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Influence of topography and hydrophilicity on initial oral biofilm formation on microstructured titanium surfaces *in vitro*

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Abstract

Objectives—The aim of this study was to analyse the influence of the microtopography and hydrophilicity of titanium (Ti) substrates on initial oral biofilm formation.

Materials and methods—Nine bacterial species belonging to the normal oral microbiota, including: *Aggregatibacter actinomycetemcomitans*, *Actinomyces israelii*, *Campylobacter rectus*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Parvimonas micra*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Streptococcus sanguinis* were tested on Ti surfaces: pretreatment (PT [$R_a < 0.2 \mu\text{m}$]), acid-etched (A [$R_a < 0.8 \mu\text{m}$]), A modified to be hydrophilic (modA), sand-blasted/acid-etched (SLA [$R_a = 4 \mu\text{m}$]), and hydrophilic SLA (modSLA). Disks were incubated for 24 h in anaerobic conditions using a normal culture medium (CM) or human saliva (HS). The total counts of bacteria and the proportion of each bacterial species were analysed by checkerboard DNA–DNA hybridization. Results: Higher counts of bacteria were observed on all surfaces incubated with CM compared with the samples incubated with HS. PT, SLA, and modSLA exhibited higher numbers of attached bacteria in CM, whereas SLA and modSLA had a significant increase in

bacterial adhesion in HS. The proportion of the species in the initial biofilms was also influenced by the surface properties and the media used: SLA and modSLA increased the proportion of species like *A. actinomycetemcomitans* and *S. sanguinis* in both media, while the adhesion of *A. israelii* and *P. gingivalis* on the same surfaces was affected in the presence of saliva.

Conclusions—The initial biofilm formation and composition were affected by the microtopography and hydrophilicity of the surface and by the media used.

Keywords

biofilm; hydrophilicity; microstructure; titanium

Biofilm formation on dental implants is a persistent problem that can cause implant failure. Once a biofilm is formed, bacterial cells become highly resistant to antibiotics and host defenses (Costerton et al. 1999), and clinical experience has shown that biofilms must be removed physically before the infection can be resolved (Costerton 2005). There is an apparent clinical and microbiological similarity between peri-implantitis and periodontitis (Papaioannou et al. 1996; Listgarten & Lai 1999). However, it remains unclear whether the first steps in biofilm formation on titanium (Ti) implants are similar to biofilm formation on teeth.

The biofilm formation process is extremely complicated and this is particularly true when multiple species are present in the biofilm as in dental plaque. This process is affected by many factors including environment, bacterial properties, and material surface characteristics, such as chemical composition, surface energy, hydrophilicity, and topography (Merritt & Chang 1991; An & Friedman 1998; Katsikogianni & Missirlis 2004). *in vitro* studies of biofilm formation on Ti surfaces have focused on the effects of surface morphology and surface chemistry (Yoshinari et al. 2000; Grossner-Schreiber et al. 2001; Barbour et al. 2007), but most biofilm models in these studies have only included one or two bacterial strains. In the oral cavity, however, the microbial ecology is complex and can consist of hundreds of species, each with a preference for specific microenvironmental properties.

Studies investigating peri-implant microbiota *in vivo* have examined the influence of oral health status on the presence of specific bacterial species. Some of these studies report similar supra- and sub-gingival microbiota on teeth and Ti implants (Groessner-Schreiber et al. 2004; Furst et al. 2007; Shibli et al. 2008). In contrast, some studies found an absence of periodontal pathogens like *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* and *Porphyromonas gingivalis* (Heuer et al. 2007).

On natural dental surfaces like enamel, the composition of the primary acquired pellicle plays an important role in determining the type and amount of bacteria that will attach (Gibbons 1996; Steinberg et al. 1998; Sela et al. 2007). Salivary and serum constituents can also adsorb onto Ti surfaces (Kohavi et al. 1995, 1997), and this is influenced by the structural and chemical properties of the surface (Katsikogianni & Missirlis 2004; Mabboux et al. 2004; Jeyachandran et al. 2006). Recent iterations of dental implant design are based on *in vitro* and *in vivo* studies showing that micron-scale and submicron-scale structural

features increase osteoblast differentiation and peri-implant bone formation, which can be further enhanced by increased hydrophilicity (Cochran 1999; Buser et al. 2004; Lossdorfer et al. 2004; Schwarz et al. 2005, 2007a, 2007b; Zhao et al. 2005; Jimbo et al. 2008). Hydrophilic surfaces have a wicking effect on tissue fluids including blood, leading to adsorption of cell attachment proteins like fibronectin. While many cells involved in tissue regeneration use these proteins to adhere to the implant, the same proteins could also possess binding sites for bacteria (Quirynen et al. 2001; Plummer & Douglas 2006). However, the effect of these surface modifications on the attachment and growth of oral microorganisms, particularly under conditions that simulate the oral environment, is not known. The purpose of this study was to evaluate how biofilm formation and composition are affected by implant surface properties like microtopography and hydrophilicity using a regular culture medium (CM) or human saliva (HS).

Material and methods

Ti-disk surfaces

Ti disks with diameters of 15 mm were prepared from 1-mm-thick sheets of grade 2 unalloyed Ti (ASTM F67). The methods used to produce the pretreatment (PT), sand-blasted and acid-etched (SLA) and hydrophilic SLA (modSLA) surfaces have been reported previously (Schwarz et al. 2007a, 2007b). The PT surface is relatively smooth with a mean peak to valley roughness (R_a) <0.2 μm . Submicron rough A surfaces were produced by treating PT with heated concentrated acid, resulting in an R_a of 0.8 μm , based on surface profilometry. PT surfaces were also sand-blasted and acid-etched to produce SLA surfaces with a R_a of 3.2 μm . Before use, PT, A, and SLA surfaces were washed in ultrasonic cleaner and sterilized in an oxygen plasma cleaner (PDC-32G, Harrick Plasma, Ithaca, NY, USA). The modA and modSLA surfaces were produced with same mechanical or chemical treatments as A and SLA surfaces, respectively. However, the modA and modSLA surfaces were rinsed under nitrogen protection to prevent exposure to air during the procedure and then stored in a sealed glass tube containing isotonic NaCl solution, reducing adsorption of atmospheric hydrocarbons, and therefore, retaining a higher surface energy (Rupp et al. 2006). These sealed disks were sterilized by g-irradiation at 25 kGy over night. All disks were fabricated by Institut Straumann AG (Basel, Switzerland) and were shipped to us ready for use. Each surface was used in triplicate for the experiments described below.

Bacterial strains

Unless otherwise noted, chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA). Nine bacterial species were tested on each surface. The reference strain for each species is shown in Table 1. The nine selected species are representative of the normal subgingival dental plaque. We used *Actinomyces israelii* and *Streptococcus sanguinis* as early colonizers. To represent the second group of bacteria that functions as a bridge between the early and late colonizers, we used *Fusobacterium nucleatum*, *Eikenella corrodens*, and *Prevotella intermedia*. Finally, *P. gingivalis* was used as a representative of the third group of species that appears at late stages of biofilm development. Lyophilized bacterial stocks (American Type Culture Collection, Rockville, MD, USA) were rehydrated in *Mycoplasma* broth base (BBL, Becton-Dickinson and Co., Sparks, MD, USA). All strains

were grown on *Mycoplasma* agar base (BBL, Becton-Dickinson and Co.) supplemented with 5% defibrinated sheep blood, 5 µg/ml hemin, and 0.3 µg/ml menadione under anaerobic conditions (80% N₂, 10% CO₂, and 10% H₂).

Initial biofilm formation assay

Bacterial growth from 5- to 7-day cultures of each strain was harvested and the optical density (OD) in each tube was adjusted to 1 at 600 nm in a spectrophotometer. Sterile disks were placed individually in 24-well plates and a total of 10⁶ cells/ml suspension of each reference strain was added, in order to obtain a mixed culture, in a total volume of 1 ml. Plates were incubated for 24 h at 35°C under anaerobic conditions using enriched *Mycoplasma* broth-media (5 µg/ml hemin and 0.3 µg/ml menadione) (CM) or HS. HS was processed as reported previously (Guggenheim et al. 2001), except that after centrifugation, supernatants were sterilized by filtration. All experiments were performed in triplicate. After anaerobic incubation, each sample was washed twice with *Mycoplasma* broth. After washing, 1 ml of enriched *Mycoplasma* broth was added and the samples were sonicated for five periods of 10 s each in order to detach the adherent bacteria from each surface.

Biofilm composition

One hundred microliters of the bacterial suspension obtained after sonication of each sample were placed in individual Eppendorf tubes with 900 µl of TE buffer pH 7.6 (10 mM Tris-HCl, 1 mM EDTA). The suspensions were mixed by vortexing and 100 µl were placed in new Eppendorf tubes with 100 µl of 0.5 M NaOH. Bacterial species were identified and quantified using the checkerboard DNA–DNA hybridization technique as described previously (Socransky et al. 1994). In brief, DNA probes were prepared using the growth from 3- to 7-day cultures of the nine reference strains used in the biofilm model (Table 1). Bacterial growth was harvested and placed in tubes containing 1 ml of TE buffer. Cells were washed twice and lysed at 37°C for 1 h with either 10% sodium dodecyl sulfate (SDS) plus proteinase K (20 mg/ml) for Gram-negative strains or lysozyme (15 mg/ml) plus achromopeptidase (5 mg/ml) for Gram-positive strains. DNA was isolated and purified using the method described by Smith et al. (1989). Whole-genomic DNA probes were prepared for each species by labeling 1 mg of DNA with digoxigenin (Roche Diagnostics, Mannheim, Germany) using a random primer technique (Feinberg & Vogelstein 1983). The specificity and sensitivity of the nine DNA probes were assessed by hybridizing each DNA probe against individual pure cultures of all of the species adjusted to 10⁴, 10⁵, 10⁶, and 10⁷ cells. The sensitivity of the assay was set to allow the detection of approximately 10⁴ cells of a given species by adjusting the concentration of each individual DNA probe. For the DNA–DNA hybridization, each sample was thawed at room temperature, boiled for 10 min, and neutralized with 800 µl of 5 M ammonium acetate. The released DNA from each sample was then placed into individual lanes (Minislot-30, Immunetics Inc., Cambridge, MA, USA), concentrated onto a 15 cm × 15 cm positively charged nylon membrane (Roche Diagnostics), and fixed to the membrane by cross-linking under ultraviolet light. Two lanes on each membrane contained standards consisting of a mixture at 10⁵ and 10⁶ cells of each bacterial species tested. The membranes were pre-hybridized at 42°C for 2 h in 50% formamide, 5 × standard saline citrate (SSC) (1 × SSC = 150 mM NaCl and 15 mM Na citrate), 1% casein (Sigma-Aldrich), 5 × Denhardt's solution, 25 mM sodium phosphate (pH

6.5), and 0.5 mg/ml yeast RNA (Roche Diagnostics). Each membrane was placed in a second device (Miniblotter-45, Immunetics) with the sample lanes rotated 90° to the channels of the apparatus. The probes were diluted to ~20 ng/ml in hybridization solution (45% formamide, 5 × SSC, 1 × Denhardt's solution, 20 mM Na phosphate (pH 6.5), 0.2 mg/ml yeast RNA, 10% dextran sulfate, and 1% casein), placed in individual channels of the device, and hybridized overnight at 42°C. Probes were hybridized in two sets of nine consecutive channels, leaving empty channels (hybridization solution only) to allow noise and background correction of signals. The membranes were washed twice at high stringency for 20 m each time at 68°C in phosphate buffer (0.1 × SSC and 0.1% SDS). Membranes were blocked by 1 h incubation in maleate buffer (100 mM maleic acid and 150 mM NaCl, pH 7.5) containing 1% casein. Hybrids were detected by exposing the membranes to a 1 : 50,000 dilution of anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Diagnostics) for 30 m, using a previously described modification (Engler-Blum et al. 1993). Signals were detected by chemiluminescence using a chemiluminescent agent (CDP-Star, Roche Diagnostics) for 30 m on the membranes at room temperature and exposed to films in autoradiographic cassettes for 30 m. Films were developed and then photographed using a digital photodocumentation system (DigiDoc, BioRad Laboratories, Hercules, CA, USA). Signals were detected with specialized software (Quantity One, BioRad Laboratories), adjusted by subtracting the average plus two standard deviations of the noise and background detected in the empty lanes, and converted to absolute counts by comparison with the standards on the membrane. Failure to detect a signal was recorded as zero.

Biofilm morphology

In order to observe biofilm morphology in each of the test substrates, samples were prepared for scanning electron microscopy (SEM). Specimens were fixed in 2% glutaraldehyde 24 h at room temperature, then washed three times with phosphate buffer solution (pH 7.4) and dehydrated through a series of graded ethanol solutions (20%, 40%, 60%, 80%, and 100%). Samples were subsequently vacuum dried, sputter-coated with Au, and observed using a scanning electron microscope (Stereoscan 440, Cambridge-Leica, Wetzlar, Germany) at 20 kV.

Data analysis

Total counts of bacteria are presented as mean standard error of the mean (SEM) × 10⁶ per ml. The proportion of each strain in the biofilms is presented as a percentage based on the total numbers of bacteria that were obtained. Data were analysed using analysis of variance (ANOVA) followed by Bonferroni's correction for multiple comparisons and significant differences were determined. Significance was set at P<0.05.

Results

The total number of bacterial counts varied with the titanium test substrate and medium used (Fig. 1). There were consistently more bacteria on disks cultivated in the CM than in HS. In addition, when CM was used, significantly lower numbers of bacteria were detected on A and modSLA substrates when compared with PT (P<0.05). SLA and modSLA substrates had greater numbers of bacteria than the A or modA surfaces in both CM and HS (P<0.05).

0.05). When bacteria were cultured with HS, the greatest adhesion of bacteria was observed on the modSLA surfaces ($P<0.05$).

The differences in bacterial distribution on the test substrates were confirmed in scanning electron micrographs of the biofilms (Fig. 2). Greater amounts of biofilm were found on the PT disks cultured in CM compared with HS. Bacteria growing in CM appeared to conform to sheet-like regions rather than to the occasional projections. Chains of bacilli-shaped bacteria predominated. In contrast, in HS bacteria coated the irregular surface and cocci filled the pits. When bacteria were grown in CM on the modA surface, the structural elements appeared to be coated with filaments with regions of cocci interspersed. This was more pronounced in cultures grown in HS. It was difficult to distinguish individual colonies or bacteria on SLA substrates. In CM, filaments, and cocci were interspersed with extracellular matrix and this was more pronounced on the modSLA surface, where the matrix formed rope-like structures along the substrate ridges. This matrix covered the bacteria in cultures grown in HS on SLA and modSLA.

The composition of the biofilm was also sensitive to substrate properties in a media-dependent manner. Figure 3 shows the proportion of each species in the biofilms formed on the test substrates. When bacteria were grown in CM, *P. gingivalis* represented the highest proportion on the biofilms in all surfaces tested, while *A. israelii* represented the lowest proportion of the total biofilm. *P. gingivalis* was found at significant higher levels on A and modA surfaces than on SLA ($P<0.05$, $P<0.01$, respectively) or on modSLA ($P<0.05$, $P<0.05$, respectively). *A. israelii*, which represented the lowest proportion of the initial biofilms formed on the test substrates, showed clear substrate specific differences, with increasing levels as the surfaces became rougher and more hydrophilic (PT vs. modSLA, $P<0.05$; A vs. modSLA, $P<0.05$). *A. actinomycetemcomitans* and *F. nucleatum* exhibited roughness-dependent increases, with reduced levels on the hydrophilic substrates. Differences in the proportion of *A. actinomycetemcomitans* were found on the PT surfaces compared with SLA or modSLA ($P<0.05$). Differences in the amount of *F. nucleatum* were found when PT was compared with modSLA. In contrast, surface topography and energy had no effect on the proportion of *C. rectus*, *E. corrodens*, *Parvimonas micra*, *P. intermedia*, and *S. sanguinis*. Different patterns of adhesion were observed when the bacteria were cultured in HS (Fig. 3). Moreover, the differential effects of substrate topography and hydrophilicity were amplified. *P. gingivalis* proportions were elevated on PT in comparison with modSLA ($P<0.01$), on A in comparison to SLA ($P<0.05$) and modSLA ($P<0.001$), and on modA in comparison with modSLA ($P<0.01$). *F. nucleatum* was higher on A when compared with modSLA ($P<0.01$). *C. rectus* was markedly elevated on modSLA in comparison to modA ($P<0.05$). *S. sanguinis* exhibited increased numbers on SLA in comparison to PT ($P<0.01$) and on modA vs. modSLA substrates. In HS, surface topography and energy had no effect on the proportion of *A. israelii*, *A. actinomycetemcomitans*, *E. corrodens*, and *P. intermedia*.

Discussion

In this study, we examined initial biofilm formation on Ti implant surfaces with different microtopography and hydrophilicity. We also tested the influence of different incubation media by growing these biofilms with normal CM or HS. Our biofilm model included nine

bacterial species representative of all the complexes of the subgingival plaque described by Socransky et al. (1998) and Socransky & Haffajee (2005).

The numbers of bacteria on all surfaces were dependent on the incubation media, revealing the strong influence of the “bulk fluid” in the initial formation and consolidation of a biofilm. The major bulk fluids in the oral cavity are saliva and the gingival crevicular fluid, and many studies indicate that these fluids are critical for the colonization of certain taxa (De Jong & Van der Hoeven 1987; Gibbons 1996). Our results showed that a higher number of bacteria attached to all surfaces when CM was used. A possible explanation could be that saliva contains some antimicrobial substances, such lysozyme, lactoferrin, lactoperoxidase, and secretory IgA (Tenovuo 1998). Scanning electron micrographs of the biofilms suggest that biofilm morphology is also affected by the CM. Coccal forms were more abundant in CM cultures and they tended to congregate in focal regions. Less special variation was evident in the HS cultures, in part because of the thick layer or extracellular matrix, which followed the contours of the micron-scale topographic features of the underlying Ti disk.

Surface microtopography is an important influence in oral bacterial colonization. A positive correlation between surface roughness and bacterial attachment *in vitro* has been shown (Quirynen et al. 1996), and a recent pilot study revealed that initial supra-gingival plaque biofilm formation *in vivo* was influenced by microtopography (Schwarz et al. 2007a, 2007b). This pilot study also suggested that hydrophilicity was less important than roughness in the formation of supra-gingival plaque. Other studies suggest that roughness appears to be a minor factor in initial biofilm formation (Bos et al. 1999; BoulangePetermann et al. 1997). In our study, based on analysis of the total number of bacteria attached to surfaces with different microtopographies (PT, A, and SLA), no differences were found when samples were incubated with saliva, supporting the hypothesis that microtexture is not a major determinant in biofilm formation. However, samples incubated with CM had a greater number of bacterial counts on PT and SLA than on the A surfaces, discounting this notion.

Regarding the surface-free energy effect on the bacterial adhesion, we observed more bacteria on modSLA than on SLA in cultures grown with HS. This correlation between surface energy and bacterial adhesion has been studied previously (van Dijk et al. 1987; Quirynen & Bollen 1995). However, we did not assess the hydrophilicity of the bacterial cell surface, which also has been reported to have an influence in bacterial adhesion (Strevett & Chen 2003), because low surface free energy materials attracted microorganisms with relatively low surface free energy (Minagi et al. 1985; Mabboux et al. 2004).

The composition of the biofilms that formed on each surface was both substrate dependent and media dependent demonstrating the interrelationship between physical and chemical properties of the surface and biological properties of the environment in initial biofilm formation. For example, *P. gingivalis*, a very important periodontal pathogen (Moore 1987; Holt & Ebersole 2005), was found in high proportions in the experimental biofilms on all surfaces tested, particularly when CM was used. This finding is consistent with those reported previously showing that *P. gingivalis* is capable of colonizing Ti surfaces at a very high rate (Yoshinari et al. 2000; Pier-Francesco et al. 2006). *S. sanguinis* is an “early colonizer” (Gibbons 1996); thus, it was not surprising to find this microorganism in high

proportions. Some studies also report significant adhesion of this microorganism on Ti surfaces (Hauser-Gerspach et al. 2007). We also found significant adhesion of *A. actinomycetemcomitans* on all surfaces, particularly on the SLA and modSLA surfaces in both CM and HS. *A. actinomycetemcomitans* is a well-known periodontal pathogen (Crossner et al. 1990; Fives-Taylor et al. 2000), and the adhesion of this microorganism to implant surfaces has also been studied (Yoshinari et al. 2000; Callan et al. 2005). Although it is intriguing that *A. actinomycetemcomitans* was found in higher numbers on SLA and modSLA surfaces, the reason for this is not known, but opens an interesting field of study.

The effects of surface microstructure and surface energy vary among the test species. This is consistent with numerous studies showing the critical role of microenvironment on attachment and growth of individual constituents of the oral microbiota (Grossner-Schreiber et al. 2001; Scarano et al. 2003; Pier-Francesco et al. 2006). The effect of the incubation media was also an important factor; differences in the bacterial adhesion and biofilm composition were observed. Using saliva as a CM could be adequate to simulate an *in vivo* situation. However, the error associated with the use of saliva could be also larger, because saliva components can vary from donor to donor and even from the same donor (Crosley et al. 2009). Therefore, for the *in vitro* study of bacterial adhesion and biofilm formation on implant surfaces, it is more reliable and convenient to use a well-controlled CM.

Taken together, the data show that initial biofilm formation and composition are affected by surface microtopography and hydrophilicity, as well as the CM used. The method used here provides a powerful tool to study bacterial interaction with biomaterials *in vitro* using a complex biofilm model. By tailoring surface properties, we may be able to control the initial biofilm formation, directing the formation of a less pathogenic biofilm on the implant surface and potentially avoiding peri-implant infections.

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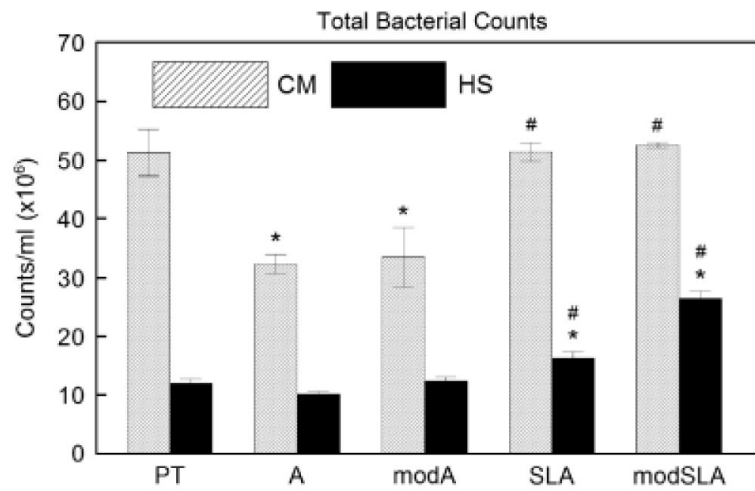


Fig. 1.

Total bacterial counts (bacterial counts/ml $\times 10^6$) on PT, A, modA, SLA, and modSLA surfaces after 24 h of anaerobic incubation in culture medium (CM) or human saliva (HS). * $P < 0.05$, vs. PT for CM or HS; # $P < 0.05$, vs. A or modA for CM or HS. PT, pretreatment; A, acid-etched; modA, A modified to be hydrophilic; SLA, sand-blasted/acid-etched; modSLA, hydrophilic SLA.

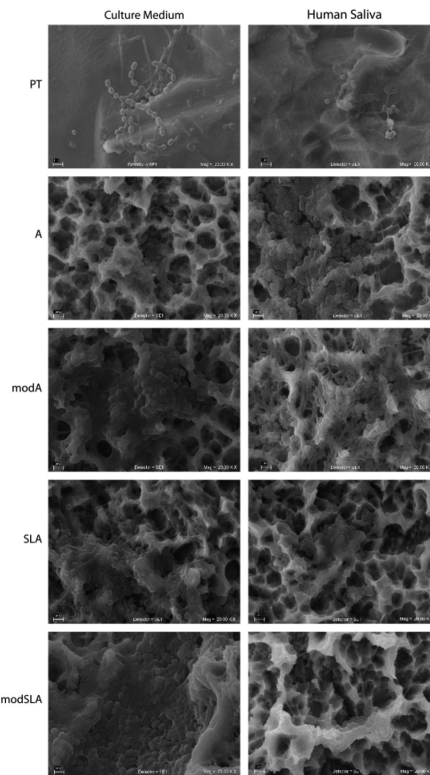


Fig. 2. Scanning electron micrographs of biofilms formed on PT, A, modA, SLA, and modSLA surfaces. Biofilms were grown in culture medium (CM) or human saliva (HS). Scale bar represents 2 μm . PT, pretreatment; A, acid-etched; modA, A modified to be hydrophilic; SLA, sand-blasted/acid-etched; modSLA, hydrophilic SLA.

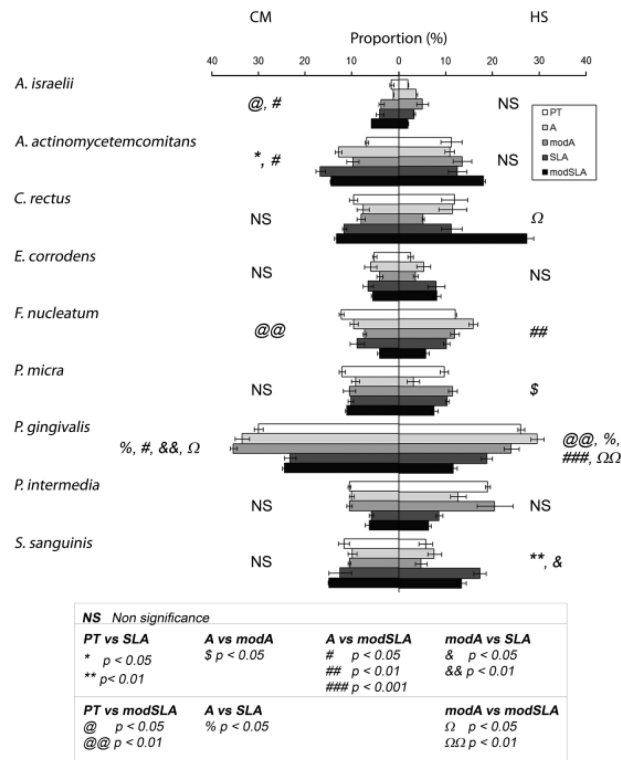


Fig. 3. Proportion of the bacterial strains in the initial biofilms formed on pretreatment (PT), A, modA, SLA, and modSLA surfaces using culture medium (CM) or human saliva (HS). PT, pretreatment; A, acid-etched; modA, A modified to be hydrophilic; SLA, sand-blasted/acid-etched; modSLA, hydrophilic SLA.

Table 1

Reference strains used for the biofilm model and for the preparation of DNA probes

Species	Strain*
<i>Actinomyces israelii</i>	12102
<i>Aggregatibacter</i> <i>actinomycetemcomitans</i> <i>serotype b</i>	43718
<i>Campylobacter rectus</i>	33238
<i>Eikenella corrodens</i>	23834
<i>Fusobacterium nucleatum ss</i> <i>nucleatum</i>	25586
<i>Parvimonas micra</i>	33270
<i>Porphyromonas gingivalis</i>	33277
<i>Prevotella intermedia</i>	25611
<i>Streptococcus sanguinis</i>	10556

* American Type Culture Collection (Rockville, MD, USA).