retention wire biofilm was significantly lower (\( p < 0.001 \)) after the use of the antibacterial, triclosan-containing toothpaste whether or not combined with an essential-oils containing mouthrinse, when compared to the use of a NaF-SLS-containing toothpaste regardless of the brushing method. Moreover, in case of an antibacterial regimen, biofilm viability was lower after brushing with a powered toothbrush than after manual brushing.
Table 1. Number and viability of bacteria retrieved from 1 cm stainless steel retainer wires after manual or powered toothbrushing with a NaF-SLS and an antibacterial, triclosan-containing toothpaste supplemented or not with the use of an essential-oils containing mouthrinse. All data represent averages ± standard deviations over 10 different volunteers.

<table>
<thead>
<tr>
<th>Oral health care regimen</th>
<th>Number of bacteria (Log-units)</th>
<th>% live bacteria</th>
<th>Powered brushing</th>
<th>Powered brushing</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaF-SLS toothpaste</td>
<td>7.9 ± 0.1</td>
<td>68 ± 12</td>
<td>60 ± 7</td>
<td></td>
</tr>
<tr>
<td>without antibacterial</td>
<td></td>
<td>a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>claims</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triclosan containing</td>
<td>7.6 ± 0.2</td>
<td>42 ± 8</td>
<td>28 ± 9</td>
<td></td>
</tr>
<tr>
<td>toothpaste</td>
<td></td>
<td>a</td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>Triclosan containing</td>
<td>7.5 ± 0.2</td>
<td>37 ± 5</td>
<td>16 ± 4</td>
<td></td>
</tr>
<tr>
<td>toothpaste + mouthrinse</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* a different from other regimens with the same brushing mode
  b different from the other brushing mode within the same regimens

Bacterial composition of biofilms formed on retention wires and enamel under the influence of the different oral hygiene regimens and brushing modes are compared in cluster trees (Figures 1A and 1B). Mode of brushing has no influence on the clustering of bacterial composition data, neither on retention wires (Figure 1A) nor on enamel surfaces (Figure 1B). However, the antibacterial regimens clearly separate from the NaF-SLS regimen although this is more clear on the retention wires than on enamel surfaces.

These changes in bacterial composition can further be exemplified from the prevalence of the marker strains applied (see Table 2), although it is difficult to find consistent patterns in effects of manual versus powered brushing. However, powered brushing yields a consistent decrease in the prevalence of *P. gingivalis*, both for biofilm collected from retention wires and enamel. Also the prevalence of *S. sanguinis* is consistently lower in case of powered brushing, but this is only the case for biofilm collected from retention wires. On the other hand, the prevalence of *S. oralis/S. mitis* increases after the use of a powered toothbrush compared to a manual toothbrush. In general, stronger effects of antibacterial regimens on the prevalence of marker stains are seen on retention wires than on enamel surfaces. *S. salivarius, Lactobacillus, S. mutans* and *P. gingivalis* decrease in prevalence on retention wires after use of the antibacterial, triclosan-containing toothpaste and these decreases become more pronounced when use of the antibacterial toothpaste is supplemented with an essential-oils containing mouthrinse. Prevalence of *S. oralis/S. mitis* on retention wires increases after the use of an antibacterial regimen.
Figure 1. Clustering trees describing the bacterial compositions of biofilm samples taken from stainless steel retention wires (A) or enamel surfaces (B) in different volunteers using manual or powered toothbrushing in combination with different healthcare regimens.
Table 2. Prevalence of marker strains in biofilm samples from stainless steel retention wires or buccal enamel surfaces in different volunteers using manual or powered toothbrushing in combination with different healthcare regimens. 100% indicates that biofilm samples from wires or enamel surfaces in all volunteers contained the indicated marker strain.

<table>
<thead>
<tr>
<th>STRAINS</th>
<th>NaF-SLS toothpaste without antibacterial claims</th>
<th>Triclosan containing toothpaste</th>
<th>Triclosan containing toothpaste + mouthrinse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>manual brushing without powered brushing</td>
<td>manual brushing</td>
<td>powered brushing</td>
</tr>
<tr>
<td></td>
<td>wire enamel</td>
<td>wire enamel</td>
<td>wire enamel</td>
</tr>
<tr>
<td>S. oralis / S. mitis</td>
<td>20 70 40 50</td>
<td>20 50 50 80</td>
<td>80 40 70 60</td>
</tr>
<tr>
<td>S. sanguinis</td>
<td>80 80 20 70</td>
<td>40 60 30 70</td>
<td>60 30 40 50</td>
</tr>
<tr>
<td>S. salivarius</td>
<td>30 20 30 10</td>
<td>10 30 10 20</td>
<td>0 10 10 10</td>
</tr>
<tr>
<td>A. naeslundii</td>
<td>0 15 0 0</td>
<td>0 10 0 0</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>20 20 20 20</td>
<td>10 30 10 10</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>S. sobrinus</td>
<td>30 10 30 30</td>
<td>30 70 30 30</td>
<td>20 40 10 10</td>
</tr>
<tr>
<td>S. mutans</td>
<td>30 10 50 0</td>
<td>10 20 10 0</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>30 10 10 0</td>
<td>20 10 10 0</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>P. intermedia</td>
<td>0 0 0 0</td>
<td>10 10 0 0</td>
<td>0 0 0 0</td>
</tr>
</tbody>
</table>
DISCUSSION

Stress-relaxation analysis of mechanically compressed biofilms has pointed out that the structure and water content of *in vitro* biofilm-left behind after powered brushing changes into a direction that makes it more amenable to penetration of chlorhexidine and cetylpiridinium chloride than after manual brushing. \(^\text{14}\) Here we demonstrate the clinical impact of these *in vitro* findings. Clinical impact involves a reduction in the viability of *in vivo* formed biofilms left-behind after powered brushing on retention sites upon the use of an antibacterial triclosan-containing toothpaste with or without supplementation with an essential-oils containing mouthrinse. Thus also clinically, a synergy between mode of brushing and antibacterial-regimen applied exists.

We chose to study *in vivo* biofilms as formed on orthodontic retention wires after different 1-week regimens of oral health care, as especially multi-strand retention wires possess multiple sites where biofilm is sheltered from mechanical and chemical attack. \(^\text{25}\) Therewith retention wires can be considered as a generalized model for biofilm-retention sites in the oral cavity, with as an additional advantage that they are easily replaceable. Biofilm will be more readily left-behind on such retention sites after brushing and in this respect it is telling that in accordance with literature, biofilm could be collected from retention wires both after manual as well as after powered brushing (see Table 1), but hardly from smooth enamel surfaces. Powered toothbrushing generates a larger energy input into a biofilm than manual toothbrushing. \(^\text{26}\) Since biofilms have visco-elastic properties, biofilm will first expand during powered brushing after which it will detach. \(^\text{27-29}\) However, biofilm left-behind will remain in its expanded, more open state enabling better antibacterial penetration, which explains why in the current study we observe a greater reduction in biofilm viability upon application of antibacterial regimens when using a powered brush versus a manual brush. Note that the use of either one of the brushing methods without the use of an oral antibacterial regimen hardly affected the viability of the biofilm compared to an unbrushed biofilm. \(^\text{25}\) This indicates that the decrease in viability is solely attributed to the oral antibacterial agents, and not to toothbrushing itself. \(^\text{30}\) Therewith this is the first time to show the existence of a synergy between mode of toothbrushing and antibacterial action with clinically demonstrable effects.

Also other clinical studies, not geared towards demonstrating a synergy between mode of brushing and antibacterial use, have shown that oral biofilm formation is reduced after the use of antibacterial toothpastes, \(^\text{31,32}\) with minor effects of the supplemental use of an essential-oils containing mouthrinse. \(^\text{33-35}\) However, we saw sizeable further reduction of biofilm viability after supplemental use of an essential-oils containing rinse (Table 1), along with changes in bacterial composition of the biofilm (Figure 1) that we earlier attributed to adsorption of triclosan to bacterial cell surfaces altering their cell surface hydrophobicity to
stimulate removal by hydrophobic ligands.\textsuperscript{17}

DGGE analysis shows that the composition of biofilm formed on stainless steel retention wires differs from biofilm formed on enamel (Table 2). Atomic force microscopy has pointed out that bacterial adhesion forces to different materials used in orthodontics, including stainless steel, differ from the ones exerted by enamel surfaces in a strain-specific fashion.\textsuperscript{36} Accordingly this explains\textsuperscript{37} why biofilms on different materials have a different bacterial composition, including the enamel and stainless steel surfaces as involved here. Furthermore, the biofilm taken from retention wires will be more mature than biofilm taken from smooth enamel surfaces, as more biofilm will be left-behind after brushing on retention wires than on smooth enamel surfaces on which biofilm has to develop newly after each brushing. The composition of a newly formed biofilm as regularly developing on smooth enamel is thus different than that from a mature biofilm as in interproximal areas and fissures,\textsuperscript{38} the latter likely being comparable with biofilm found on the retention wires.

**CONCLUSIONS**

This is the first study to show that a synergy exists between powered toothbrushing and antibacterial regimen with clinically demonstrable effects, most notably on the viability of biofilm left-behind after brushing. Enhancing this synergy may be a goal of further research, either by changing the design of powered toothbrushes or use of different oral antibacterials. Since oral sites where biofilm is most frequently left-behind are also most susceptible to disease, this approach may proof to have major impact on oral health.

**ACKNOWLEDGEMENTS**

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**CONFLICT OF INTERESTS**

The authors declare no potential conflicts of interest with respect to authorship and/or publication of this article. HJB is also director of a consulting company SASA BV, Thesinge, The Netherlands. Opinions and assertions contained herein are those of the authors and are not construed as necessarily representing views of the companies who donated the different wires or their respective employees.
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