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Antibacterial measures for biofilm control

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Chapter 3

**Synergy of
brushing mode and antibacterial use
on in vivo biofilm formation**

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Abstract

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

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 (Marsh 2012). Mechanical biofilm removal by powered toothbrushing has been demonstrated to be superior to manual brushing (Yaacob et al. 2014). 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% (Paraskevas et al. 2006; Van der Weijden et al. 2008), 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 (Sheiham and Sabbah 2010). 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 (Ren et al. 2014).

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 (Marsh 2012). However, the general structure and composition of oral biofilm hampers penetration of oral antibacterials through the depth of an entire biofilm (Van Leeuwenhoek 1684). 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 (Flemming and Wingender 2010; Marsh 2010). 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 (He et al. 2014), especially when the bristles of the brush have not been able to touch the biofilm and remove it (Busscher et al. 2010). This more open structure is caused by a high energy transfer from a powered toothbrush into the biofilm through strong fluid flows (Van der Mei et al. 2007), air bubble inclusion (Parini and Pitt 2006) and acoustic waves (Busscher et al. 2010). 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 (He et al. 2014). Also, once oral antibacterials

have penetrated the biofilm, the biofilm left-behind might act as a reservoir for the oral antibacterial agents ensuring a prolonged action of the agent (Otten et al. 2012). However, the impact of these in vitro findings for the clinical situation has never been demonstrated and could only be speculated upon.

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 (Levin et al. 2008; Jongsma et al. 2014) 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 and 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®, 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 males, age ranging from 24 to 31, 5 females, age ranging from 20 to 37) in agreement with the rules set out by the Ethics Committee at the University Medical Centre Groningen (letter June 23rd, 2011). A power analysis indicated that 10 volunteers would be sufficient to achieve 80% power at an alpha level of 0.0500. The outcome for the sample calculation was bacterial counts in a logarithmic scale which was treated as a continuous variable. The expected difference between groups was set at 0.3, the standard deviation at 0.3, and the correlation coefficient at 0.5. 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 were not pregnant. All volunteers were dental students, dentists, orthodontists or dental hygienists. 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®, Philips Nederland B.V., Eindhoven, The Netherlands) on the other side. Proper use of the different toothbrushes was demonstrated to the volunteers. Volunteers were furthermore instructed to use a NaF-sodium lauryl sulphate (NaF-SLS) containing toothpaste without antibacterial claims (Prodent Softmint®, Sara Lee Household & Bodycare, Exton, USA), or a triclosan-containing toothpaste (Colgate Total®, 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®, Pfizer Consumer Healthcare, Morris Plains, NJ,

USA) (Fig. 1). The oral hygiene products were presented to the volunteers in their original packaging. The order in which the regimens were applied in the different volunteers was determined at random. Volunteers were asked to pick a number corresponding to a certain order of toothpaste/mouthrinse regimens. 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 including 5 volunteers 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. Enamel biofilms were removed with a sterile cotton swab and in order to obtain enough biofilm for evaluation, the entire buccal enamel surface surrounding the brackets was swabbed. Wires were removed in the morning after breakfast and regular brushing by the volunteers. Wires and cotton swabs containing enamel biofilms were stored in an Eppendorf tube containing 1.0 ml filter sterile reduced transport fluid (RTF) (Syed and Loesche 1972) for transportation from the orthodontic clinic to the laboratory. The collection and evaluation of the biofilm was performed blinded. All samples were given a number and the researchers were not told which type of oral hygiene regimen corresponded with that number. This code was broken for the statistical analysis of the results.
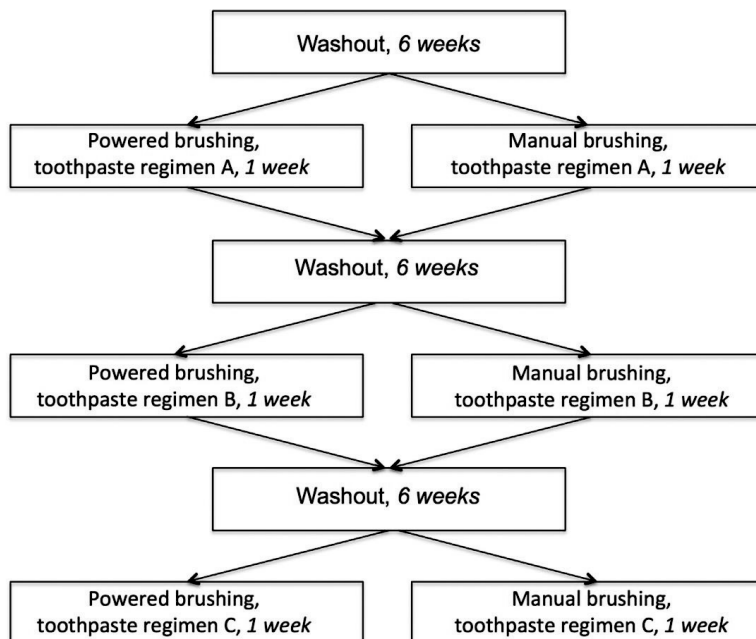
Figure. 1. Schematic representation of the experimental protocol. The study was performed as a split-mouth design. One of the wires, placed on the upper arch of the volunteers, was brushed manually, while the other wire was brushed with a powered toothbrush. The order in which the different toothpaste/mouthrinse regimens were applies was determined at random. The different regimens that were applied consisted of—A NaF-SLS containing toothpaste without antibacterial claims; A triclosan containing toothpaste; A triclosan containing toothpaste combined with a essentals oils containing mouthrinse. During the washout period a NaF-SLS containing toothpaste without antibacterial claims was used by all volunteers.

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 (*Bac*Light™, Invitrogen, Breda, The Netherlands) of the 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. 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,

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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 (Shi et al. 2007; Netuschil et al. 2014). 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 (Jongsma et al. 2014). 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 (Muyzer et al. 1993). The PCR products were applied on 0.08 g/ml polyacrylamide gel in 0.5 × TAE buffer (20 mM Tris acetate, 10 mM sodium acetate, 0.5 mM EDTA, pH 8.3). The denaturing gradient consisted of 30–80% 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 ºC. Gels were stained with silver nitrate (Zijnge et al. 2006). Each DGGE gel was normalized according to a marker consisting of 7 reference species comprising common bacterial species associated with oral health and disease (Marsh 2006). 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 (Otten et al. 2012).

Statistical analysis

Data were analyzed with the Statistical Package for Social Sciences (Version 16.0, SPSS Inc., Chicago, IL, USA). A log transformation was used on the data concerning number of bacteria. The distribution of the number of bacteria and the percentage live bacteria were tested for normality. Both number of bacteria and percentage live bacteria were found to be distributed normally. Multiple paired t-test were used to assess pairwise comparisons on the number of bacteria and their percentage viability with brushing modes and oral care regimen as variables. 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 dendograms indicating similarities in composition (Signoretto et al. 2010).

Results

When powered toothbrushing was applied, 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, only the use of an antibacterial, triclosan-containing toothpaste supplemented with an essential-oils containing mouthrinse yielded a significant decrease in the number of bacteria (Table 1). When powered toothbrushing was applied however, significantly less bacteria 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.

Viability of retention wire biofilm was significantly lower 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 significantly lower after brushing with a powered toothbrush than after manual brushing.

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 (Fig. 2A and B). Mode of brushing has no influence on the clustering of bacterial composition data, neither on retention wires (Fig. 2A) nor on enamel surfaces (Fig. 2B), as can be seen from the proximity of similarly coloured dots to one another. 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. Prevalences of *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.

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, treiclosan-containing toothpaste supplemented or not with the use of an essential-oils containing mouthrinse. The data represent averages ± standard deviations over 10 different volunteers and p-values for the comparisons between different regimes, accounting for a split-mouth design and considering multiple measurements per patient due to the cross-over design (pair-wise comparison).

| | | Average ± S.D | | p-values for number of bacteria | | | | | |
| | | | | Manual brushing | | | Powered brushing | | |
		Number of bacteria (Log-units)	%live bacteria	1 NaF-SLS toothpaste	2 Triclosan toothpaste	3 Triclosan toothpaste + mouthrinse	4 NaF-SLS toothpaste	5 Triclosan toothpaste	6 Triclosan toothpaste + mouthrinse
Manual brushing	1 NaF-SLS toothpaste	7.9 ± 0.1	68 ± 12		0.333	0.001	0.333	<0.001	<0.001
	2 Triclosan toothpaste	7.6 ± 0.2	42 ± 8	<0.001		0.728	1.000	0.015	<0.001
	3 Triclosan toothpaste + mouthrinse	7.5 ± 0.2	37 ± 5	<0.001	1.00		0.728	1.000	<0.001
Powered brushing	4 NaF-SLS toothpaste	7.6 ± 0.1	60 ± 7	0.58	<0.001	<0.001		0.015	<0.001
	5 Triclosan toothpaste	7.3 ± 0.3	28 ± 9	<0.001	0.004	0.228	<0.001		0.011
	6 Triclosan toothpaste + mouthrinse	7.0 ± 0.2	16 ± 4	<0.001	<0.001	<0.001	<0.001	0.027	
				p-values for % live bacteria					
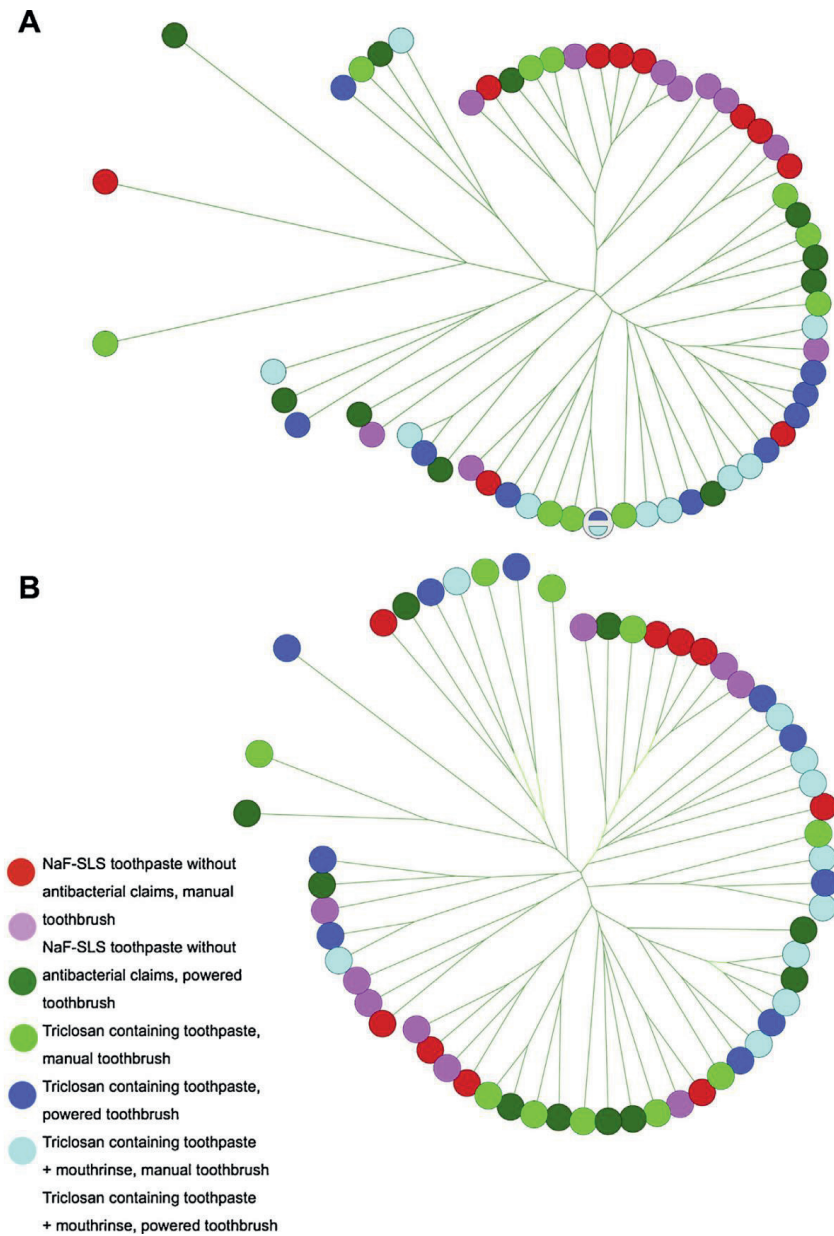
Figure 2. Clustering trees describing the bacterial compositions of biofilm samples taken from stainless steel retention wires (A) or enamel surfaces (B) in different volunteers using manual or powered toothbrushing in combination with different healthcare regimens. The closer the proximity of similarly coloured dots to one another, the more the composition is alike.

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 (n = 10 volunteers for all samples).

STRAINS	NaF-SLS toothpaste without antibacterial claims				Triclosan containing toothpaste				Triclosan containing toothpaste + mouthrinse			
	Manual brushing		Powered brushing		Manual brushing		Powered brushing		Manual brushing		Powered brushing	
	Wire	Enamel	Wire	Enamel	Wire	Enamel	Wire	Enamel	Wire	Enamel	Wire	Enamel
S.oralis/S. mitis	20	70	40	50	20	50	50	80	80	40	70	60
S. sanguinis	80	80	20	70	40	60	30	70	60	30	40	50
S. salivarius	30	20	30	10	10	30	10	20	0	10	10	10
A. naeslundii	0	15	0	0	0	10	0	0	0	0	0	0
Lactobacillus	20	20	20	20	10	30	10	10	0	0	0	0
S. sobrinus	30	10	30	30	30	70	30	30	20	40	10	10
S. mutans	30	10	50	0	10	20	10	0	0	0	0	0
P. gingivalis	30	10	10	0	20	10	10	0	0	0	0	0
P. intermedia	0	0	0	0	10	10	0	0	0	0	0	0

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 (He et al. 2014). 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 (Jongsma et al. 2013). 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 (Praskevas et al. 2006; Van der Weijden et al 2008), 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, amounting around 0.1 mW for a manual brush and 110 mW for sonic brushing (Veeregowda et al. 2012). Since biofilms have visco-elastic properties, biofilm will first expand due to energy input during powered brushing after which it will detach (Cense et al. 2006; Rmaile et al. 2014; Peterson et al. 2015). 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 (Jongsma et al. 2013). This indicates that the decrease in viability is solely attributed to the oral antibacterial

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agents, and not to toothbrushing itself (MacNeill et al.1998). This shows the existence of a synergy between mode of toothbrushing and antibacterial action with clinically demonstrable effects. General long-term (>2 months) benefits of powered toothbrushing and antibacterial regimens have been described in the literature (Stoeken et al. 2007; Cortelli et al. 2013; He et al. 2013; Riley and Lamont 2013). Although our study only extends over a time period of one week, with a relatively small sample size and involving volunteers with a high level of oral hygiene awareness predominantly, we believe that the clinical effects observed can be extrapolated to longer-term effects in the general population, as structural changes in the biofilm are underlying to the mechanisms of enhanced penetration of antibacterials in biofilm left-behind.

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 (He et al. 2013; Riley and Lamont 2013), with minor effects of the supplemental use of an essential-oils containing mouthrinse (Cortelli et al. 2013; Stoeken et al. 2007; Tufekci et al. 2008). 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 (Fig. 2) that we earlier attributed to adsorption of triclosan to bacterial cell surfaces altering their cell surface hydrophobicity to stimulate removal by hydrophobic ligands (Jongsma et al. 2014).

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 (Mei et al. 2009). Accordingly this explains (Wessel et al. 2014) 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 (Marsh 2004), the latter likely being comparable with biofilm found on the retention wires.

Further enhancing the synergy between powered toothbrushing and oral antibacterials may be a goal of future 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.

Conclusions

This study shows 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, but also with regard to the amount and composition of the biofilm.

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