Adhesion and biofilm formation by oral streptococci on different commercial brackets

ABSTRACT

Aim To compare early bacterial adhesion and biofilm formation in vitro by different oral streptococci on a variety of commercial brackets.

Methods Adhesion and biofilm formation in vitro of 6 Streptococcus spp. on 15 different commercial brackets, in standard culture medium and in human saliva were evaluated by the MTT reduction assay.

Results Significant differences were evidenced in both early adhesion and biofilm formation among the studied brackets and between the two conditions of growth. Gold brackets resulted less prone to colonisation compared to composite brackets. The growth rates of the tested species on the different tested materials were significantly different.

Conclusion The adopted experimental plan, dissecting the two phases of plaque formation on different brackets in different conditions, showed that composite brackets are more susceptible to adhesion and colonisation by streptococci, while the remaining tested brackets did not show differences that could be clinically relevant. Data suggest that different personal behaviours affecting the oral environment could significantly affect colonisation of brackets by oral streptococci.

Keywords Bacterial adhesion; biofilm formation; brackets.

Introduction

In recent decades orthodontics has undergone a great increase in its diffusion, mostly due to patients seeking orthodontic treatment to improve their dentofacial aesthetics, while only a minority require treatment for medical or dental reasons [Shaw, 1991; Harris 2011]. Such a situation, in turn, greatly improved the necessity for orthodontic treatment to be safe and free from side effects. Among short- and long-term complications of fixed orthodontic treatment, those related to increased formation of dental plaque are of great relevance [vanGastel, 2007]. Placement of fixed orthodontic appliances induces significant ecological changes affecting the composition, metabolic activity, and pathogenicity of the oral microbiota, thus favouring the incidence of periodontal inflammation and incipient carious lesions [Atack et al., 1996; Naranjo et al., 2006; Ahn et al., 2007; van Gastel et al., 2008].

While ecological alterations and periodontal inflammation are considered to be largely reversible in children and adolescents [Ristic et al., 2007], incipient caries results at least in the formation of persisting white spot lesions [Øgaard, 1989; Sallum et al., 2004] that contrast with aesthetic requirements of patients and their parents. Enamel decalcification and caries formation, due to increased prevalence of Streptococcus mutans and Lactobacillus species in dental plaque have been extensively studied as a complication of fixed orthodontic treatment in children [Forsberg, 1991; Rosenbloom, 1991]. Although once believed to be essentially a consequence of quantitative alteration of the dental microbiota [Boyd, 1983], this side-effect is also caused by the specific properties of bracket materials, affecting the quality of dental microbiota [Fournier et al., 1998; Anhoury et al., 2002; Brusca et al., 2007; Petti et al., 1997].

These evidences prompted researchers to try to develop materials less susceptible to bacterial colonisation thus minimizing plaque accumulation around brackets and other fixed appliances [vanGastel et al., 2007; van Gastel et al., 2009; Faltermeier et al., 2008; Ahn et al., 2007; Papaioannou et al., 2007].

With few exceptions, these studies addressed the problem of adhesion of cariogenic bacteria to bracket materials, while less attention was dedicated to other oral streptococci. This study, in consequence, was aimed to compare early bacterial adhesion and biofilm formation in vitro by different oral streptococci on a variety of different commercial brackets.

Materials and methods

Brackets

Fifteen commercially available brackets, made of different materials, were used (Table 1). All brackets
were maxillary premolar brackets, with the Roth prescription and a 0.022-inch slot. Twelve brackets for each bacterial strain were tested.

**Bacterial strains and cultures**

Six reference strains of different species of oral streptococci were used: *Streptococcus salivarius* DSM20560, *Streptococcus gordonii* DSM6777, *Streptococcus sanguinis* DSM20567, *Streptococcus oralis* DSM20627, *Streptococcus mutans* DSM20523 and *Streptococcus sobrinus* DSM20742. All strains were maintained in stock cultures freezed at -80°C in Tryptic Soy broth (TSB) containing glycerol (20% v/v). For adhesion assays, isolated colonies of each strain were inoculated in TSB and incubated at 37°C with mild shaking till the mid logarithmic phase of growth. Bacterial cells were then collected by centrifugation and suspended in fresh sterile 0.5xTSB (i.e. TSB diluted 1/2 in PBS pH 7.2) or sterile 0.5xSaliva (i.e. human pooled saliva diluted 1/2 in TSB) at an OD600nm = 0.1. Saliva was obtained by paraffin stimulation from 15 healthy volunteers (having refrained from eating and drinking in the previous 2 hours) and checked for pH being in the range 7.0 to 7.3. Saliva samples underwent sonication (1 minute at 30W with refrigeration), the were filtered through a 70µm filter (Cell Strainer, Becton Dickinson Italia, Buccinasco, Italy) and centrifuged at 22,000 x g for 60 minutes at 4°C. Supernatants were pooled, sterlised by sequential filtration through 0.45 µm and 0.2 µm filters, stored at 4°C and used within the next 48 hours.

**Adhesion assays**

In order to perform standardised adhesion assays, brackets were mounted on 0.6 x 0.6 cm polished clear acrylic blocks (K-Mac Plastics Wyoming, MI, USA) stucked to the cover of a 24-well polystyrene plate. The entire mounting process was performed by a single operator inside a sterile class II biohazard cabinet. The central region of each block, in the exact position were a bracket had to be fixed, was roughened with a diamond coated bur in such a manner that these areas were completely covered by the bracket bases. The brackets were then bonded with Transbond Plus color change adhesive (3M Unitek, Monrovia, CA, USA). Excesses of adhesive were carefully removed and the composite was light-cured for 30 seconds from both sides. Brackets mounted this way were completely immersed when each well was filled with 1.1 ml of bacterial suspension.

Before contact with the bacterial cultures, brackets were placed in 24-well plates containing either sterile 0.5xTSB or sterile 0.5xSaliva and incubated at 37°C for 1 hour. Pre-conditioned brackets were then transferred to a new plate with wells filled with the bacterial suspension in the same medium, and incubated for 4 and 48 hours at 37°C on an orbital shaker at 60 RPM. Following contact with the different bacterial suspensions, the brackets were removed with a sterile pliers and transferred into an adequately coded well with a flat bottom 96-well plate containing 0.1 ml of sterile PBS. Brackets were then washed 5 times with sterile PBS and further processed for the enumeration of adherent bacteria by the MTT reduction assay.

**Quantification of adherent bacteria by the MTT reduction assay**

The amount of bacteria adherent to each bracket was determined by the MTT-reduction assay [Kairo et al., 2007].

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**TABLE 1** List of brackets used during the study, their identification keys in the text and results section, manufacturer and construction material.

<table>
<thead>
<tr>
<th>IDENTIFICATION</th>
<th>BRACKET</th>
<th>MANUFACTURER</th>
<th>MATERIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Clarity Advanced Ceramic</td>
<td>3M Unitek</td>
<td>Ceramic</td>
</tr>
<tr>
<td>B</td>
<td>Ceramic Bracket</td>
<td>Dentsply</td>
<td>Ceramic</td>
</tr>
<tr>
<td>C</td>
<td>Fascination 2</td>
<td>Dentaureum</td>
<td>Ceramic</td>
</tr>
<tr>
<td>D</td>
<td>Enhance Ceramic</td>
<td>Ortho Specialties</td>
<td>Ceramic</td>
</tr>
<tr>
<td>E</td>
<td>Victory Series</td>
<td>3M Unitek</td>
<td>Stainless Steel</td>
</tr>
<tr>
<td>F</td>
<td>Stainless Steel Bracket</td>
<td>Dentsply</td>
<td>Stainless Steel</td>
</tr>
<tr>
<td>G</td>
<td>Equilibrium 2</td>
<td>Dentaureum</td>
<td>Stainless Steel</td>
</tr>
<tr>
<td>H</td>
<td>Gold Victory Series</td>
<td>3M Unitek</td>
<td>Gold</td>
</tr>
<tr>
<td>I</td>
<td>Regency Gold</td>
<td>Ortho Specialities</td>
<td>Gold</td>
</tr>
<tr>
<td>J</td>
<td>Clear Brackets</td>
<td>Dentsply</td>
<td>non-polycarbonate plastic</td>
</tr>
<tr>
<td>K</td>
<td>Elegance</td>
<td>Dentaureum</td>
<td>Polycarbonate</td>
</tr>
<tr>
<td>L</td>
<td>Comp Plus T</td>
<td>Ortho Specialties</td>
<td>Composite</td>
</tr>
<tr>
<td>M</td>
<td>Equilibrium Ti</td>
<td>Dentaureum</td>
<td>Titanium</td>
</tr>
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<td>GEM Monocrystalline</td>
<td>Ortho Specialties</td>
<td>Monocrystalline Sapphire</td>
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<tr>
<td>P</td>
<td>Pure</td>
<td>Ortho Technology</td>
<td>Monocrystalline Sapphire</td>
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</tbody>
</table>
al., 1999; Walencka et al., 2006]. Briefly, each bracket-containing well was carefully emptied of any residual liquid, and 0.15 ml of PBS were added, followed by 0.05 ml of MTT (Sigma Chemical Co. USA) (0.3% v/v in PBS). Samples were then incubated 2 hours at 37°C and MTT was replaced with 0.15 ml of dimethyl sulfoxide and 0.025 ml of glycine buffer (0.1 M, pH 10.2) for 15 minutes at room temperature. In this assay, bacteria with an active electron transport system reduce the pale yellow tetrazolium salt to water soluble purple formazan. The amount of formazan produced by each reaction was determined using a BioRad model 680 microplate reader at A\textsubscript{550}. Quantitative analysis was performed following construction of species specific standard curves for each tested strain.

**Statistics**

Statistic evaluation of the significance of differences among results of adhesion assays was performed by the Student T test available in the Microsoft Excel software. Differences yielding values of \( P \) in the range >0.01 to \( \leq 0.05 \) were considered significant while differences yielding values of \( P \leq 0.01 \) were considered very significant.

**Results**

Mean values of adherent streptococci detected at the surface of different brackets in adhesion and biofilm formation assays performed in TSB or saliva are reported in Figure 1. The 15 tested brackets were divided into 6 groups depending on material they were made of:

- ceramic (brackets A, B, C and D);
- stainless steel (brackets E, F and G);
- gold (brackets H and I);
- composites (brackets J, K and L);
- titanium (bracket M);
- monocrystalline sapphire (brackets N and P).

Results showed that streptococci are significantly different in their adhesiveness to the studied materials.

**FIGG. 1** Adherent bacteria detected at the surface of different brackets in adhesion (4h) and biofilm formation (48h) assays performed in Tryptic Soy broth (TSB) or in human saliva. Results are reported as means for brackets grouped according to construction material. Individual standard deviations are reported.
Streptococcus mutans and Streptococcus sobrinus constantly yielded among the highest values with all tested materials and conditions, while Streptococcus gordonii and Streptococcus sanguinis showed to be less adhesive. Adhesion assays performed in the presence of human saliva yielded significantly higher values for all tested species and materials, although the influence of saliva was different depending on both bacteria and materials. In fact, comparison of cumulative results of adhesion assays for the different materials showed values of \( P \) (obtained by the Student T test) for the comparison TSB vs. Saliva ranging 0.01 to 7.91x10^{-7} (ceramic \( P \)= 7.91x10^{-7}; stainless steel \( P \)= 3.25x10^{-3}; gold \( P \)= 7.70x10^{-4}; composites \( P \)= 1.07x10^{-5}; titanium \( P \)= 0.01; monocrystalline sapphire \( P \)= 2.62x10^{-3}).

Biofilm formation, assessed by counting adherent bacteria after 48h of growth in TSB or saliva, showed that the studied bacteria have different capacities to form biofilms in the studied conditions and that results obtained after growth in TSB are significantly different from those obtained in saliva (Fig. 1).

When results obtained at 4h and 48h were analysed cumulatively for each group of brackets, differences in the possibility of the different materials to favour biofilm growth appeared evident in both TSB and saliva (Fig. 2). In both media composites resulted as the worst performing brackets, while gold brackets showed the best performances.

Analysis of ratios (saliva/TSB) of adherent bacteria detected at the surface of the studied brackets showed that ratios differed significantly between 4h and 48h for all studied species (Figure 3a), but not for all tested brackets (Figure 3b). In fact, biofilm formation at 48h was significantly greater on gold, stainless steel and ceramic brackets, but not on the remaining ones (Figure 3b). Values of standard deviation reported in Figure 3 (panels A and B) show that biofilm formation at the surface of brackets is strongly influenced by both the material and the industrial process. These differences are not evident from adhesion assays at 4h.

**Discussion**

The present study was aimed to evaluate the susceptibility of 15 different brackets to adhesion by 6 different species of oral streptococci and to assess the ability of these brackets, made of 6 classes of materials,
to support biofilm formation by the studied bacteria, in different in vitro conditions. The rationale of this study is to support further information on differences existing among commercially available brackets as to their capacity to favour formation of streptococcal biofilms that can give rise to initial caries. In fact, data from the international literature show that fixed orthodontic appliances induce increased plaque accumulation [Balenseifen and Madonia, 1970; Årtun and Brobakken, 1986] and help colonisation by the cariogenic mutans streptococci and lactobacilli [Rosenbloom and Tinanoff, 1991; Forsberg et al., 1991], thus increasing the risk of decalcification, which can involve up to 50% of patients, and lead to the development of caries.

Brackets are per se an obstacle to correct oral hygiene, but constructive material is believed to play a relevant role in determining the amount and quality of bacterial plaque accumulating around teeth of patients undergoing fixed orthodontic treatment. Colonisation of a site depends on two distinct phases, early adhesion, mostly due to electrostatic and hydrophobic interactions [Papaioannou et al., 2007] and subsequent biofilm formation, that is influenced by a variety of environmental and surface properties [Anhoury et al., 2002; Demling et al., 2010].

Surface free energy is believed to play a relevant role in bacterial adhesion to surfaces and materials with high surface free energies, as stainless steel [Eliades et al., 1995] should favor adhesion of mutans streptococci more than other materials [An and Friedman, 1998; Ahn et al., 2007; Faltermeier et al., 2008]. Our data, nevertheless, indicate that although stainless steel effectively favors initial bacterial adhesion, as compared to other materials, with the exception of composites, it subsequently does not support biofilm formation to the same extent, so that final values of bacterial accumulation are substantially comparable to those obtained with other materials, including ceramic, titanium and sapphire. This is particularly true for data obtained with streptococci of the mutans group. These discrepancies were already evident from data of other studies [Fournier et al., 1998; Ahn et al., 2002], and could possibly depend on the role played in biofilm formation by the acquired pellicle and on material dependent differences in its composition [Ahn et al., 2002; 2003] as demonstrated in our experiments by different rates of adhesion and biofilm formation obtained in TSB as compared to saliva containing medium. Although significant discrepancies can be found between our data and data by other authors concerning relative performances of the different materials [Fournier et al., 1998; Ahn et al., 2002; 2003], this is a consequence of the different experimental protocols that have been adopted.

Overall, our experimental data show that the type of bracket material is relevant for plaque formation around teeth treated with fixed orthodontic appliances. Although statistical analyses suggest that significant differences exist among results obtained with almost all tested materials, an overview to obtained results suggests clearly that composite brackets are evidently characterized by a higher susceptibility to colonisation by the different tested streptococci and particularly by those of the mutans group, while all the other tested brackets have better performances. Although the non-composite brackets yielded significantly different results in the adhesion and biofilm formation assays, these differences are not so relevant to be considered clinically important. Gold brackets appear to be the better performing ones in all tests, while a cumulative evaluation of the remaining groups suggests that although less prone to initial colonisation, titanium and monocry stalline sapphire brackets must overall be considered comparable to ceramic and stainless steel brackets.

Data evaluating the influence of the medium on adhesion and biofilm formation suggest that in vivo the bacterial colonisation of brackets, and consequently the formation of incipient caries, could be influenced by food and other personal behaviours.

Our experimental plan allowed us to dissect the two basic moments of bacterial colonisation of orthodontic appliances and could constitute the basis for further competitive colonisation studies.

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References

- Balenseifen JW, Madonia JV. Study of dental plaque in orthodontic patients.


Papaioannou W; Gizani S; Nassika M; Kontou E; Nakou M. Adhesion of Streptococcus mutans to different types of brackets. Angle Orthod, 2007; 77: 1090–1095.


Walenczka E, Sadrowska B, Różańska S, Hryniewicz W, Różańska B. Staphylococcus aureus biofilm as a target for single or repeated doses of oxacillin, vancomycin, linezolid and/or lysostaphin. Folia Microbiol (Praha) 2006; 51:381-B.