Oral biofilms
Verkaik, Martinus Johannes

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CHAPTER 2

COMPARISON OF DIFFERENT MODES OF CONTACT BRUSHING ON REMOVAL OF EARLY COLONIZING ORAL BACTERIA -AN IN VITRO STUDY WITH SINGLE STRAINS-
Introduction

Prevention of caries and periodontal diseases requires a strict plaque control (Axelsson and Lindhe, 1981). When performed with an adequate technique and duration of time, manual brushing is highly effective and there is strong evidence that toothbrushing reduces gingivitis (Lang et al., 1973). However, for most patients, neither of the former mentioned criteria are fulfilled. Despite the fact that a healthy dentition is a precious asset, the average time people spend on a daily basis to brush their teeth is 33 - 60 seconds (Kleber et al., 1981; Macgregor and Rugg-Gunn, 1985), which is far less than the two times 2 min considered adequate for regular oral hygiene. Sometimes, people try to compensate short brushing by vigorous brushing and exert high forces. Whereas the regular force applied for manual toothbrushing is around 3 N (Danser et al., 1998), examples of gingival damage and enamel abrasion have been documented due to forces less than 3 N (Ganss et al., 2009). The immediate effect of brushing is removal of bacteria, but brushing also influences the re-deposition of new bacteria. Re-deposition of new bacteria is stimulated by bacterial remnants or ‘footprints’ (Neu and Marshall, 1991) left on the tooth surface after brushing. Effective removal of these footprints depends on the type of toothbrush and applied forces (Van der Mei et al., 2004). Powered toothbrushes were introduced in the early 1960’s, amongst other reasons, to compensate for a poor brushing technique (Fischman, 1997; Sicilia et al., 2002). Powered toothbrushes have decreased the need to exert high forces and electric-rotating brushing is already effective at forces of about 1.5 N (McCracken et al., 2003).

Powered toothbrushes with a rotating-oscillating or sonic action remove plaque and reduce gingivitis significantly more than manual brushes (Tritten and Armitage, 1996; Ho and Niederman, 1997; Moritis et al., 2002; Biesbrock et al.,
2008; Rosema et al., 2008). Other forms of powered brushes (side to side, counter oscillation, circular and ultrasonic) produce a less consistent reduction of plaque and gingivitis (Robinson et al., 2005).

The human oral cavity may be inhabited by thousands of different bacterial species, bringing about a multitude of interactions between bacteria as well as between bacteria and oral surfaces (Kolenbrander et al., 2006; Ten Cate, 2006; Keijser et al., 2008). Next to the microbiological complexity, saliva is rather complex as well. Salivary composition is not only dependent on the individual, but depends on the time of the day as well (Nieuw Amerongen et al., 2004). When choosing an appropriate biofilm model for mechanical plaque removal and re-deposition studies, the model needs to be reproducible and straightforward. The main purpose of any model would be to distinguish the removal efficacy and influence on re-deposition of different removal devices and identify differences in removal or re-deposition between strains. To this case, a single strain oral biofilm model is an excellent model. In addition, the choice of the parallel plate flow chamber for biofilm growth is based on the presence of shear during growth and temperature control.

The aim of the present in vitro study was to compare the efficacies of three different modes of contact-brushing on bacterial removal and re-deposition in single strain biofilm models on a saliva-coated surface.

Materials & Methods

Bacterial Strains, Culture Conditions and Harvesting

*Streptococcus oralis* J22, *Streptococcus mutans* NS and *Actinomyces naeslundii* T14V-J1 were used in this study. *Streptococci* were cultured in Todd Hewitt broth (THB, OXOID, Basingstoke, UK) in ambient air and *A. naeslundii* in Schaedler’s
broth (SB) supplemented with 0.01 g/L hemin under anaerobic conditions (10% H₂, 85% N₂ and 5% CO₂), both at 37°C. Stocks were kept in growth media with 7% DMSO (dimethylsulfoxide, Merck, Darmstadt, Germany) at -80°C, and from these stocks, bacteria were precultured on blood agar plates. One colony was used to inoculate a 24 h batch culture. This culture was used to inoculate a second culture, which was grown for 16 h. These bacteria were harvested by centrifugation for 5 min at 6,500 g and washed twice with adhesion buffer (2 mM potassium phosphate, 50 mM potassium chloride and 1 mM calcium di-chloride, pH 6.8). Subsequently, in order to break up bacterial chains or aggregates, bacteria were sonicated during 3 x 10 s for *S. oralis* J22 and *S. mutans* NS and 4 x 10 s for *A. naeslundii* T14V-J1 intermittently while cooling on ice at 30 W (Vibra Cel model 375; Sonics and Materials, Danbury, CT, USA). Bacteria were diluted to a density of 3 x 10⁸ per mL, in adhesion buffer with 2% growth medium added and the streptococcal suspension was supplemented with 1.5 mg/mL lyophilized human whole saliva.

**Saliva Collection and Preparation**

In order to form a salivary conditioning film, human whole saliva from at least 20 healthy volunteers of both genders was collected into ice-cooled beakers after stimulation by chewing Parafilm®. The saliva was pooled and centrifuged for 5 min at 10,000 g at 10°C. Saliva was treated by adding phenylmethylsulfonyl fluoride (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) to a final concentration of 1 mM as a protease inhibitor in order to inhibit salivary protein denaturation. The solution was again centrifuged, dialyzed (Molecular Weight Cut Off, 6-8 kD, Spectra/Por 1, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) overnight at 4°C against demineralized water, and lyophilized for storage. For experiments,
lyophilized saliva was dissolved at a concentration of 1.5 mg/mL in adhesion buffer. A glass plate was saliva-coated by incubating in saliva for 16 h at room temperature. All volunteers gave their informed consent to saliva donation and with approval of the Medical Ethical Committee at UMCG, Groningen (M09.069162), The Netherlands.

**Biofilm Formation and Brushing**

Bacterial adhesion experiments were performed in a parallel plate flow chamber (dimensions: l x w x h = 175 x 17 x 0.75 mm), as previously described (Busscher and Van der Mei, 2006) and shown in Figure 1A. The flow chamber was mounted on the stage of a phase contrast microscope equipped with a 40x ultra-long working distance objective (Olympus ULWD-CD Plan 40 PL). Images were taken from the bottom plate of the parallel plate flow chamber, using Matlab based imaging software connected to a camera (1392 x 1040 pixels). Each image was obtained after summation of 15 consecutive images (time interval 1 s) in order to enhance the signal to noise ratio and to eliminate moving bacteria from the analysis. The plates used in the chamber were microscope glass slides.

Before each experiment, the flow chamber was cleaned by washing with a detergent (Extran®, Merck, Darmstadt, Germany), thoroughly rinsed with tap water and finally with demineralized water. The glass slides were cleaned in a 2% RBS 25 (Omniclean, Breda, The Netherlands) detergent solution under simultaneous sonication for 3 min, and alternately rinsed with tap water, methanol, tap water again and finally demineralized water. The flow chamber, glass slides and all tubes were sterilized before use. In order to create a salivary pellicle on the bottom plate, glass slides were submerged in saliva for 16 h at room temperature.
Figure 1. A) Parallel plate flow chamber consisting of bottom plate, spacer, top plate and in-/out-let. The open space in the flow chamber is designed for a substratum (glass) plate. Temperature sensors and heating element are attached as well B) Basic design of the entire flow system used, shown with one flow chamber.
Two different protocols were used: (A), 2 h adhesion followed by brushing and 2 h re-deposition and (B), 2 h adhesion continued by overnight growth, followed by brushing and 2 h re-deposition. In protocol A, one flow chamber was used per experiment, while in protocol B, three flow chambers, linked in series, were used for each experiment. The flow system is depicted in Figure 1B.

Prior to each experiment, all tubes and the flow chamber(s) were filled with adhesion buffer, while care was taken to remove all air bubbles from the system. Once the system was filled, and prior to the addition of a bacterial suspension, adhesion buffer was pre-flowed for 30 min through the system in order to remove remnants of saliva and allow the system to warm up to 33°C, a relevant oral surface temperature (Spierings et al., 1984), at which temperature all experiments were performed. The flow rate (Q), represented moderate oral shear (Dawes et al., 1989) and was kept constant during the experiment at 1 mL/min, corresponding with a wall shear rate (σ) of 10 s⁻¹ according to

\[
\sigma = \frac{3Q}{2d^2w}
\]

in which d is the half-depth and w is the width of the flow chamber. Subsequently, flow was switched to a bacterial suspension and was circulated through the system for 2 h. The viability of the bacterial suspensions during the first 2 h of adhesion was checked using Live/Dead stain (BacLightTM, Invitrogen, Breda, The Netherlands) and amounted 97% on average.

In protocol A, after 2 h, 10 images were taken from three different areas on the saliva coated bottom plate, corresponding with the areas to be brushed by a manual (Oral-B soft indicator Regular 40; Oral-B laboratories, Belmont, CA, USA), electric rotating (Oral-B Professional Care 7850 DLX; Braun GmbH, Kronberg, Germany) or sonic (Oral-B Sonic Complete; Braun GmbH) brush. After taking images, the flow
was stopped, the flow chamber dismantled and the bottom plate removed. The three selected areas were brushed in a wetted state (i.e. with a thin film of water on the pellicle but not immersed), for 20 s with the brushes attached to a moving tray (Van der Mei et al., 2004) (Figure 2), involving 20 single strokes back and forth over a distance of 5.5 (manual), 4.0 (electric rotating) or 5.0 (sonic) cm. Each brush crosses the complete glass plate (2.6 cm) and under a clinically relevant weight of 220 g for the manual, 150 g for the electric rotating and 90 g for the sonic brush (Danser et al., 1998; McCracken et al., 2001; Van der Mei et al., 2004; Van der Weijden et al., 2004). After brushing, the glass plates were mounted again in the parallel plate flow chamber, the flow chamber was filled with buffer and rinsed for 10 min. Subsequently, 10 images of the selected areas were taken and re-deposition was started by flowing with the same bacterial suspension for another 2 h. After re-deposition, again 10 images were taken at the same places as after brushing.

Figure 2. Brushing machine showing the manual brush mounted with a weight attached to the brush. The electric rotating and sonic brush are depicted in the figure as well (left to right).
In protocol B, after 2 h, flow was switched to growth medium (THB for *Streptococci* and SB for *A. naeslundii*). The growth medium was perfused through the system without recirculation. After overnight growth, flow was stopped, the flow chambers dismantled and the bottom plates removed. Each flow chamber was used for one brush, the glass slide was divided by a bar in two parts: one side for brushing while the other side served as a non-brushing control. The dividing bar prevented influences of brushing on the control side. Brushing was done as described in protocol A. After brushing, the glass plates were again mounted in the flow chambers, which were filled with buffer and rinsed for 30 min. Subsequently, 10 images of the selected areas were taken and re-deposition was started by flowing with a fresh bacterial (same strain as used in first 2 h of the experiment) suspension for another 2 h. After re-deposition, the flow chambers were rinsed with adhesion buffer for 30 min and again 10 images were taken in the same selected areas as after brushing. As a control, one glass plate (for each protocol) was taken through the entire procedure in the absence of brushing, in order to account for potential detachment processes during handling of the flow chambers.

Images were analyzed with a Matlab based counting program, to determine the fractional surface coverage (A) of the substratum by adhering bacteria. The percentage removal was subsequently calculated by:

\[
\text{\% removal} = \frac{A_b - A_a}{A_b} \times 100
\]

where \(A_b\) and \(A_a\) denote the fractional surface coverage before and after brushing, respectively.
**Statistical Analysis**

Statistical analysis and comparison of the different groups was performed with Student’s paired samples t-test for comparison before and after brushing and Students’ independent samples t-test for comparisons between the different brushes. Two way ANOVA was used for comparison between 2 h adhesion and growth, taking the three brushing modes together. A significance level of \( p < 0.05 \) was used.

**Results**

**Brushing and Re-deposition after 2 h Adhesion**

After 2 h adhesion but prior to brushing and opening of the flow chamber, the fractional surface coverage of bacteria on the surface for *S. mutans* NS was \( 0.03 \pm 0.02 \), for *S. oralis* J22 it amounted \( 0.09 \pm 0.02 \) and for *A. naeslundii* T14V-J1 it was \( 0.18 \pm 0.05 \). Note by comparison with the control data in Table 1, that opening and closing of the flow chamber yielded severe detachment for *S. mutans* and *S. oralis*, corresponding with a removal of 91% of the adhering *S. mutans* NS, and 57% of the adhering *S. oralis* J22 bacteria. All brushes yielded major removal of initially adhering single bacterial strains. The removal by the three different modes of brushing (manual, electric rotating and sonic) was on average 93%, for *S. mutans* NS as well as for *S. oralis* J22 and 95% for *A. naeslundii* T14V-J1 (Table 1). The differences between the three modes of brushing were not statistically significant. Single bacterial strains adhering to the pellicle surfaces prior to and after brushing are shown in Figure 3. After re-deposition, the fractional surface coverage by newly deposited bacteria per unit area and percentage regained were independent of the brush type (Table 1).

Despite the fact that there was little difference between the different modes of brushing, huge differences were observed between the bacterial strains. The fractional
surface coverage by bacteria after 2 h adhesion was significantly different for the
*S. mutans* NS (0.03 ± 0.02), *S. oralis* J22 (0.09 ± 0.02) and *A. naeslundii* T14V-J1
(0.18 ± 0.05). The percentage regained was statistically significant lower for
*S. oralis* J22 (75%) than for *S. mutans* NS (102%) or *A. naeslundii* T14V-J1 (94%).
Moreover, re-deposition showed significant differences between the bacteria, with the
highest fractional surface coverage after re-deposition for *A. naeslundii* T14V-J1 and
the lowest for *S. mutans* NS (Table 1).

**Brushing and Re-deposition after Overnight Growth**

For both *S. oralis* J22 and *A. naeslundii* T14V-J1 the fractional surface coverage after
growth, but prior to brushing and opening of the flow chamber, was 100%. Both
strains showed no significant differences between the brushes in the percentage
removal after growth, fractional surface coverage after re-deposition or the percentage
fractional surface coverage regained during re-deposition (Table 2). However,
remarkable differences were observed between the two strains. After growth, the
percentage removal was significantly higher for *S. oralis* J22 (on average 94%) than
for *A. naeslundii* T14V-J1 (on average 68%). Single bacterial strains adhering to the
pellicle surfaces prior to and after brushing are shown in Figure 4. Re-deposition also
gives a significantly lower fractional surface coverage as well as percentage fractional
surface coverage regained for *S. oralis* J22 than for *A. naeslundii* T14V-J1. Note that
the *S. mutans* NS strain could not be used for growth experiments since the fractional
surface coverage after growth was only 0.02, which is similar as after 2 h adhesion.
Table 1. Fractional surface coverage (FSC) by adhering bacteria after brushing on saliva coated glass and the percentage removal after brushing by three different brushing modes. The percentage removal is not corrected for handling of the flow chamber and includes brushing, opening and closing of the flow chamber. Furthermore the FSC by newly deposited bacteria and the percentage FSC of adhering bacteria regained after re-deposition is shown.

%Removal and %regained was expressed with respect to the coverage by bacteria after 2 h of adhesion. After 2 h adhesion, the FSC of bacteria on the surface for S. mutans NS was 0.03 ± 0.02, for S. oralis J22 it amounted 0.09 ± 0.02 and for A. naeslundii T14V-J1 it was 0.18 ± 0.05.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Toothbrush</th>
<th>After brushing</th>
<th>Redeposited</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FSC</td>
<td>% removal</td>
</tr>
<tr>
<td>S. mutans NS</td>
<td>control*</td>
<td>0.003</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>Manual</td>
<td>0.001 ± 0.001</td>
<td>97 ± 1</td>
</tr>
<tr>
<td></td>
<td>Electric rotating</td>
<td>0.003 ± 0.002</td>
<td>89 ± 7</td>
</tr>
<tr>
<td></td>
<td>Sonic</td>
<td>0.002 ± 0.001</td>
<td>92 ± 5</td>
</tr>
<tr>
<td>S. oralis J22</td>
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<td>0.04</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Manual</td>
<td>0.008 ± 0.002</td>
<td>92 ± 1</td>
</tr>
<tr>
<td></td>
<td>Electric rotating</td>
<td>0.006 ± 0.004</td>
<td>94 ± 2</td>
</tr>
<tr>
<td></td>
<td>Sonic</td>
<td>0.007 ± 0.004</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>A. naeslundii T14V-J1</td>
<td>control*</td>
<td>0.18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Manual</td>
<td>0.004 ± 0.003</td>
<td>98 ± 1</td>
</tr>
<tr>
<td></td>
<td>Electric rotating</td>
<td>0.005 ± 0.004</td>
<td>97 ± 1</td>
</tr>
<tr>
<td></td>
<td>Sonic</td>
<td>0.020 ± 0.009</td>
<td>89 ± 6</td>
</tr>
</tbody>
</table>

# = control experiment involving opening and closing of the flow chamber only.

Figure 3. Images of initially adhering A. naeslundii T14V-J1 after 2 h adhesion (A) and after 2 h adhesion followed by removal using a manual brush (B). Bar denotes 10µm.
Table 2. The fractional surface coverage (FSC) by *S. oralis* J22 and *A. naeslundii* T14V-J1 after overnight growth and brushing on saliva-coated glass and the percentage removal after brushing with three different brushing modes. The percentage removal is not corrected for handling of the flow chamber and includes brushing, opening and closing of the flow chamber. Furthermore the FSC by newly deposited bacteria and the percentage FSC regained after re-deposition is shown. %Removal and %regained are expressed with respect to the coverage by adhering bacteria after growth. For both bacterial strains, the FSC was 100% after growth.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Toothbrush</th>
<th>After brushing</th>
<th>Redeposited</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FSC</td>
<td>% removal</td>
</tr>
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<td><em>S. oralis</em> J22</td>
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<td>57</td>
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<tr>
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<td>Manual</td>
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<td>95 ± 1</td>
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<td></td>
<td>Electric rotating</td>
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<td>93 ± 3</td>
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<td></td>
<td>Sonic</td>
<td>0.08 ± 0.04</td>
<td>92 ± 4</td>
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<tr>
<td><em>A. naeslundii</em> T14V-J1</td>
<td>control*</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Manual</td>
<td>0.24 ± 0.13</td>
<td>76 ± 13</td>
</tr>
<tr>
<td></td>
<td>Electric rotating</td>
<td>0.34 ± 0.02</td>
<td>66 ± 2</td>
</tr>
<tr>
<td></td>
<td>Sonic</td>
<td>0.39 ± 0.14</td>
<td>61 ± 14</td>
</tr>
</tbody>
</table>

* = control experiment involving opening and closing of the flow chamber only.

Figure 4. Images of adhering *A. naeslundii* T14V-J1 after growth (A) and after growth followed by removal using a electric rotating brush (B). Bar denotes 10µm.
Discussion

In this study, we compared the efficacy of three different modes of brushing on bacterial removal and re-deposition of single strain bacteria from salivary pellicle surfaces, after 2 h adhesion as well as after 2 h adhesion followed by growth. No differences in percentage removal were observed between the brushes which could be explained by the fact that brushing was done in a contact mode, which is usually highly effective in all modes (Van der Mei et al., 2007). Re-deposition did not result in differences between the brushes, suggesting that the effect of footprints is probably not of influence in single strain experiments. This is in contrast to the influence found in the co-adhesion study by Van der Mei et al. (Van der Mei et al., 2004), where re-deposition increased with increasing weight and was influenced by the type of brush.

Interestingly, despite the fact that no differences were observed between the different brushing techniques, remarkable differences in binding strength between the bacterial strains were observed, as measured by the percentage removal, shown in Tables 1 and 2. The lower the percentage removal, the stronger the binding strength. The binding strength increased in the following order: *A. naeslundii* T14V-J1 > *S. oralis* J22 > *S. mutans* NS. Moreover, after re-deposition the percentage regained was significantly lower for *S. oralis* J22 than for *S. mutans* NS or *A. naeslundii* T14V-J1, which both return to around 100%. The lower surface coverage for *S. oralis* J22 after 2 h re-deposition compared to 2 h deposition before brushing, suggests the removal of specific adhesins in the pellicle by brushing.

Adhesion of bacteria to a pellicle-coated surface is modulated by a multitude of proteins present in whole saliva. Bacteria express different strain specific adhesins, entailing the complexity of bacterial colonization. Although the adhesion of *S. mutans* is promoted by high molecular-weight proteins, agglutinins, these are also responsible
for aggregation (Carlen and Olsson, 1995; Carlen et al., 1996), yielding weak hydrophobic interactions as the main adhesion force (Nieuw Amerongen et al., 2004). In our experiments we have observed large surface aggregates for the *S. mutans* strain and these aggregates were easily removed from the surface, even by opening of the flow chamber. This can be explained by the fact that the binding strength within the aggregates is stronger than their adhesion to the pellicle. The weak binding strength of *S. mutans* NS to salivary pellicles may reflect its characteristic as a late colonizer of dental hard surfaces *in vivo* and therewith its absence in the composition of initial plaque (Nyvad and Kilian, 1990). *S. oralis* J22 and *A. naeslundii* T14V-J1 are early colonizers. In a very early stage of the oral biofilm formation, *Actinomyces* are predominantly present. After 2 h, *Streptococci* as e.g. *S. oralis* appear, and increase their relative presence at the expense of *Actinomyces*, although the absolute level of *Actinomyces* remains unaltered (Ramberg et al., 2003; Li et al., 2004). Interaction with the salivary pellicle mucin MG2, proline rich proteins and proline rich glycoproteins promotes adhesion of *S. oralis* and *A. naeslundii* (Murray et al., 1992; Ruhl et al., 2004). In addition, *A. naeslundii* possesses phosphoprotein-binding type 1 fimbriae, which are important adhesins in modulating adhesion (Cisar et al., 1984; Carlen et al., 2004; Ruhl et al., 2004) and binding strength (Tang et al., 2004). These are likely to be involved in the stronger adhesion of *Actinomyces* (see Tables 1 and 2) as compared with adhesins of *Streptococci* (Prakobphol et al., 1995; Sharma et al., 2005).

In conclusion, the choice of a given bacterial strain is of great importance in *in vitro* studies on mechanical plaque removal, as different strains of early colonizing bacteria clearly have different binding strengths to the salivary pellicle. In the present study no significant difference could be demonstrated between manual and powered
contact-brushing in the contact removal or re-deposition of bacteria on pellicle coated surfaces.
Reference List


