Adhesion and biofilm formation by periodontopathogenic bacteria on different commercial brackets.

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Abstract

Objective.

To compare early bacterial adhesion and biofilm formation in vitro by common and uncommon periodontal pathogens on a variety of commercial brackets.

Matherials and Methods

Adhesion and biofilm formation in vitro of 4 bacterial strains on 15 different commercial brackets, in standard culture mediums complemented or not with either serum or human saliva was evaluated by quantitative real time PCR after extraction of bacterial DNA.

Results.

Materials significantly influenced bacterial adhesiveness in a species specific way. Titanium and gold brackets constantly yielded the lowest values with all tested bacteria and in all tested conditions. Bracket materials and medium of growth significantly influenced biofilm formation.

Conclusions.

Materials and environmental conditions significantly influence biofilm formation by periodontal pathogens at the surface of brackets. Whenever possible brackets should be kept far from the gingival margin and if this is not possible, brackets made of gold, titanium, and ceramic should be preferentially used.

Introduction

The number of subjects recurring to orthodontic treatment has increased enormously over the last few decades, together with the mean age of patients. In parallel, the reasons inducing subjects to require treatment have changed and in a number of cases they only want to improve their dentofacial esthetics, in the absence of any subjective functional limitation or defect [Harris, 2011]. A fixed orthodontic appliance, once placed in the mouth of a patient, significantly interferes with oral hygiene procedures. The consequent abnormal accumulation of dental plaque, cumulated with procedures that may alter enamel integrity leads to increased incidence of complications of microbial origin, including caries [Årtun and Brobakken, 1986; Rosenbloom and Tinanoff, 1991], reversible gingival inflammation [van Gastel et al., 2007; Bollen et al., 2008], and periodontal damage [Aass et al., 1988; Hongyan et al., 2011].

Although morphologic alterations of dental profiles due to the application of brackets and ligatures have a first line role in favoring the accumulation of plaque in these patients, materials used to construct appliances and laboratory techniques used to assemble them are also important. Much attention has been dedicated in the recent past to the relationship existing between the presence of orthodontic appliances, constructive materials and the accumulation of cariogenic bacteria[van Gastel et al., 2007; Ahn et al., 2007; Papaioannou et al., 2007], but several reports have pointed out that fixed orthodontic treatment favors colonization of dental sites by potentially periodontopathogenic bacteria[Paolantonio et al., 1996; Petti et al., 1997].

In spite of the above mentioned evidences on the relationship existing between orthodontic appliances and the incidence of gingival-periodontal pathologies, most researches intended to identify materials that are less susceptible to bacterial colonization have considered mainly adhesion of cariogenic bacteria. This consideration prompted us to perform a comparative study evaluating the adhesion of different periodontopathogenic bacteria to a variety of brackets representing the main commercially available categories.

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. Four strains of different species of common and uncommon periodontal pathogens were used (Table 2). All strains were maintained in stock cultures freezed at -80°C in the adequate culture medium (Table 2), containing glycerol (20% v/v). For adhesion assays, isolated colonies of each strain were inoculated in the corresponding culture medium 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 medium, diluted 1/2 with sterile phosphate buffered saline pH7.2 (PBS), or sterile heat inactivated foetal bovine serum (FBS) or sterile saliva, at an OD_{600nm} = 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 were subjected to sonication (1 minute at 30W with refrigeration), 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, sterilized 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 standardized adhesion assays, brackets were mounted on 0.6 x 0.6 cm polished clear acrylic blocks (K-Mac Plastics Wyoming, MI, USA) sticked to the cover of a 24 wells polystyrene plate. All the mounting process was performed by a single operator inside a 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 burr 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 removed carefully 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 the sterile medium diluted 1/2 in PBS, or FBS or saliva 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 corresponding medium and incubated for 4 and 48 hours at 37°C on a orbital shaker at 60 RPM.

Following incubation with the different bacterial suspensions, the brackets were removed with a sterile pliers and transferred into an adequately coded well of a flat bottom 96 well plate containing 0.1 ml of sterile PBS. Brackets were then washed five times with sterile PBS and further processed for the enumeration of adherent bacteria by quantitative Real Time PCR

Bacterial DNA extraction

To extract bacterial DNA from lysates of adherent bacteria the Nucleospin Genomic DNA purification Kit (Macherey-Nagel GmbH Düren, Germany) was used. To obtain lysis of bacteria adherent to the surface of brackets, 0.2 ml of lysis buffer (20 mM Tris-HCl; 2 mM EDTA; 1% Triton X-100; pH 8.0 supplemented with 20 mg/ml lysozyme and 0.2 mg/ml lysostaphin) were added to each bracket that was then incubated at 37°C for 60 minutes. Proteinase K was then added and samples were incubated at 56°C until complete lysis was obtained. Following lysis total DNA was purified according to the instructions of the manufacturer. Purified DNA was recovered and stored at -80°C as the template for Real Time PCR reactions. All chemicals were purchased from Sigma-Aldrich (Milan, Italy)

Quantitation of bacterial DNA by Real Time PCR.

Bacterial DNA was extracted, as described above for samples from adhesion assays, from 1ml of a pure culture of each tested strain at a density of 10^8 CFU/ml. Total DNA was serially diluted to construct a series of samples containing DNA from different amounts of bacteria for each tested species in the range $5x10^2$-$5x10^5$ cells/sample. Quantitative determination of bacterial DNA in standards and samples was performed by a quantitative real time PCR using the 16SrRNA gene universal primers 357F and 907R [Lane, 1991; Yamaura et al., 2005] using the Maxima® SYBR

Green/Fluorescein qPCR Master Mix (Fermentas Life Sciences) according to the instructions of the manufacturer. Cycling conditions were performed as described previously [Yamaura et al., 2005] [16] and were undertaken using an Applied Biosystems 7300 apparatus. Purity of amplification products was assessed following construction of melting curves. Data were reported as number of bacteria detected for each bracket.

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 ≤ 0.05 were considered significant while differences yielding values of $P \leq 0.01$ were considered very significant.

Results

Mean values of adherent periodontopathogenic bacteria detected at the surface of different brackets in adhesion and biofilm formation assays performed in culture medium alone or complemented with either FBS or saliva are reported in Figure 1. The 15 tested brackets were divided in 6 groups depending on material they were made of: i) ceramic (brackets 1 to 4), stainless steel (brackets 5 to 7), gold (brackets 8 and 9), composites (brackets 10 to 12), titanium (bracket 13) and monocrystalline sapphire (brackets 14 and 15). Results showed that materials significantly influenced bacterial adhesiveness in a species specific way. In fact, *A. actinomycetemcomitans* and *S. aureus* adhered overall better than the two strict anaerobes *P. gingivalis* and *P. intermedia* although significant differences were evident in adhesiveness to the different groups of brackets. Titanium and gold brackets constantly yielded the lowest values with all tested bacteria and in all tested conditions, while brackets made of composites always resulted more susceptible to bacterial adhesion (Figure 1). The medium used to perform adhesion assays did not influence results significantly. In fact, results of adhesion assays at 4h for all tested materials in medium, FSB and saliva were comparable (Figures 1 and 2). Biofilm formation, assessed by counting adherent bacteria after 48h of growth in medium alone or containing either FBS or saliva yielded different results in a strain, material and medium dependent manner. In fact, *S. aureus* formed much greater biofilms in all tested conditions as compared to the other tested bacteria (Figure 1). Moreover, *A. actinomycetemcomitans* formed greater biofilms as compared to *P. gingivalis* and *P. intermedia* (Figure 1). Overall, saliva significantly stimulated biofilm growth in *S. aureus*, *P. gingivalis* and *P. intermedia* but not in *A. actinomycetemcomitans* (Figures 2).

The presence of saliva significantly enhanced biofilm growth on all tested materials (Figure 2, 3). Composites resulted significantly more susceptible than other tested materials to growth of bacterial biofilms in all tested conditions (Figure 3).

Discussion

The present study was aimed to evaluate the susceptibility of 15 different brackets to adhesion and biofilm formation by 4 different bacterial species selected among common and occasional periodontopathogens. In fact, fixed orthodontic appliances are known to promote gingival inflammation, potentially biasing the periodontal health of patients[Aass et al., 1988; Hongyan et al., 2011] although further epidemiologic evidences are needed for this. In recent years the number of young adults and adults requiring orthodontic treatment, mostly for aesthetic reasons has greatly increased, making it necessary to have more information on the materials that are best to be used in these cases that are naturally more susceptible to periodontitis than children. Results of this study confirm that different materials used in the construction of brackets greatly influence adhesion by different important periodontopathogens and by the oral colonizer *S. aureus*, that is recently receiving attention as a possible cause of periodontal damage [Passariello et al., 2012a]. More interestingly, results have shown that the presence of saliva greatly influence the capacity of some of these microorganisms to form biofilms at the surface of all tested brackets. This observation suggests the necessity to keep brackets away from the gingival margin, because their presence stimulates plaque overgrowth not only due to space hindrance but possibly also as a consequence of adsorption of salivary components. Consequently, the choice of brackets made of gold, titanium, ceramic and to a lesser extent sapphire could be strongly indicated in the presence of reduced clinical crown dimensions. The application of aesthetic plastic brackets in these cases appears not to be recommended due to higher risk to promote inflammation as a consequence of heavy plaque accumulation.

Our results demonstrate moreover that analysis of the susceptibility of biomaterials to bacterial colonization should include not only adhesion assays, that are poorly influenced by the assaying conditions, but also biofilm formation assays, due to their higher capability to show differences in the susceptibility of materials to colonization.

In the light of recent reports demonstrating that oral colonization by *S. aureus* is influenced by oral conditions [Passariello et al., 2012a,b] and that the presence of this microorganism in the oral cavity of humans may constitute a threat to their general health [Zuanazzi et al., 2010], specific studies should be performed to address this point and evaluate if selection of specific materials for orthodontic appliances may influence *S. aureus* oral carriage rates and constitute a way to reduce the circulation of this dangerous opportunistic pathogen, particularly in at risk patients.

Data presented in this paper demonstrate that fixed orthodontic appliances may be differently colonized by periodontal pathogens and other oral bacteria according to their position with respect to the gingival margin. The orthodontist should do his best to keep brackets far from the gingival margin and if this is not possible, he should preferentially use brackets made of gold, titanium, and ceramic.

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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.

Identification	Bracket	Manufacturer	Material
1	Clarity Advanced Ceramic	3M Unitek	Ceramic
2	Ceramic Bracket	Dentsply	Ceramic
3	Fascination 2	Dentaurum	Ceramic
4	Enhance Ceramic	Ortho Specialities	Ceramic
5	Victory Series	3M Unitek	Stainless Steel
6	Stainless Steel Bracket	Dentsply	Stainless Steel
7	Equilibrium 2	Dentaurum	Stainless Steel
8	Gold Victory Series	3M Unitek	Gold
9	Regency Gold	Ortho Specialities	Gold
10	Clear Brackets	Dentsply	non-polycarbonate plastic
11	Elegance	Dentaurum	Polycarbonate
12	Comp Plus T	Ortho Specialities	Composite
13	Equilibrium Ti	Dentaurum	Titanium
14	GEM Monocrystalline	Ortho Specialities	Sapphire
15	Pure	Ortho Technology	Sapphire

Table 2. Bacterial strains and culture conditions.

Species	Strain	Medium	Culture conditions
Aggregatibacter actinomycetemcomitans	DSM8324	TSB	37°C - M
Porphyromonas gingivalis	DSM20709	DSM medium 104	37°C - AN
Prevotella intermedia	DSM20706	DSM medium 104	37°C - AN
Staphylococcus aureus	SA1448	TSB	37°C-AE

TSB: Trypticase Soy Broth; DSM medium 104: formula available at www.DSMZ.de; M: incubation in 5%CO_2 enriched atmosphere; AN: incubation in atmosphere of 80% N_2, 10% CO_2, and 10% H_2; AE: incubation in air.

Figure legends

Figure 1. Adherent bacteria detected at the surface of different brackets in adhesion (4h) and biofilm formation (48h) assays performed in culture medium alone or complemented with either foetal bovine serum (FBS) or saliva. Results are reported as means for brackets grouped according to construction material. A: ceramic, B: stainless steel, C: Gold, D: plastic or composite, E: titanium, F: monocrystalline sapphire. Standard deviation bars are reported.

Figure 2. Ratios of adherent bacteria detected at 4 and 48h of incubation at the surface of the studied brackets after incubation in foetal bovine serum (FBS) (panels a and c) or saliva (panels b and d) as compared to results obtained in culture medium. Results are grouped according to tested strain (panels a and b) and bracket material (panels c and d). A: ceramic, B: stainless steel, C: Gold, D: plastic or composite, E: titanium, F: monocrystalline sapphire. * indicates significant differences.

Figure 3. Mean biofilm growth curves obtained at the surface of different types of brackets with the tested bacteria grown in culture medium alone or complemented with either foetal bovine serum (FBS) or saliva.

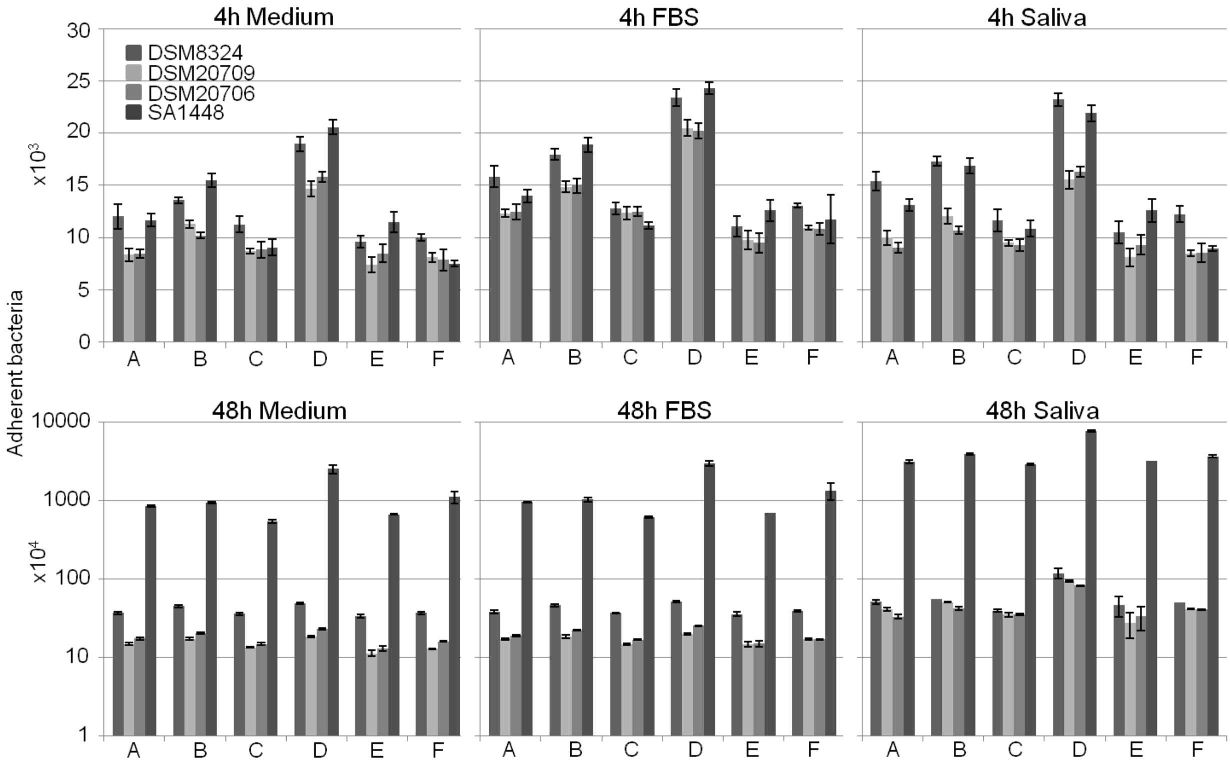
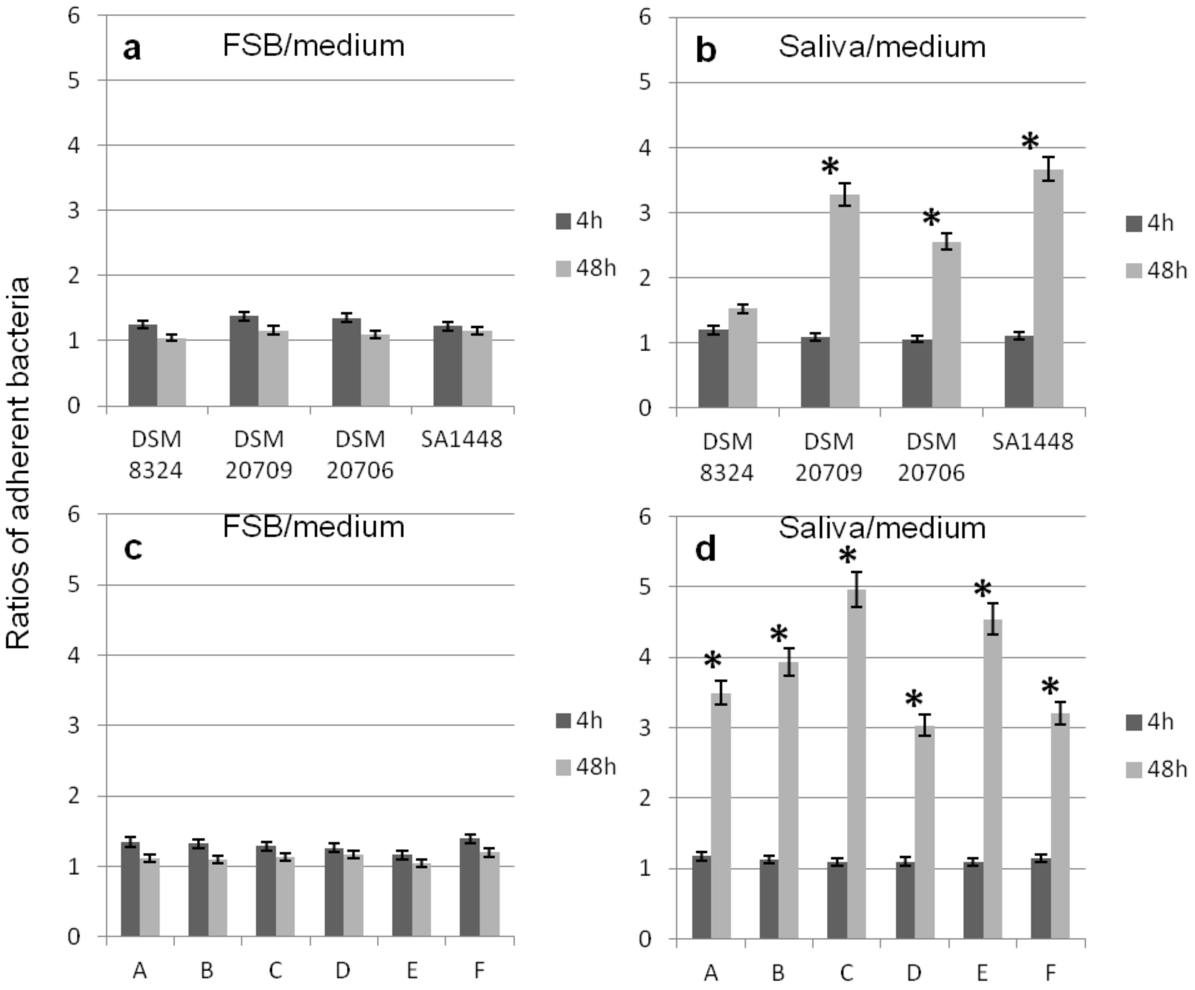

4h Medium
4h FBS
4h Saliva
DSM8324
DSM20709
DSM20706
SA1448
30
25
20
15
10
5
0
x10³
A
B
C
D
E
F
Adherent bacteria
48h Medium
48h FBS
48h Saliva
10000
1000
100
10
1
x10⁴

Figure 1.



Ratios of adherent bacteria
a
FSB/medium
b
Saliva/medium
c
FSB/medium
d
Saliva/medium
4h
48h
DSM 8324
DSM 20709
DSM 20706
SA1448
A
B
C
D
E
F
0
1
2
3
4
5
6

Figure 2.

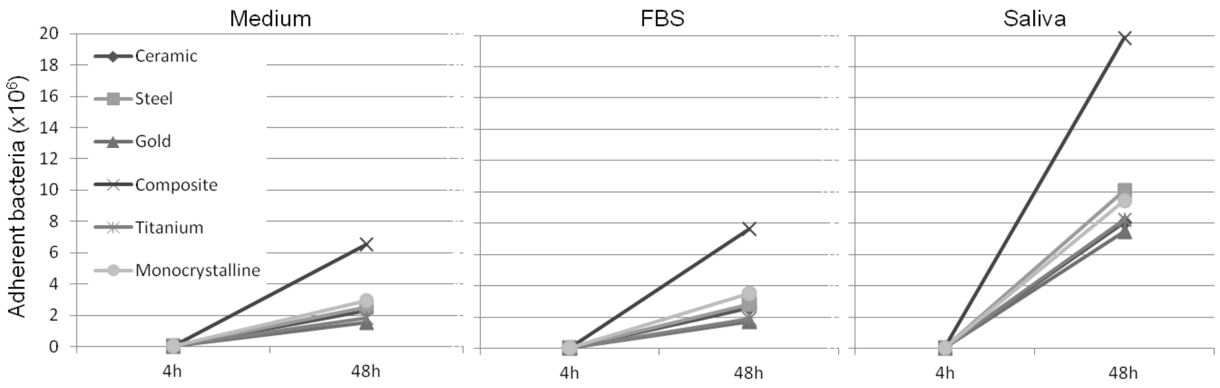

Medium
FBS
Saliva
Adherent bacteria (x10^6)
20
18
16
14
12
10
8
6
4
2
0
Ceramic
Steel
Gold
Composite
Titanium
Monocrystalline
4h
48h

Figure 3.