Biofilm on orthodontic retention wires
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Chapter 4

*In vivo* biofilm formation on stainless steel bonded-retainers during different regimens of oral health care

Marije A. Jongsma, Henny C. van der Mei, Jelly Atema-Smit, Henk J. Busscher, Yijin Ren.

ABSTRACT

Permanent bonded retention wires to anterior teeth are used after orthodontic treatment to prevent teeth from relapsing to pre-treatment positions. A drawback of bonded retainers is biofilm accumulation along the wires, yielding greater incidence of gingival recession, increased pocket depth and bleeding-on-probing.

This study compares in vivo biofilm formation on single-strand and multi-strand retention wires during different regimens of oral healthcare. Two-cm wires were placed in brackets bonded to the buccal side of first molars and second premolars in the upper arches of 22 volunteers. Volunteers used a selected toothpaste with or without additional use of an essential-oils containing mouthrinse. Brushing was performed manually. Regimens were maintained for one week, after which wires were removed and oral biofilm was collected for enumeration of the number of organisms and their viability, microbial composition and electron microscopic visualization. Six weeks wash-out was applied in between regimens. Less biofilm was formed on single-strand wires than on multi-strand wires, on which bacteria were observed adhering in between strands. Use of antibacterial toothpastes marginally reduced the amount of biofilm on both wire types, but viability of biofilm organisms was significantly reduced by use of antibacterial toothpastes. No significant effects were observed on amount or viability of biofilms upon additional use of the mouthrinse.

However, major shifts in biofilm composition were induced by combining a stannous-fluoride or triclosan containing toothpaste with the essential-oils containing rinse. Tentatively, these shifts are attributed to small changes in bacterial cell surface hydrophobicity after adsorption of toothpaste components, that stimulate bacterial adhesion to hydrophobic oil, as illustrated for a Streptococcus mutans strain.
INTRODUCTION

A major challenge in orthodontics is to retain treatment results after removal of orthodontic appliances. Long-term results of orthodontic treatment show relapse of crowding without use of retention devices. To prevent relapse, permanent retention wires are often bonded to the anterior teeth. Different types of retention wires can be used, including single-strand retainers, bonded only to the canines, or multi-strand retainers that are bonded to all six anterior teeth. The downside of placing retention wires is that biofilm and calculus accumulate along the wires, which may cause a greater incidence of gingival recession, increased pocket depth and bleeding on probing.

Previous in vitro results have shown that wire morphology has an influence on the number of viable organisms in biofilm formed on retention wires. Biofilms pre-formed on single-strand wires harvested less viable organisms than biofilms formed on multi-strand wires after a single exposure to a NaF-sodium lauryl sulphate containing toothpaste slurry and an essential-oils containing mouthrinse, demonstrating that biofilms on multi-strand wires are less susceptible to oral antimicrobials than biofilms formed on single-strand wires. The biofilm mode of growth is indeed known to protect its inhabitants against penetration of antimicrobials agents, an effect that may be enhanced when the biofilm is formed in crevices and niches of a retention wire. It is unknown however, how these differences in the susceptibility of oral biofilms pre-formed on different wire morphologies in vitro, translate to biofilm formation in vivo during the use of antibacterial health care products, such as toothpastes or mouthrinses with antibacterial claims.

In the great majority of the population not all biofilm is removed by mechanical means, and as a consequence, despite the difficulty for antimicrobials to penetrate a biofilm, oral antimicrobials generally have a favourable effect on biofilm inhibition in vivo. Biofilm-left-behind after brushing, either dead or alive, can play an important role in making an antimicrobial action substantive as it can absorb antimicrobials to become released over time in antimicrobially effective amounts. It is unknown however, whether this is a mechanism that is clinically operative to a degree that it yields measurable effects on biofilm formation.

The aim of this study is to compare biofilm formation in vivo on both single-strand and multi-strand retention wires during different regimens of oral health care and to evaluate whether use of oral antimicrobials affects the composition of the biofilm. Regimens included manual brushing. Two different toothpastes with antibacterial claims were used, containing either stannous fluoride or triclosan or a fluoridated toothpaste without antibacterial claims. Toothpastes were employed with or without the additional use of an essential-oils containing mouthrinse.
MATERIALS AND METHODS

Retainers, volunteers and inclusion criteria
Two different types of retainers were evaluated in this study, a single-strand wire (Forestanit®, Forestadent, Pforzheim, Germany) and a multi-strand wire (Quadcat®, PG Supply, Inc., Avon, USA). Brackets (SPEED System Orthodontics, Cambridge, Canada) were bonded to the buccal side of the first molar and the second premolar in the upper arch of 22 healthy volunteers in agreement with the rules set out by the Ethics Committee at the University Medical Centre Groningen (letter June 23rd, 2011). Wires had a length of 2 cm between the brackets in which they were placed. The wires were sterilized in 70% ethanol before use and stayed in situ for one week during which the volunteers were instructed to brush for 2 min twice a day with a manual toothbrush (Lactona iQ X-Soft, Lactona Europe B.V., Bergen op Zoom, The Netherlands) and use a toothpaste with antibacterial claims (Oral-B Pro Expert®, Procter & Gamble, Cincinnati, USA or Colgate Total®, Colgate-Palmolive Company, Piscataway, USA) or a toothpaste without antibacterial claims that contains only NaF-sodium lauryl sulphate (Prodent Softmint®, Sara Lee Household & Bodycare, Exton, USA). Toothpastes were used either without additional oral hygiene measures or in combination with an essential-oils containing mouthrinse (Cool Mint Listerine®, Pfizer Consumer Healthcare, Morris Plains, NJ, USA).

In between regimens, a washout period of 6 weeks was applied during which only the NaF-sodium lauryl sulphate containing toothpaste without antibacterial claims was used. The duration of the washout period was based on the results of a pilot study which indicated that the composition of the oral biofilm returned to base line values within 5 weeks after use of an antibacterial toothpaste.

Volunteers were included in the study, provided that they had a healthy and complete dentition, no bleeding upon probing, did not use any medication and did not smoke. All volunteers granted a written informed consent. After inclusion, volunteers were randomly divided into two groups. The first group successively used 3 different types of toothpaste, the second group combined the same toothpastes with an antimicrobial mouthrinse (see Figure 1).
Regimens were maintained for 1 week, after which wires were removed and oral biofilm was collected from the buccal enamel surfaces for reference using a cotton swab, while also unstimulated salivary samples were taken. The wires, collected enamel biofilms and salivary samples were stored in an Eppendorf tube containing 1.0 mL filter sterile reduced transport fluid. Saliva samples were stored on ice.

For enumeration of the numbers of organisms, retention wires with adhering biofilm, cotton swabs with oral biofilm collected from enamel, both stored in Eppendorf tubes containing 1.0 mL filter sterile reduced transport fluid and saliva samples were separately sonicated three times for 10 s with 30 s intervals on ice chilled water, to disperse bacteria. Bacteria were then enumerated in a Bürker-Türk counting chamber. In addition, the percentage viability of the biofilms was evaluated after live/dead staining (BacLight™, Invitrogen, Breda, The Netherlands) of dispersed biofilms. Live/dead stain was prepared by adding 3 μL of SYTO®9/propidium iodide (1:3) to 1 mL of sterile, demineralised water. 15 μL of the stain was added to 10 μL of the undiluted biofilm 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. Scanning electron micrographs of the biofilms on wires were taken, as described below.

**DGGE analysis of in vivo biofilms**

All samples of in vivo formed biofilms and saliva were stored at -80°C until use for PCR-Denaturing Gradient Gel Electrophoresis (DGGE) in order to compare their microbial
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5415D, Hamburg, Germany) and subsequently washed and vortexed with 200 μL TE-buffer (10 mM Tris-HCl, 1 mM EDTA pH 7.4), again followed by centrifugation for 5 min at 13,000 g. Next, the supernatant was removed and the pellet was placed in a microwave (500 W, 5 min), after which it was suspended in 50 μL TE-buffer, vortexed and placed on ice. The quality and quantity of DNA samples were measured with a NanoDrop® spectrophotometer (ND-1000, NanoDrop Technologies, Inc, Wilmington, DE, USA) at 230 nm. The final concentration of each DNA sample was adjusted to 100 ng DNA for PCR amplifications.

PCR was performed with a Tgradient thermocycler (Bio-rad I-cycler, GENOtronics BV, USA). For amplification of the 16S rRNA gene, the following bacterial primers were used: F357-GC (forward primer, 5'–GC clamp-TACGGGAGGCAGCAG-3')17 containing a GC clamp (5'–CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC-3')18 to make it suitable for DGGE, and R-518 (reverse primer, 5'–ATTACCGCGGCTGCTGG-3').19 Twenty-five μL of each PCR mixture contained 12.5 μL PCR Master Mix (0.05 units/μL Taq DNA polymerase in reaction buffer, 4 mM MgCl₂, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, 0.6 mM dTTP (Fermentas Life Sciences)), 1 μL of both forward and reverse primer (1 μM), and 100 ng DNA (in a volume of 10.5 μL). The temperature profile included an additional denaturing step of 5 min at 94°C, followed by a denaturing step at 94°C for 45 s, a primer annealing step at 58°C for 45 s, an extension step at 72°C for 1 min and a final extension step of 72°C for 5 min. 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.,20 using system PhorU (INGENY, Goes, The Netherlands). The PCR products were applied on 8% (w/v) 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). Gels were poured using a gradient mixer. 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.18 Each DGGE gel was normalized according to a marker consisting of 7 reference species comprising common bacterial species associated with oral health and disease21 and stored at 4°C. The reference strains included Lactobacillus sp., Streptococcus oralis ATCC 35037, Streptococcus mitis ATCC 9811, Streptococcus sanguinis ATCC 10556, Streptococcus salivarius HB, Streptococcus sobrinus ATCC 33478 and S. mutans ATCC 10449.14

Scanning electron microscopy

Biofilms on the different wires were visualized using scanning electron microscopy (SEM). Wires were fixed overnight in 2% glutaraldehyde and post-fixed for 1 h with 1%
osmiumtetroxide. After dehydration through a water-ethanol series, wires were incubated in tetramethylsilane, air-dried and sputter-coated with a gold-palladium alloy, after which they were fixed on SEM-stub-holders using double-sided sticky carbon tape and visualized in a field emission scanning electron microscope (FE-SEM), type 6301F (JEOL Ltd., Tokyo, Japan) at 2 kV with a working distance of 39 mm and a small spot size.

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 and salivary samples were analysed using a band based similarity coefficient and a non-weighted pair group method with arithmetic averages was used to generate dendograms indicating similarities in composition.\textsuperscript{22} and a non-weighted pair group method with arithmetic averages was used to generate dendograms indicating similarities in composition.\textsuperscript{22}

RESULTS
The total number of bacteria collected from the multi-strand wire was slightly but significantly higher than from single-strand, regardless of the oral health care regimen applied ($p < 0.01$, Table 1). The percentage viability of the bacteria adhering to the different types of wires was significantly higher on single-strand wires compared to multi-strand wires ($p < 0.05$) and buccal enamel surfaces ($p < 0.001$) when using a standard, fluoridated toothpaste without antibacterial claims, regardless of the additional use of an essential-oils containing mouthrinse.

The use of antibacterial toothpastes not complemented with the mouthrinse, hardly affected the total number of bacteria retrieved from the wires but their viability was significantly reduced ($p < 0.001$). The viability on the wires remained higher than on buccal enamel surfaces. The combined use of a triclosan containing toothpaste with the mouthrinse yielded the lowest number and viability of adhering bacteria on either wire.
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Microbial composition of biofilms adhering to the different wires and buccal enamel surfaces and of the salivary microbiome are compared in a cluster tree (Figure 2A), combining the different oral hygiene regimens. The composition of the salivary microbiome separates from the composition of the different adhering biofilms, mainly through a higher prevalence of *S. salivarius* and a lower prevalence of *S. mutans* in saliva (Table 2). Biofilms adhering on the wires have a higher prevalence of *Lactobacilli* and *S. sobrinus* than biofilms adhering on buccal enamel surfaces (Table 2).

Microbial composition of biofilms adhering to the different wires and buccal enamel surfaces and of the salivary microbiome are compared in a cluster tree (Figure 2A), combining the different oral hygiene regimens. The composition of the salivary microbiome separates from the composition of the different adhering biofilms, mainly through a higher prevalence of *S. salivarius* and a lower prevalence of *S. mutans* in saliva (Table 2). Biofilms adhering on the wires have a higher prevalence of *Lactobacilli* and *S. sobrinus* than biofilms adhering on buccal enamel surfaces (Table 2).

When combining results for the different biofilms, an influence of the oral health care regimens becomes evident (Figure 2B). Regimens involving only the triclosan containing toothpaste, and the different individual toothpastes combined with the mouthrinse, form clear clusters. The regimen involving only the stannous fluoride toothpaste, yields a decrease in prevalence of *Lactobacilli, S. oralis/S. mitis* and *S. sanguinis* in comparison with the toothpaste without antibacterial claims and this decrease continues when the stannous fluoride containing paste is used together with the mouthrinse. In the latter, combined regime, also *S. salivarius* is found less prevalent (Table 2). The prevalence of *S. sobrinus* and *S. mutans* in biofilms adhering to wires and buccal enamel surfaces is similar as for the paste without antibacterial

Table 1. The number of bacteria retrieved from 1 cm retainer wires treated with the different toothpastes and with or without the essential-oils containing mouthrinse and their viability. For reference, the viabilities on buccal enamel surfaces is also provided, but because of experimental reasons no comparative data could be given on the total numbers of adhering bacteria. All data represent averages ± standard deviations over 11 different volunteers.

<table>
<thead>
<tr>
<th></th>
<th>Number of bacteria (Log-units)</th>
<th>% live bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single strand</td>
<td>Multi strand</td>
</tr>
<tr>
<td>Toothpaste without antibacterial claims</td>
<td>7.5 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.0 ± 0.2</td>
</tr>
<tr>
<td>Toothpaste without antibacterial claims + mouthrinse</td>
<td>7.5 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70.9 ± 14.5</td>
</tr>
<tr>
<td>Stannous fluoride containing toothpaste</td>
<td>7.3 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.8± 0.3</td>
</tr>
<tr>
<td>Stannous fluoride containing toothpaste</td>
<td>7.0 ± 0.1&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>7.5 ± 0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Triclosan containing toothpaste</td>
<td>7.1 ± 0.2&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>7.7 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Triclosan containing toothpaste + mouthrinse</td>
<td>6.6 ± 0.2&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>7.4 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different from multi-strand wire  
<sup>b</sup> Significantly different from a toothpaste without antibacterial claims  
<sup>c</sup> Significantly different from toothpaste only  
<sup>d</sup> Significantly different from enamel  
<sup>e</sup> Significantly different from a toothpaste without antibacterial claims, with or without the use of mouthrinse

When combining results for the different biofilms, an influence of the oral health care regimens becomes evident (Figure 2B). Regimens involving only the triclosan containing toothpaste, and the different individual toothpastes combined with the mouthrinse, form clear clusters. The regimen involving only the stannous fluoride toothpaste, yields a decrease in prevalence of *Lactobacilli, S. oralis/S. mitis* and *S. sanguinis* in comparison with the toothpaste without antibacterial claims and this decrease continues when the stannous fluoride containing paste is used together with the mouthrinse. In the latter, combined regime, also *S. salivarius* is found less prevalent (Table 2). The prevalence of *S. sobrinus* and *S. mutans* in biofilms adhering to wires and buccal enamel surfaces is similar as for the paste without antibacterial

Microbial composition of biofilms adhering to the different wires and buccal enamel surfaces and of the salivary microbiome are compared in a cluster tree (Figure 2A), combining the different oral hygiene regimens. The composition of the salivary microbiome separates from the composition of the different adhering biofilms, mainly through a higher prevalence of *S. salivarius* and a lower prevalence of *S. mutans* in saliva (Table 2). Biofilms adhering on the wires have a higher prevalence of *Lactobacilli* and *S. sobrinus* than biofilms adhering on buccal enamel surfaces (Table 2).

When combining results for the different biofilms, an influence of the oral health care regimens becomes evident (Figure 2B). Regimens involving only the triclosan containing toothpaste, and the different individual toothpastes combined with the mouthrinse, form clear clusters. The regimen involving only the stannous fluoride toothpaste, yields a decrease in prevalence of *Lactobacilli, S. oralis/S. mitis* and *S. sanguinis* in comparison with the toothpaste without antibacterial claims and this decrease continues when the stannous fluoride containing paste is used together with the mouthrinse. In the latter, combined regime, also *S. salivarius* is found less prevalent (Table 2). The prevalence of *S. sobrinus* and *S. mutans* in biofilms adhering to wires and buccal enamel surfaces is similar as for the paste without antibacterial
Table 2. Prevalence of marker strains in microbial samples from biofilms adhering to the different wires and buccal enamel surfaces and taken from the salivary microbiome for different oral health care regimens and different volunteers.

<table>
<thead>
<tr>
<th>STRAINS</th>
<th>COMBINING ORAL HEALTH CARE REGIMENS</th>
<th>Single-strand wire</th>
<th>Multi-strand wire</th>
<th>Enamel</th>
<th>Saliva</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus</td>
<td></td>
<td>20</td>
<td>25</td>
<td>13</td>
<td>22</td>
</tr>
<tr>
<td>S. oralis/mitis</td>
<td></td>
<td>55</td>
<td>56</td>
<td>57</td>
<td>65</td>
</tr>
<tr>
<td>S. sanguinis</td>
<td></td>
<td>63</td>
<td>60</td>
<td>65</td>
<td>57</td>
</tr>
<tr>
<td>S. salivarius</td>
<td></td>
<td>16</td>
<td>21</td>
<td>20</td>
<td>57</td>
</tr>
<tr>
<td>S. sobrinus</td>
<td></td>
<td>45</td>
<td>52</td>
<td>39</td>
<td>46</td>
</tr>
<tr>
<td>S. mutans</td>
<td></td>
<td>57</td>
<td>48</td>
<td>57</td>
<td>35</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>STRAINS</th>
<th>COMBINING BIOFILMS ADHERING TO WIRES AND BUCCAL ENAMEL SURFACES</th>
<th>Toothpaste without antibacterial claims</th>
<th>Toothpaste without antibacterial claims + mouthrinse</th>
<th>Stannous fluoride containing toothpaste</th>
<th>Stannous fluoride containing toothpaste + mouthrinse</th>
<th>Triclosan containing toothpaste</th>
<th>Triclosan containing toothpaste + mouthrinse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus</td>
<td></td>
<td>30</td>
<td>45</td>
<td>21</td>
<td>5</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>S. oralis/mitis</td>
<td></td>
<td>53</td>
<td>95</td>
<td>29</td>
<td>20</td>
<td>86</td>
<td>71</td>
</tr>
<tr>
<td>S. sanguinis</td>
<td></td>
<td>67</td>
<td>45</td>
<td>50</td>
<td>10</td>
<td>82</td>
<td>95</td>
</tr>
<tr>
<td>S. salivarius</td>
<td></td>
<td>23</td>
<td>35</td>
<td>31</td>
<td>10</td>
<td>32</td>
<td>38</td>
</tr>
<tr>
<td>S. sobrinus</td>
<td></td>
<td>39</td>
<td>80</td>
<td>43</td>
<td>70</td>
<td>34</td>
<td>33</td>
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<tr>
<td>S. mutans</td>
<td></td>
<td>30</td>
<td>70</td>
<td>43</td>
<td>85</td>
<td>68</td>
<td>5</td>
</tr>
</tbody>
</table>

claims, regardless of whether complemented with the use of the mouthrinse. The triclosan containing toothpaste yields major increases in the prevalence of adhering *S. oralis/S. mitis, S. sanguinis* and *S. mutans*. However, when combining the triclosan containing toothpaste with the essential-oils containing mouthrinse, we see the lowest prevalences of *Lactobacilli, S. sobrinus* and *S. mutans* developing over the different regimens applied.

Scanning electron micrographs (Figure 3) show the protected location of bacteria adhering to multi-strand wires. On the multi-strand wires, bacteria are mostly located in the crevices between strands, while on the single-strand wires bacteria are present as a thin scattered film, attached mainly to irregularities on the wire surface.
Figure 2. Clustering trees describing the bacterial compositions of the microbial samples taken from the different volunteers included in this study. The corresponding circles in Figures 2A and 2B represent the same sample. (A) Colours indicate different locations of microbial sampling, i.e. enamel, retention wires or saliva. (B) Colours indicate the use of different oral health care regimens.
Figure 3. Scanning electron micrographs of 1 week old biofilms formed in vivo, during use of a toothpaste without antibacterial claims. 
(A), (B), (C) Single-strand wire. 
(D), (E), (F) Multi-strand wire.

DISCUSSION

Biofilm formation \textit{in vivo} on both single-strand and multi-strand retention wires during the use of antibacterial toothpastes and a mouthrinse was evaluated. Although statistically significant differences were found in the numbers of bacteria adhering to retention wires upon use of different toothpastes with and without antibacterial claims, and when complemented or not with the use of an essential-oils containing mouthrinse, these differences are likely too small to be of clinical significance. This coincides with results of clinical studies, showing that antibacterial toothpastes, including the two included in this study, yield reduced amounts of oral biofilm formed.\cite{23,24} Clinical studies also confirm a small, if any effect of the additional use of an essential-oils containing mouthrinse on oral biofilm formation.\cite{12,25,26} More interestingly from a clinical perspective, the use of antibacterial toothpastes reduced the percentage viability of the adhering organisms. Statistically significant, but likely clinically irrelevant differences in the number of bacteria adhering to single- and multi-strand wires were found too, but more importantly antibacterial regimens caused a stronger drop in the viability of the adhering organisms on single- than on multi-strand wires, indicating better penetration of antimicrobials in biofilms forming on single-strand wires. This coincides with a higher viability of biofilms forming on single-strand wires compared to multi-strand ones during use of a
toothpaste without antibacterial claims. This is probably caused by the fact that, similar to antimicrobials, also nutrients have better access to bacteria adhering on single-strand than on multi-strand wires.27

Biofilms on both types of retention wires have roughly the same microbial composition (Table 2), with some differences with respect to the composition of oral biofilm on enamel surfaces. Adhering biofilms have a very different composition than the salivary microbiome. These substratum-dependent microbial compositions confirm recent work28 that the surface dictates the composition of the biofilm it attracts through differential adhesion forces exerted on different strains of bacteria. The largest differences in microbial composition in biofilms adhering to retention wires and enamel surfaces are seen after regimens of antibacterial toothpastes combined with the essential-oils containing rinse. Most strikingly and of clinical importance, a regimen comprising the use of a triclosan containing toothpaste complemented with an essential-oils containing mouthrinse yielded a reduction in the prevalence of S. mutans from 30% to 5%. Other combination regimens, increased the prevalence of S. mutans in retainer biofilms. It is intriguing why the combination of a triclosan containing toothpaste with an essential-oils containing mouthrinse causes such a drastic shift in the composition of the oral microbiome into a direction that could be perceived as being less cariogenic, i.e. comprising less S. mutans. Oil containing mouthrinses have the ability to remove bacteria from the oral cavity through adhesion to the hydrophobic oil, which requires a certain degree of hydrophobicity of the bacterial cell surface.29 Moreover, certain concentrations of cationic antibacterial agents such as cetylpyridinium chloride and chlorhexidine have been demonstrated to promote binding of oral microorganisms to oil droplets.30

Hypothetically, exposure to the non-polar triclosan31 could make S. mutans cell surfaces more hydrophobic which would facilitate their removal by hydrophobic oils. In order to verify this hypothesis, we exposed a S. mutans strain used in this study to supernatants of the different toothpastes, and examined its removal by a hexadecane in the so-called kinetic MATH assay,32 as described in the Supplementary information. S. mutans possessed a low removal rate by hexadecane (Figure S1), classifying its surface as very little hydrophilic (Table S1, Supplementary information). Only exposure to the triclosan containing toothpaste supernatant however, increased the removal by hexadecane of S. mutans (see also Figure S1 and Table S1). This finding supports our hypothesis that exposure to triclosan can make S. mutans cell surfaces more hydrophobic facilitating their removal by oil containing mouthrinses and corresponds with the observation that S. mutans strains grown in the presence of triclosan formed more extensive biofilms.33 However, the authors of the latter paper ruled out effects of triclosan on streptococcal cell surface hydrophobicity, probably because they did not use the MATH assay in its more sensitive kinetic mode as advocated by Ligtenberg et al.32 Pathways to influence the composition of oral biofilms towards a “healthy” composition are
still in its infancy. Adsorption of toothpaste components to create more hydrophobic surfaces of selected oral pathogens and making use of hydrophobic oil-containing mouthrinses for their subsequent removal from the oral cavity seems like a clinically feasible approach to this end. The present results warrant more research into components that alter the cell surface hydrophobicity of selected oral bacterial strains.

At this stage it is impossible to say whether the compositional changes observed have any beneficial clinical effect. However, it has been shown that the use of an antibacterial toothpaste containing sodium lauryl sulphate and stannous fluoride or triclosan, increases the pH of oral biofilm and decreases its viability\textsuperscript{34, 35} to yield a less cariogenic biofilm. Clearly such changes in biofilm properties may be taken as an indication of an altered microbial composition if not of a reduced prevalence of \textit{S. mutans} and \textit{Lactobacilli} in the biofilm. Most studies on oral biofilm composition, including the present one, make use of a control regimen, like in our study the use of a NaF-sodium lauryl sulphate containing toothpaste with mint flavour. This paste was chosen as a control, because it has no antimicrobial claims, but at the same time it cannot be ruled out that it affects both oral biofilm viability as well as composition. Both fluoride, sodium lauryl sulphate as well as mint flavouring agents are known to have antibacterial properties,\textsuperscript{15, 36, 37} while fluoride is known to inhibit calcium-bridging between co-adhering pairs of oral bacteria.\textsuperscript{38}

In summary, oral biofilm formation \textit{in vivo} is slightly less on single-strand retention wires than on multi-strand wires. Orthodontic patients with a fixed bonded retainer benefit from use of an appropriate regimen of an antibacterial toothpaste and mouthrinse, not so much through reduction of the amount of biofilm formed, but rather through reduction of its viability. Moreover, appropriate regimens may make the selected members of the oral microbiome more hydrophobic through adsorption of non-polar components from toothpastes to subsequently enhance their removal by oil containing mouthrinses, yielding less pathogenic biofilms. This pathway to restoring a healthy oral microbiome needs to be explored further though.

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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. 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 employers.
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SUPPLEMENTARY INFORMATION: MICROBIAL ADHESION TO HYDROCARBONS (MATH)

*S. mutans* ATCC 10449 grown on blood agar plates from a frozen stock, was used to inoculate 10 mL Tryptone Soya Broth (TSB) and cultured for 24 h at 37°C. This culture was used to inoculate 100 mL TSB, which was grown overnight. Bacteria were harvested by centrifugation and washed twice with potassium phosphate buffer (pH 7.0) and suspended to an optical density A0 (at 600 nm) of between 0.4 and 0.6. Next, half of the suspension was mixed with the supernatant of a toothpaste slurry (25% by weight) in water used after centrifugation, 5 min at 10,000 g to remove particulate matter for 2 min, centrifuged, washed and resuspended in potassium phosphate buffer to an optical density A0 (at 600 nm) of between 0.4 and 0.6. In order to measure the hydrophobicity of the bacterial cell surfaces before and after exposure to a toothpaste supernatant, 150 µL hexadecane was added to 3 mL of each suspension and the suspension was vortexed for 10 s, allowed to settle for 10 min for phase separation and finally the optical density At of the aqueous phase was measured. This was repeated 6 times and log (At/A0 x100) was plotted against the vortexing time (Figure S1). Initial removal rates R0 (min⁻¹) were calculated as the slopes of the tangent of the curves obtained and used to compare effects of adsorption of toothpaste components on the hydrophobicity of the streptococcal cell surface (Table S1).

Supplementary Table S1. Cell surface hydrophobicity of *S. mutans* ATCC 10449 before and after exposure to slurries of the different toothpastes involved in this study, as measured by the kinetic MATH assay and expressed in terms of their initial removal rates. All data represent the average ± SD of three experiments with

<table>
<thead>
<tr>
<th>Toothpaste used</th>
<th>Initial removal rate (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>Toothpaste without antibacterial claims</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Stannous fluoride containing toothpaste</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Triclosan containing toothpaste</td>
<td>0.05 ± 0.011*</td>
</tr>
</tbody>
</table>

*Significantly different from all other data at p<0.000 (A One-Way ANOVA was used with a Bonferroni test for post-hoc multiple comparisons. Statistical significance was set at p< 0.05.
Figure S1. Optical density log ($\log (A_t/A_0 \times 100)$) as a function of the vortexing time for the removal of *S. mutans* ATCC 10449 prior to or after its exposure to a toothpaste slurry by hexadecane. Each data point represents the average over three experiments with different bacterial cultures. Standard deviations are smaller than the data points.
Effects of antimicrobials on oral biofilms