Bacterial adhesion forces and biofilm prevention on orthodontic materials

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Chapter 2

Oral bacterial adhesion forces to biomaterial surfaces constituting the bracket-adhesive-enamel junction in orthodontic treatment

Abstract

Bacterial adhesion to biomaterial surfaces constituting the bracket-adhesive-enamel junction represents a growing problem in orthodontics, because bacteria can adversely affect treatment by causing demineralization of the enamel surface around the brackets. It is important to know the forces with which bacteria adhere to the surfaces of these junction materials, as the strength of these forces will determine how easy it will be to remove the bacteria. We compared the adhesion forces of five initially colonizing and four cariogenic strains of bacteria to an orthodontic adhesive, stainless steel, and enamel, with and without a salivary conditioning film. Adhesion forces were determined using atomic force microscopy and a bacterial probe. In the absence of a salivary conditioning film, the strongest bacterial adhesion forces occurred to the adhesive surface (-2.9 to -6.9 nN), while adhesion forces to the enamel surfaces were lowest (-0.8 to -2.7 nN). In the presence of a salivary conditioning film, adhesion forces were reduced strongly, to less than 1 nN, and the differences between the various materials were reduced. Generally, however, initial colonizers of dental hard surfaces presented stronger adhesion forces to the different materials (-4.7 and -0.6 nN in the absence and presence of a salivary conditioning film, respectively) than cariogenic strains (-1.8 and -0.5 nN).
Introduction

In the last decades, orthodontic treatment has received increasing popularity in juveniles and adults alike. A variety of materials has been introduced into the orthodontic practice in order to improve clinical efficiency of the treatment and patient comfort. Stainless steel and light-cured composite resin are the most frequently used materials for brackets and adhesives in orthodontics\(^1\)-\(^3\). Despite recent advances in the development of orthodontic materials, the prevalence of enamel demineralization at the bracket-adhesive-enamel junction has not decreased\(^4\)-\(^7\) and orthodontic materials still create a favorable substratum for biofilm formation\(^8\). The formation of biofilms exacerbates pre-existing periodontal diseases, and causes enamel decalcification, affecting about 50% of all patients undergoing orthodontic treatment\(^9\)-\(^11\).

Initial bacterial adhesion to the bracket-adhesive-enamel junction is a crucial step in orthodontic-induced enamel decalcification\(^12\). Subsequent growth of initially adhering bacteria incompletely removed by tooth brushing, eventually leads to a pathogenic oral biofilm. Organic acids produced by adhering bacteria result in enamel demineralization and compromise orthodontic treatment outcome. A number of previous studies have investigated bacterial adhesion and biofilm formation in orthodontics\(^2\)-\(^4\),\(^8\),\(^13\)-\(^18\). However, these studies mainly focused on quantitative and qualitative assessment of biofilm accumulation on orthodontic appliances. Till date, the direct adhesion forces of oral bacteria to the different materials constituting the bracket-adhesive-enamel junction are poorly understood.

In order to prevent and control biofilm accumulation during orthodontic treatment, it is essential to understand the mechanisms of oral bacterial adhesion to orthodontic biomaterial surfaces. Therefore, the present study was aimed to investigate the adhesion forces of both initial colonizers (\textit{Streptococcus mitis}, \textit{Streptococcus sanguinis}, \textit{Streptococcus oralis} and \textit{Actinomyces naeslundii})\(^19\) and cariogenic bacterial strains (\textit{Streptococcus sobrinus}, \textit{Streptococcus mutans} and
Lactobacillus acidophilus to orthodontic materials, including enamel using atomic force microscopy (AFM), a powerful tool to measure microbial adhesion forces with substratum surfaces. In addition, the hydrophobicities of the orthodontic materials constituting bracket-adhesive-enamel junction were determined from water contact angle measurements. Orthodontic materials and enamel were used with and without an adsorbed salivary conditioning film.

Materials and Methods

Orthodontic materials

Transbond™ XT, used for bonding brackets to enamel surfaces, is a light-cured adhesive (3M Unitek, California, USA). The adhesive was made into 1 cm diameter disks with a thickness of 1 mm. Adhesive discs were cured following the manufacturers’ instructions and light activated with a halogen lamp (Optilux 501 Curing Light, Danbury, CT 06810-4153, USA) for 20 s. For maximal flatness of the adhesive surface and convenience of removal after setting, the adhesive was shaped between two glass plates, covered with copier overhead films (MC 110, Oce’, The Netherlands). No additional surface polishing procedure was carried out.

Stainless steel 316 (Stryker Corp, Kiel, Germany), used for orthodontic brackets, was machined into 1 cm diameter discs. Subsequently, surfaces were polished with a diamond polishing paste of decreasing particle size from 14 to 0.05 μm. After polishing, stainless steel samples were cleaned by demineralized water and ultrasonication.

Enamel slabs were cut from labial surfaces of bovine incisors. First, an incisor was grind under running tap water with 220 to 1200 grit sandpapers into 0.6 x 0.6 cm² samples, with a thickness of 2 mm. Subsequently, the enamel surfaces were micropolished on a polishing pad with wet, 0.05 μm alumina particles (Buehler Ltd., USA) for 3 min. Polished enamel discs were cleaned by 2 min ultrasonication in 35
kHz ultrasonic bath (Transsonic TP 690-A, Elma, Germany), and thoroughly rinsed with demineralized water.

**Oral bacterial strains and growth conditions**

Nine different bacterial strains, listed in Table 1 were used in this study. Strains numbered 1 to 5 are initial colonizers of dental hard surfaces; strains numbered 6 to 9 are considered cariogenic. For each experiment, bacteria were first grown from a frozen stock on blood agar plates by incubation during 24 h at 37°C in ambient air. Then, a fresh colony was inoculated into 10 mL of the appropriate growth medium, as also listed in Table 1, at 37°C for 24 h. Subsequently, bacteria were inoculated into 200 mL growth medium at 37°C for 16 h. Bacteria were harvested by centrifugation (5 min, 5000 g, 10°C), washed twice with demineralized water, and re-suspended in demineralized water. Finally, the bacterial suspensions were intermittently sonicated to break the chains or aggregates in an ice/water bath for 3 x 10 s at 30 W.

**Saliva collection and preparation**

Human whole-saliva from 20 healthy volunteers of both sexes was collected into ice-chilled Erlenmeyer flasks after stimulation induced by chewing Parafilm® (Pechiney, Plastic Packaging, Menasha, USA), as described previously. After the saliva was pooled and centrifuged two times (10,000 g, 15 min, 4°C), phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM as a protease inhibitor. Afterwards, the solution was centrifuged again, dialyzed (24 h, 4°C) against demineralized water, and freeze-dried for storage. All volunteers gave their informed consent for saliva donation, in agreement with the rules set out by the Ethics Committee at the University Medical Center Groningen. For each experiment, the lyophilized saliva was dissolved in adhesion buffer (50 mM potassium chloride, 2 mM potassium phosphate, 1 mM calcium chloride, pH 6.8) at
<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Growth Medium</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. acidophilus</td>
<td>Todd Hewitt Broth</td>
<td>Own clinical isolate JP</td>
</tr>
<tr>
<td>S. mutans</td>
<td>Todd Hewitt Broth</td>
<td>Own clinical isolate NS</td>
</tr>
<tr>
<td>S. mutans</td>
<td>Todd Hewitt Broth</td>
<td>American Type Culture Collection ATCC700610</td>
</tr>
<tr>
<td>S. sobrinus</td>
<td>Todd Hewitt Broth</td>
<td>American Type Culture Collection ATCC9070610</td>
</tr>
<tr>
<td>A. naeslundii</td>
<td>Todd Hewitt Broth</td>
<td>Clinical isolate ACTA, The Netherlands HG1025</td>
</tr>
<tr>
<td>S. oralis</td>
<td>Todd Hewitt Broth</td>
<td>American Type Culture Collection ATCC10966</td>
</tr>
<tr>
<td>S. sanguinis</td>
<td>Todd Hewitt Broth</td>
<td>American Type Culture Collection ATCC9088</td>
</tr>
<tr>
<td>S. mitis</td>
<td>Todd Hewitt Broth</td>
<td>Own clinical isolate BMS</td>
</tr>
<tr>
<td>S. mitis</td>
<td>Todd Hewitt Broth</td>
<td>Clinical isolate ACTA, The Netherlands HG1025</td>
</tr>
</tbody>
</table>

Table 1. Bacterial strains involved in this study together with their respective growth media and cultures conditions. Strains 1-5 are initial colonizers of dental hard surfaces, while strains 6-9 are considered cariogenic strains.

1) Todd Hewitt Broth: OXOID, Basingstoke, United Kingdom.
2) Schaedler’s broth: in one liter: 5.6 g tryptone, 1.0 g proteose peptone, 5.0 g phytonpe peptone, 5.0 g yeast extract, 1.7 g NaCl, 1.5 g K2HPO4, 1.0 g KH2PO4, 0.4 g L-cysteine-HCl-2H2O, 2.5 g (D+)glucose, pH 7.3 - 7.6.
3) Tryptone Soya Broth: OXOID; supplemented with 0.01 g hemin, pH 7.3 - 7.6.
4) MRS Broth: Merck, Germany.
5) Todd Hewitt Broth: OXOID; supplemented with 0.01 g hemin, pH 7.3 - 7.6.
6) Todd Hewitt Broth: Todd Hewitt Broth; pH 7.3 - 7.6.
7) Todd Hewitt Broth: Todd Hewitt Broth; pH 7.3 - 7.6.
8) Todd Hewitt Broth: Todd Hewitt Broth; pH 7.3 - 7.6.
9) Todd Hewitt Broth: Todd Hewitt Broth; pH 7.3 - 7.6.

| Table 1. | Bacterial strains involved in this study together with their respective growth media and cultures conditions. Strains 1-5 are initial colonizers of dental hard surfaces, while strains 6-9 are considered cariogenic strains. |
a concentration of 1.5 g/L. The experimental discs, made out of the orthodontic materials studied, including enamel, were immersed into the reconstituted saliva for 16 h to create a salivary conditioning film. After 16 h, all saliva-conditioning film-coated materials were dipped three times in demineralized water and used immediately in the experiments.

**AFM adhesion force and surface roughness measurements**

A tipless cantilever, model no. CSC12/tipless/no Al (Ultrasharp, µ-Masch, Estonia), was immersed in a drop of 0.01% (w/v) sterile filtered poly-L-lysine (Sigma-Aldrich, UK) solution for 1 min with a micromanipulator (Leica, Wetzlar, Germany). Subsequently, the cantilever was dried in air for 2 min. Then, cantilevers were immersed into a drop of a bacterial suspension for 1 min to immobilize bacteria onto the cantilever\(^{24}\), to form a bacterial probe. All procedures were viewed under an optical microscope. All AFM cantilevers with immobilized bacteria were used for experiments immediately after preparation.

Adhesion force measurements were performed at the room temperature in adhesion buffer (pH 6.8) using a Dimension 3100 system (Nanoscope IIIA Digital Instrument, Woodbury, NY) in the contact mode. Force measurements were initiated by engaging the AFM bacterial probe with the substratum surface at a scan rate of 0.5 Hz and ramp size 1.5 µm. The loading force was applied by setting the trigger mode to relative, *i.e.* a trigger threshold of 1 V. Ten force measurements were made at each site; three locations were selected for the force measurement on each material surface. Cantilever deflection data were converted to force values (nN) by multiplying with the cantilevers spring constant according to Hooke’s law:

\[
F = K_{sp} \cdot D
\]

in which \(K_{sp}\) is the cantilever spring constant; \(D\) is the cantilever deflection. For each experiment, the cantilever spring constant was determined using the thermal method\(^{25}\).
In addition, the AFM was used to determine the surface roughness of the orthodontic materials, including enamel, with and without a salivary conditioning film. This was done in the contact mode with a silicon nitride cantilever tip (DNP, Veeco, Woodbury, NY). Five samples of each material were measured. Each sample was imaged at three randomly selected sites and surface plots were made to provide a three-dimensional perspective of the surface, from which the mean surface roughness ($R_a$) was calculated. $R_a$ represents the average distance from the roughness profile to the center plane of the profile.

**Water contact angles**

Water contact angles were measured on all materials at 25°C using the sessile drop technique in combination with a home-made contour monitor. The monitor registers the contour of a water droplet based on grey-value threshold. Contact angles are calculated from the height and base width of the droplet. For water contact angle measurements on saliva-coated materials, samples were first air-dried to a so-called plateau level until the contact angles became constant in time. For biological surface, like protein-coated surface, it was assumed to correspond to removal of all free water and to represent physiologically relevant contact angle\(^{26}\).

**Statistics**

Contact angles and surface roughnesses were normally distributed (Shapiro-Wilk test, $P > 0.05$) and therefore are presented as mean ± SD; between-group comparisons were performed using ANOVA. Adhesion forces, which are not normally distributed (Shapiro-Wilk test, $P < 0.01$), are presented as median and interquartile range; and compared using non-parametric analyses (Kruskal-Wallis test), followed by Dunn's multiple-comparison post hoc analysis, when overall differences were significant. Differences were considered significant when the $P$-value was $< 0.05$. 

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Results

**Physicochemical characteristics of the orthodontic materials with and without saliva-coating**

Water contact angles ($\theta_w$) on the different materials in the absence of a salivary conditioning film varied between 30 ± 1 degrees for enamel to 71 ± 1 degrees for the adhesive (Table 2). The $\theta_w$ of three materials are in line with data published \(^{27,28}\). For stainless steel the values are in between the ones reported for as received and chemically cleaned 316L stainless steel. Note that stainless steel aggressively cleaned by plasma treatment is much more hydrophilic \(^{29}\). However, such cleaning conditions were not employed because they are clinically unrealistic. The $\theta_w$ changed significantly upon saliva-coating (P < 0.01), yielding non-significant difference of 23 ± 2 degrees for stainless steel and 26 ± 1 degrees for enamel.

A comparison of the surface roughness ($R_a$) showed that the adhesive had the highest $R_a$ (Table 2), followed by enamel and stainless steel. The $R_a$ of the adhesive was 28.5 ± 2.9 nm in the absence of a salivary conditioning film. All rough features of the adhesive surface disappeared upon saliva coating, which significantly decreased the $R_a$ of adhesive and enamel surfaces to 11.8 and 5.1 nm, respectively and increased the $R_a$ of stainless steel to 6.1 nm (P < 0.01).

**Table 2.** Water contact angles ($\theta_w$) and surface roughnesses ($R_a$) of orthodontic adhesive, stainless steel, and enamel with and without salivary conditioning film \(^1\).

<table>
<thead>
<tr>
<th></th>
<th>$\theta_w$ (degrees) (^\circ)</th>
<th>$R_a$ (nm) (^\circ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No saliva</td>
<td>With saliva</td>
</tr>
<tr>
<td>Adhesive</td>
<td>71 ± 1 (^{S,E})</td>
<td>25 ± 3 (^*)</td>
</tr>
<tr>
<td>Stainless steel</td>
<td>68 ± 1 (^{A,E})</td>
<td>23 ± 2 (^*)</td>
</tr>
<tr>
<td>Enamel</td>
<td>30 ± 1 (^{S,A})</td>
<td>26 ± 1 (^*)</td>
</tr>
</tbody>
</table>

\(^*\) Indicates significant difference (P < 0.01) between uncoated and saliva-coated materials. \(^1\) All data represent mean ± standard deviation from five samples. \(^2\) The superscript A, M, and E denote that data for these materials are significantly (P < 0.01) different from those of adhesive (A), stainless steel (S), or enamel (E).
**Force-distance curves and adhesion forces using bacterial probes**

Fig. 1 shows examples of force-distance curves between a bacterial probe (S. mitis BMS) upon approach and retract from the three materials studied, in the absence and presence of a salivary conditioning film. Differences between the three materials and effects of the saliva coating become clear upon retract. Adhesion forces are strongest for the adhesive and weakest for enamel in the absence of a salivary coating, while upon saliva coating of the materials these differences disappear and the adhesion forces become weaker.

![Force-distance curves](image)

**Figure 1.** Example of force-distance curves between Streptococcus mitis BMS with different materials constituting bracket-adhesive-enamel junction, in the absence (panel A) and presence (panel B) of a salivary conditioning film.

Fig. 2 summarizes the distribution of the adhesion forces between S. oralis J22 and the different biomaterial surfaces in the absence and presence of a salivary conditioning film, and clearly indicates a non-parametric, skewed distribution. All other strains displayed such skew distributions. Therefore, median values for the adhesion forces and their interquartile ranges were summarized for all strains and materials and are presented in Fig. 3. It is unknown whether the non-parametric distribution of the adhesion forces is caused by an inherent heterogeneity on the surface of a single cell\(^{30}\), or whether it represents population heterogeneity.
Adhesion forces of bacteria on material surfaces without a salivary conditioning film were almost 10-fold higher than in the presence of a conditioning film \( (P < 0.01) \), except for \textit{S. mutans} NS and \textit{L. acidophilus} JP on enamel. In the absence of a salivary conditioning film (Fig. 3A), the adhesive surface exerted the strongest adhesion forces, followed by stainless steel and enamel surfaces in all nine bacterial strains \( (P < 0.05) \). In the presence of a salivary coating, differences between the adhesion forces exerted by the three materials became smaller; although significant strain-specific differences were still present (Fig. 3B).

\textbf{Figure 2.} Example of skew distributions of adhesion forces between \textit{Streptococcus oralis} J22 and the different materials in the absence (A, B, C) and presence (D, E, F) of a salivary conditioning film. (A, D) adhesive, (B, E) stainless steel, (C, F) enamel. Note the scales of X-axes in (A, B, C) are 10 times larger than in (D, E, F).
**Figure 3.** Adhesion forces between different bacteria and different materials in the absence (A) and presence (B) of a salivary conditioning film. *, # indicate significant differences at P<0.01 and P<0.05 with the other two materials for the same strain unless indicate differently. Note the scale of Y-axis in (A) is 10-time larger than (B).

**Adhesion forces of initial colonizers and cariogenic bacteria**

Adhesion forces of the initial colonizers (strains 1-5, Table 1) and the cariogenic bacteria (strains 6-9) were pooled for each bacterial group and all materials, yielding the skewed distributions (Shapiro-Wilk test, P < 0.01) presented in Fig. 4. In the absence of a salivary conditioning film, the initial colonizers had significantly stronger (P < 0.01) adhesion forces (-4.7 nN) than cariogenic bacteria (-1.8 nN). A similar statistical significant difference (P < 0.01) existed in the presence of a salivary conditioning film, although the difference between initial colonizers (-0.6 nN) and cariogenic strains (-0.5 nN) had become much smaller (Table 3).
Table 3. Adhesion forces (nN) between initial colonizers and cariogenic bacteria with the surfaces involved in the bracket-adhesive-enamel junction †.

<table>
<thead>
<tr>
<th></th>
<th>Initial colonizers</th>
<th>Cariogenic strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*,**,§</td>
<td>*,**,§</td>
</tr>
<tr>
<td>No salivary coating</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adhesive</td>
<td>-6.9 (5.5)</td>
<td>-2.9 (2.9)</td>
</tr>
<tr>
<td>Stainless steel</td>
<td>-5.2 (5.3)</td>
<td>-1.8 (1.5)</td>
</tr>
<tr>
<td>Enamel</td>
<td>-2.7 (3.3)</td>
<td>-0.8 (1.1)</td>
</tr>
<tr>
<td>A + S + E ‡</td>
<td>-4.7 (5.2)</td>
<td>-1.8 (2.1)</td>
</tr>
<tr>
<td>With salivary coating</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adhesive</td>
<td>-0.5 (0.3)</td>
<td>-0.4 (0.3)</td>
</tr>
<tr>
<td>Stainless steel</td>
<td>-0.5 (0.3)</td>
<td>-0.5 (0.2)</td>
</tr>
<tr>
<td>Enamel</td>
<td>-0.7 (0.5)</td>
<td>-0.6 (0.5)</td>
</tr>
<tr>
<td>A + S + E ‡</td>
<td>-0.6 (0.4)</td>
<td>-0.5 (0.3)</td>
</tr>
</tbody>
</table>

* indicates significant difference (P < 0.01) between uncoated and saliva-coated materials. ** indicates significant difference between initial and cariogenic strains. † All data represent median and interquartile range (within parenthesis) over 90 force-distance curves, taken with nine bacterial probes and nine samples. ‡ A+S+E indicates the pooled data for the adhesive, stainless steel, and enamel. § The superscript letters A, S, and E denote that data for these materials are significantly different from adhesive (A), stainless steel (S), and enamel (E).

Figure 4. Skew distribution of adhesion forces of initial colonizers (A, C) and cariogenic bacteria (B, D) as obtained by averaging the results over all materials included in the study, but split for the absence (A, B) or presence (C, D) of a salivary conditioning film. Note the scales of X-axis in panels A, and B are 10 times larger than in panels C, and D.
Discussion

The development of orthodontic biomaterials that attract less biofilm has been a goal for decades, but is hampered by a lack of knowledge of the fundamental aspects of bacterial adhesion to the different materials constituting the bracket-adhesive-enamel junction. The present study, for the first time, directly measured the adhesion forces of different oral bacterial strains to the different materials making up the bracket-adhesive-enamel junction. In the absence of a salivary conditioning film, the strongest bacterial adhesion forces were measured to the adhesive surface, while adhesion to stainless steel was slightly weaker. Bacterial adhesion forces were lowest to enamel surfaces in the absence of a salivary conditioning film. In the presence of a salivary conditioning film, adhesion forces were strongly reduced, and the differences between the various materials became small, despite their statistical significance. However, in general, strains considered to be initial colonizers of dental hard surfaces yielded stronger adhesion forces to the different materials than more cariogenic strains, both in the absence and presence of a salivary conditioning film.

The observation that the presence of a salivary conditioning film reduced the oral bacterial adhesion forces with the underlying surfaces with respect to adhesion forces with a bare surface, is in line with the protective function generally ascribed to salivary conditioning films, and is despite the fact that initial colonizers continue to have the ability to adhere to salivary conditioning films. Pratt-Terpstra et al. compared oral bacterial adhesion to substrata with different surface free-energies and also noticed that the presence of a salivary conditioning film strongly reduced the numbers of adhering bacteria, probably as a corollary of the reduced adhesion forces in the presence of a salivary conditioning film measured in the current study. Moreover, the differences between the numbers of bacteria adhering for different strains became smaller in the presence of a salivary conditioning film,
in line with the convergence in adhesion forces among different strains found in this study after formation of the salivary conditioning film.

The influence of the different surface chemistries of the bare materials on bacterial adhesion forces not only becomes evident from the different adhesion forces found on stainless steel, adhesive and enamel, but also from the impact that the formation of salivary conditioning film has on the bacterial adhesion forces. In a clinical situation, it is generally accepted that bacteria never adhere in the absence of a salivary conditioning film. Along the same lines, although it is known that brushing does not fully remove the salivary conditioning film, it is unknown to what extent the conditioning film becomes thinner and whether regions more or less devoid of conditioning films arise locally with an influence on bacterial adhesion forces. Moreover, the composition of salivary conditioning films is also affected by the chemistry of the underlying surface with an impact on the bacterial adhesion forces. In the current study, for instance, this can be inferred from the fact that the adhesion forces on enamel, stainless steel, and adhesive surfaces are slightly different despite their salivary coating. It is noticeable that, depending on the strains involved, the AFM loading force, and thus the contact area created between the bacterial cell and substratum surfaces, highly specific interactions may occur between oral bacteria and a salivary conditioning film. S. mutans LT11 with antigen I/II on its outer cell surface, for instance, showed interaction forces of -3.0 nN with a salivary coating identical to the ones used in this study, whereas the strains in our current study had interaction forces limited to around -0.5 nN, indicating the absence of specific interactions.

Apart from possessing different chemistries, the three materials involved in this study also possessed different roughness and hydrophobicity (Table 2), and the strongest adhesion force in the absence of a saliva conditioning film was found on the roughest and most hydrophobic surface. In line with this, adhesion forces became smaller upon coating the materials with a salivary film, creating a more
hydrophilic surface. Stronger adhesion forces may arise for more hydrophobic surfaces, because water is more easily removed from the area between the cell surface and a hydrophobic material than from the area between the cell surface and a hydrophilic material, enabling a closer approach and thus stronger adhesion forces. Similarly, it can be envisaged that a rougher surface will have more extensive contact points with the cell surface, which will also contribute to stronger adhesion forces.

Regardless of the absence or presence of a salivary conditioning film, initial colonizers adhered with stronger adhesion forces than the more cariogenic strains included in this study. *In vivo*, oral biofilm development follows a well-defined spatio-temporal pattern\(^3\), in which later colonizers, like the more cariogenic strains involved here, do not adhere directly to the substratum surface but to already adherent initial colonizers. In order to ensure adhesion of such a multispecies biofilm on oral surfaces, the initial colonizers must adhere more strongly to the substratum than later colonizers which, judging from the results of this study, they indeed do. It might appear surprising that differences in the sub-nN range, as existing between the adhesion forces of the current collection of strains with a salivary conditioning film, might be related to their classification as initial and later colonizers *in vivo*. Indeed, we do not fully understand the relevance of these minor adhesion force differences. However, it has been suggested, based on force differences observed in a collection of *Staphylococcus aureus* strains\(^4\), that a microorganisms “force taxonomy” may provide a fundamental and practical indicator of the pathogen-related risk that infections pose to patients. Invasive strains distinguished themselves from control strains by a sub-nN force difference that was only 0.28 nN higher than found in the control strains.

In conclusion, initial colonizers of dental hard surfaces generally presented stronger adhesion forces than more cariogenic strains, appearing later in the process of oral biofilm formation, regardless of the absence or presence of a salivary conditioning film or the material involved. Bacteria adhered more strongly
to adhesive and stainless steel surfaces than to enamel surface in the absence of a salivary conditioning film. These differences in bacterial adhesion forces to the biomaterials making up the bracket-adhesive-enamel junction in orthodontic treatment became smaller after the salivary conditioning film was formed, while bacterial adhesion forces measured on adhesive and stainless steel were slightly smaller than on enamel. These types of measurements have not yet been described in the literature and may provide a framework for understanding the adhesive events leading to oral biofilm formation and its prevention. The results, however, clearly demonstrate that preventive measures based on material surface modification will be hampered by the formation of a salivary conditioning film, which makes the difference between the materials surface properties smaller and therewith the adhesion forces with oral bacteria.
References