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Polyspecies biofilm formation on implant surfaces with different surface characteristics

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ABSTRACT

Objective: To investigate the microbial adherence and colonization of a polyspecies biofilm on 7 differently processed titanium surfaces. Material and Methods: Six-species biofilms were formed anaerobically on 5-mm-diameter sterilized, saliva-preconditioned titanium discs. Material surfaces used were either machined, stained, acid-etched or sandblasted/acid-etched (SLA). Samples of the latter two materials were also provided in a chemically modified form, with increased wettability characteristics. Surface roughness and contact angles of all materials were determined. The discs were then incubated anaerobically for up to 16.5 h. Initial microbial adherence was evaluated after 20 min incubation and further colonization after 2, 4, 8, and 16.5 h using non-selective and selective culture techniques. Results at different time points were compared using ANOVA and Scheffé *post hoc* analysis. Results: The mean differences in microorganisms colonizing after the first 20 min were in a very narrow range (4.5 to 4.8 log CFU). At up to 16.5 h, the modified SLA surface exhibited the highest values for colonization (6.9±0.2 log CFU, $p<0.05$) but increasing growth was observed on all test surfaces over time. Discrepancies among bacterial strains on the differently crafted titanium surfaces were very similar to those described for total log CFU. *F. nucleatum* was below the detection limit on all surfaces after 4 h. Conclusion: Within the limitations of this *in vitro* study, surface roughness had a moderate influence on biofilm formation, while wettability did not seem to influence biofilm formation under the experimental conditions described. The modified SLA surface showed the highest trend for bacterial colonization.

Key words: Dental implants. Titanium. Biofilms. Surface properties. Wettability.

INTRODUCTION

The use of dental implants has become a routine procedure in dentistry to replace one or more missing teeth. Given implant survival as the main criteria for success, the majority of clinical studies show high success rates for dental implants[4]. However, there is also evidence of chronic inflammation, in the range of 8.6%-9.7%, in soft and hard tissues neighboring implants[12,15], and is commonly observed about ten years after implantation[19]. These pathologic conditions termed "mucositis" and "peri-implantitis", are considered major complications in dental implantology and their clinical manifestations such as gingival bleeding, swelling and bone loss, strongly resemble

periodontal inflammation[12]. Numerous studies have shown a bacterial etiology with a similar spectrum of putative pathogens[13] and a high concordance of bacterial species[14,16]. It is noteworthy that the colonization of "pristine" peri-implant pockets with periodontal disease associated bacteria has been shown to occur within 2 weeks[17] and that some of these bacteria were found to be present as early as 30 min after insertion[8]. Biofilm formation on implant surfaces is therefore similar in composition and mechanisms known from teeth[17], but may be additionally influenced by their special micro- and macroscopic design features.

Implants with smooth surfaces have been shown to exhibit a higher incidence of early failure, whereas implants with rougher surfaces display a lower incidence of early failure, but show increased failure rates over time[8]. Rough implant surfaces - while being beneficial for initial bone formation and osseointegration - have thus been generally considered to enhance initial adhesion and the subsequent colonization of oral bacteria[18]. Interactive energy effects, e.g. substratum hydrophobicity, surface-free energy and charge, however, must also be taken into consideration[5].

This *in vitro* study assessed the extent of early biofilm colonization up to 16.5 h on titanium surfaces with different surface roughness and wetting characteristics. The hypothesis tested was that there would be no or only minute differences in the quantity and quality of biofilm formed over time. In this context, changes below 1 log step were considered to be irrelevant.

MATERIAL AND METHODS

Disc preparation

Round test specimens (5 mm diameter and 1 mm thickness) were manufactured from commercially pure (c.p.) grade 2 titanium (Straumann AG, CH-4002, Basel, Switzerland) according to one of seven procedures, as described in Figure 1. Visual details of the microstructure of each surface are depicted in Figure 2. Modified surfaces (labeled "modSLA" and "modA") represent chemical modifications and were stored in glass ampoules containing the storage liquid, whereas all other samples were kept in air at room temperature. All specimens displayed different surface characteristics, i.e. surface roughness and wetting potentials, which were assessed as described below.

Measurement of surface roughness and wetting ability

Surface topography and roughness were analyzed by scanning electron microscopy (SEM) and white light confocal microscopy, respectively. Samples for SEM (Leo 1430, LEO Elektronenmikroskopie GmbH, Oberkochen, Germany) were sputter coated (BAL-TEC SCD 050, BAL-TEC AG, Balzers, Liechtenstein) by a thin Au-Pd layer and examined at an accelerating voltage of 20 kV. The confocal three-dimensional (3D) white light microscope (µSurf, NanoFocus AG, Oberhausen, Germany) was used for surface topography measurements. An area of 798 µm x 770 µm was measured to calculate the 3D roughness parameter Sa (arithmetic mean deviation of the surface) using a moving average Gaussian filter with a cut-off wavelength of 30 µm. Five samples from each surface type were investigated.

Contact angle hysteresis was tensiometrically examined by the Wilhelmy method by means of an electrobalance (Sigma 70; KSV Instruments, Ltd., Helsinki, Finland). Dynamic contact angle analysis (DCA) was described in detail elsewhere[20,21]. The immersion velocity was set to 10 mm/min for all experiments; the immersion depth was 15 mm. All

	Surface modification
Machined Sa>0.2 µm	Mechanically polished samples were prepared by using SiC gringing paper
Machined Sa<0.2 µm	Mechanically polished samples were prepared by using SiC gringing paper
PT	Degreasing by washing in acetone, processing through 2% ammonium fluoride/2% hydrofluoric acid/10% nitric acid solution at 55°C for 30 s
A	Acid-etching with a hot solution of HCl/H2SO4 according to a proprietary process of Institut Straumann AG
ModA	New developed A surface. A surface will be rinsed under nitrogen protection and then stored in a sealed glass tube containing isotonic NaCl solution at pH 4 to 6
SLA	Alumina blasting with a large grit particles (average particle size 250 µm) and subsequent acid-etching with a hot solution of HCl/H2SO4 according to a proprietary process of Institut Straumann AG
ModSLA	Further development of SLA surface. SLA will be rinsed under nitrogen protection and then stored in a sealed glass tube containing isotonic NaCl solution at pH 4 to 6

Figure 1- Surfaces and surface modifications investigated in this study

multi-loop experiments have been repeated at least fourfold at room temperature. Five samples from each surface type were investigated.

Biofilm preparation

The experiments were repeated 3 times in triplicates for each surface, resulting in a sample size of 9 specimens *per* group.

Actinomyces oris OMZ 745, *Veillonella dispar* ATCC 17748T (OMZ 493), *Fusobacterium nucleatum* KP-F2 (OMZ 596), *Streptococcus sobrinus* OMZ 176, *Streptococcus oralis* SK248 (OMZ 607), and *Candida albicans* OMZ 110 were used as inocula for biofilm formation[10,22,24]. In brief, all microorganisms were grown to the mid logarithmic phase using a strictly controlled incubation period for each species. In numerous preliminary experiments, growth curves of all microorganisms using culture techniques and optical density measurements were made. Thus, the incubation time of each strain of consortium to reach the mid logarithmic phase could be determined. The density of aliquots of the cultures was measured and adjusted to an absorption of 1.0 (λ 550 nm). The density of the cultures was adjusted accordingly and 1 mL of each culture was used to prepare the final inoculum[10]. The inoculum contained reproducibly 10^7-10^8 microorganisms of each species *per* ml. Biofilms were grown in 24-well polystyrene cell culture plates[25]. For this purpose, discs were preconditioned (pellicle-coated) in processed whole unstimulated pooled human saliva and were cultured as shown in Figure 3. The collection of saliva and the preconditioning of discs have been previously described[10]. Whole, un-stimulated saliva was obtained over several days from volunteers (with informed consent) at least 1.5 h after eating, drinking, or tooth cleaning. Saliva samples, collected in sterile 50 mL polypropylene tubes chilled in an ice bath, were frozen at -20°C. When a total of ca. 500 mL saliva had been collected, it was pooled and centrifuged (30 min, 4°C, 27,000 xg), and the supernatant was pasteurized (60°C, 30 min) and re-centrifuged in sterile bottles; the resulting supernatant was dispensed into sterile 50-mL polypropylene tubes and stored at 20°C. The efficacy of pasteurization was assessed by plating processed saliva samples onto CBA; after 72 h at 37°C, no colony forming units (CFU) were observed on either aerobically or anaerobically incubated plates.

To allow formation of a salivary pellicle, titanium disc were incubated in sterile 24-well polystyrene cell culture plates (NuncA/S, Roskilde, Denmark), with processed saliva (for 4 h, gently shaken, at room temperature). Saliva was aspirated from each well and replaced with 800 mL saliva and 800 mL mFUM medium containing 0.15% glucose and 0.15% sucrose. The wells were inoculated with the

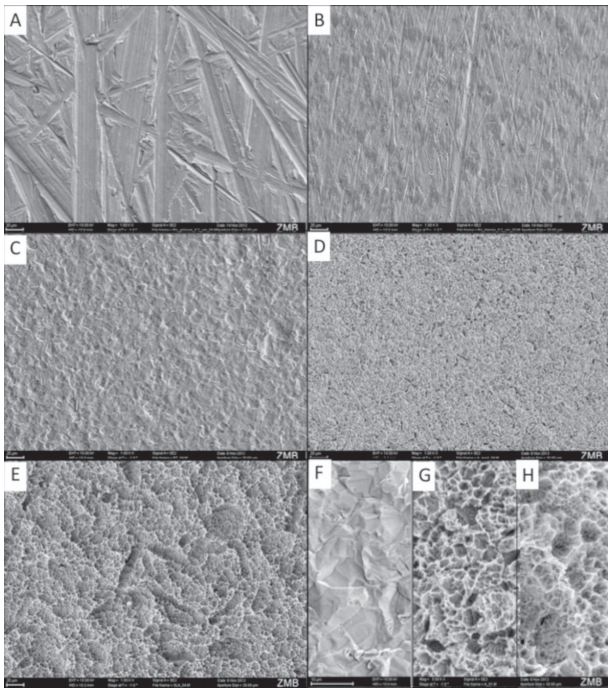

Figure 2- Scanning electron microscopy images of the different titanium surfaces at magnifications of 500x (A-E) and 1,000x (E-G), respectively. A: machined Sa>0.2 µm; B: machined Sa<0.2 µm; C: stained; D: acid-etched; E: sandblasted/acid-etched (SLA). Modified surfaces did not differ in their microscopic surface characteristics. Details at higher magnification of images C-E are given in F: stained PT; G: acid-etched and H: SLA

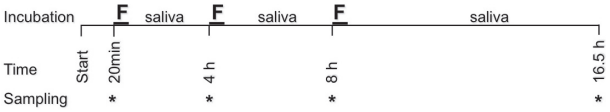

Figure 3- Schematic timeline of the experimental conditions (F represents 45 min feeding periods with saliva/mFUM 30/70). Between feedings, the discs were incubated in saliva

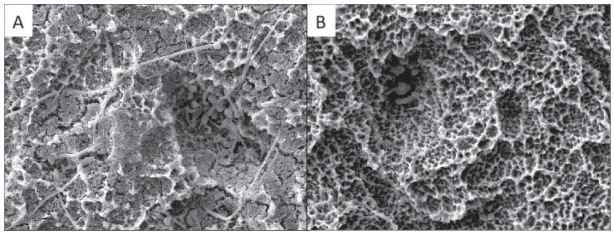

Figure 4- Scanning electron microscophy images of sandblasted/acid-etched (SLA) surfaces (before treatment see figure 2 E and H) after 16.5 h biofilm formation before (A) and after vortexing (B). Coherent mats of microorganisms cover the surface (A). Only few bacteria remain on the surface after harvest by vortexing (B). The surface is comparable to the original surface (Figure 2 E/H)

pooled oral microbiota (200 mL) for 20 min.

Following an initial adherence period of 20 min, the biofilms were fed with the same medium for 45 min and again after 4 and 8 h incubation. After each feeding, the discs were 3x dip-washed in saline to remove non-adhering microorganisms. During the remaining time, the biofilms were incubated in saliva. Biofilms were harvested after 20 min, 2, 8 and 16.5 h (Figure 4).

Harvesting and examination of the biofilm microbiota

To harvest adherent microorganisms, each disc was transferred to a sterile 50 ml polypropylene tube containing physiological saline (1 mL, room temperature) and vortexed vigorously for 2 min. The suspensions were then transferred to sterile 6-ml polystyrene tubes and sonified for 5 s at 30 W. Serial dilutions (10^{-2}-10^{-5}) of sonified cells were prepared in physiological saline and aliquots (50 mL) were spirally plated (Spiral System, Model D, Spiral Systems, Inc., Cincinnati, USA) onto CBA plates (Columbia Blood Agar, Oxoid, CM 331 + 5% whole human blood) for assessing total CFU and as well for identifying and counting *A.naeslundi* and *V.dispar*. *F.nucleatum* was counted using an FAA [Fastidious Anaerobe Agar (lab m, UK, BAG, 7621)] supplemented with erythromycin (1 mg/L) Sigma, E-6376/dissolved in distilled H_2O, vancomycin (4 mg/L) Lilly, 657/dissolved in distilled H_2O and norfloxacin (1 mg/L) Sigma, N-9890/dissolved in absolute ethanol. *S. sobrinus* and *S. oralis* colonies were assessed on MS agar (Mitis-Salivarius agar, Difco 0298-17-2, Heidelberg, Germany) and *C. albicans* on Biggy-Agar (Difco 0635-17-4). After 72 h incubation, CFUs were counted with the assistance of a stereomicroscope.

Data presentation and statistical analysis

Statistical analysis was performed with a commercially available program (StatView, Version 5, Abacus Concepts Inc., Berkeley, USA). For the determination of the surface roughness (S_a, µm) and contact angle (q, °) measurements, mean values and standard deviations were calculated.

For the biofilm evaluation, mean values and corresponding 95% confidence intervals (95% CI) were calculated. To determine the differences

Table 1- Arithmetic mean deviation of the surface (S_a, µm) types and respective contact angle values (q, °). SLA: Sandblasted/acid-etched

Roughness Parameter	Machined S_a>0.2 µm	Machined S_a>0.2 µm	Stained	Acid-etched	Modified Acid-etched	SLA	Modified SLA
Sa	0.3±0.06	0.1±0.02	0.4±0.07	0.6±0.01	0.6±0.02	1.3±0.06	1.2±0.09
q (°)	92±5	92±5	93±5	121±4	0	134±5	0

Table 2- Total log colony forming units (CFU) (mean values and 95% confidence intervals in parentheses; N=9 *per* group) on the various surfaces at the given time points

	20 min	4 h	8 h	16.5 h
Machined	4.5 ^{A}	3.9 ^{A}	4.8 ^{AB}	5.9 ^{ABCD}
S_a<0.2 µm	(4.3, 4.7)	(3.6, 4.2)	(4.6, 5.0)	(5.6, 6.3)
Stained	4.3 ^{A}	3.9 ^{A}	4.6 ^{A}	5.4 ^{AB}
	(4.1, 4.5)	(3.7, 4.1)	(4.4, 4.7)	(5.2, 5.7)
Machined Sa>0.2 µm	4.5 ^{A}	3.7 ^{A}	4.9 ^{AB}	5.9 ^{ABCD}
	(4.4, 4.7)	(3.4, 4.0)	(4.6, 5.2)	(5.7, 6.2)
Acid-etched	4.5 A	3.8 ^{A}	4.9 ^{AB}	5.7 ^{BD}
	(4.4, 4.7)	(3.5, 4.2)	(4.7, 5.4)	(5.3, 6.2)
Modified acid-etched	4.5 ^{A}	4.3 ^{A}	5.4 ^{BC}	6.5 ^{CE}
	(4.3, 4.7)	(4.0, 4.5)	(5.3, 5.4)	(6.4, 6.6)
SLA	4.7 ^{AB}	4.2 ^{A}	5.6 ^{C}	6.5 ^{DE}
	(4.6, 4.8)	(3.9, 4.5)	(5.4, 5.8)	(6.3, 6.6)
Modified SLA	4.8 ^{B}	4.8 ^{B}	6.2 ^{D}	6.9 ^{E}
	(4.8, 4.9)	(4.7, 4.9)	(5.9, 6.5)	(6.7, 7.0)

Different superscript letters represent significant differences (ANOVA and Scheffé *post hoc* analysis, read vertically). SLA: Sandblasted/acid-etched

between smooth and rough surfaces at respective biofilm formation times, an unpaired t-test was used. One-way analysis of variance (ANOVA), together with the *post hoc* Scheffé test, was applied to establish the differences between the different materials. The significance level was set at 95%.

Table 3- Colony forming units (log CFU, mean values and 95% confidence intervals in parentheses) of the different species determined on the various surfaces at the given time points (N=9 *per* group; ANOVA and Scheffé *post hoc* analysis)

		20 min	4 h	8 h	16.5 h
A. naeslundii	S_a<0.2µm	3.1 (2.8; 3.4)	1.3 (1.2; 1.5)	2.5 (2.2; 2.8)	2.3 (2.3; 2.4)
	Stained	3.1 (2.9; 3.2)	2.0 (1.4; 2.7)	2.2 (1.8; 2.7)	2.4 (2.2; 2.6)
	S_a>0.2µm	2.9 (2.8; 3.0)	-	2.5 (2.3; 2.7)	2.4 (2.2; 2.7)
	acid-etched	3.3 (3.2; 3.5)	1.5 (1.3; 1.8)	2.2 (1.6; 2.8)	2.4 (2.2; 2.7)
	mod. acid-etched SLA	3.4 (3.3; 3.6)	1.8 (1.3; 2.3)	2.8 (2.4; 3.2)	3.4 (3.3; 3.6)
	SLA	3.7 (3.6; 3.9)	1.7 (1.4; 2.0)	3.2 (2.9; 3.6)	3.6 (3.2; 3.9)
	mod. SLA	3.9 (3.7; 4.0)	2.1 (1.7; 2.6)	3.7 (3.5; 4.0)	4.2 (3.7; 4.7)
V. dispar	S_a<0.2µm	4.4 (4.2; 4.6)	3.8 (3.5; 4.1)	4.3 (4.2; 4.5)	5.9 (5.6; 6.3)
	Stained	4.2 (4.0; 4.4)	3.8 (3.7; 4.0)	4.1 (4.0; 4.3)	5.4 (5.1; 5.6)
	Sa>0.2 µm	4.2 (3.9; 4.6)	3.5 (3.1; 4.0)	4.6 (4.3; 4.9)	5.9 (5.6; 6.2)
	acid-etched	4.4 (4.2; 4.6)	3.7 (3.4; 4.0)	4.4 (4.1; 4.7)	5.7 (5.2; 6.1)
	mod. acid-etched SLA	4.5 (4.2; 4.7)	4.2 (3.9; 4.4)	4.9 (4.7; 5.2)	6.5 (6.3; 6.6)
	SLA	4.5 (4.3; 4.7)	4.0 (3.8; 4.3)	5.3 (5.1; 5.5)	6.5 (6.3; 6.6)
	mod. SLA	4.6 (4.6; 4.7)	4.7 (4.5; 4.8)	5.9 (5.7; 6.1)	6.8 (6.5; 7.0)
F. nucleatum	S_a<0.2 µm	1.4 (1.2; 1.6)	-	-	-
	Stained	1.4 (1.3; 1.5)	-	-	-
	S_a>0.2µm	-	-	-	-
	acid-etched	1.7 (1.2; 2.1)	-	-	-
	mod. acid-etched SLA	1.5 (1.3; 1.7)	-	-	-
	SLA	1.5 (1.2; 1.7)	1.5 (1.3; 1.7)	-	-
	mod. SLA	1.7 (1.2; 2.1)	-	-	-
S. sobrinus	S_a<0.2 µm	2.1 (1.6; 2.5)	2.3 (2.0; 2.7)	4.3 (3.8; 4.8)	4.4 (4.1; 4.7)
	Stained	1.6 (1.4; 1.8)	2.2 (1.6; 2.7)	4.0 (3.7; 4.4)	4.0 (3.8; 4.2)
	S_a>0.2 µm	1.7 (1.5; 2.0)	2.1 (1.8; 2.4)	4.3(3.7; 4.9)	4.3 (4.1; 4.5)
	acid-etched	1.9 (1.6; 2.2)	2.6 (2.0; 3.2)	4.3 (3.4; 5.3)	4.5 (4.0; 4.9)
	mod. acid-etched SLA	2.0 (1.6; 2.3)	3.1 (2.6; 3.6)	4.6 (4.0; 5.1)	4.8 (4.5; 5.1)
	SLA	2.2 (1.9; 2.5)	3.1 (2.5; 3.7)	5.0 (4.4; 5.6)	5.1 (4.8; 5.4)
	mod. SLA	2.6 (2.3; 2.9)	3.5 (2.8; 4.2)	5.5 (5.0; 5.9)	5.6 (5.4; 5.9)
S. oralis	S_a<0.2 µm	2.0 (1.8; 2.3) 2.1 (1.9; 2.3)	2.1 (1.9; 2.3)	2.9 (2.6; 3.2)	3.9 (3.6; 4.2)
	Stained	2.0 (1.7; 2.3)	2.1 (1.6; 2.5)	2.6 (2.2; 3.0)	3.4 (3.0; 3.7)
	S_a>0.2 µm	2.1 (1.9; 2.3)	1.9 (1.5; 2.2)	3.1 (2.7; 3.4)	3.6 (3.3; 3.8)
	acid-etched	2.0 (1.8; 2.6)	2.6 (2.3; 2.8)	3.3 (2.9; 3.6)	3.7 (3.4; 4.0)
	mod. acid-etched SLA	2.6 (2.0; 3.2)	2.5 (2.2; 2.9)	3.5 (3.1; 3.8)	4.0 (3.8; 4.2)
	SLA	2.4 (2.2; 2.5)	2.6 (2.1; 3.0)	3.7 (3.4; 4.0)	4.2 (4.0; 4.4)
	mod. SLA		2.5 (2.2; 2.9)	4.2 (4.0; 4.4)	4.7 (4.4; 5.1)
C. albicans	S_a<0.2 µm	3.0 (2.6; 3.3)	1.8 (1.6; 2.1)	2.4 (2.0; 2.7)	2.3 (2.1; 2.5)
	Stained	3.1 (2.9; 3.2)	2.4 (2.0; 2.8)	2.2 (2.0; 2.4)	2.1 (1.8; 2.4)
	S_a>0.2 µm	3.2 (3.0; 3.4)	2.0 (1.7; 2.4)	2.7 (2.4; 2.9)	2.1 (1.7; 2.4)
	acid-etched	3.6 (3.3; 3.9)	2.0 (1.6; 2.5)	2.0 (1.6; 2.4)	2.0 (1.7; 2.3)
	mod. acid-etched SLA	3.2 (3.1; 3.4)	2.3 (1.8; 2.9)	2.3 (1.9; 2.8)	2.4 (2.1; 2.7)
	SLA	3.9 (3.8; 4.0)	3.1 (2.7; 3.5)	3.5 (3.1; 3.9)	3.0 (2.8; 3.1)
	mod. SLA	3.9 (3.8; 4.0)	3.6 (3.4; 3.9)	3.7 (3.5; 4.0)	3.2 (3.2; 3.3)

RESULTS

Surface roughness and wetting ability

The measured values are presented and summarized in Table 1.

S_a values >1 µm were only obtained for the SLA surfaces. The acid-etched surfaces had mean S_a values of 0.6 µm. The chemically modified and activated surfaces showed no statistically significant difference as compared to their un-activated counterparts. All other samples showed a mean surface roughness below 0.5 µm, with the more polished surface reaching mean S_a values of 0.1 µm.

With regard to the contact angle measurements, it can be summarize that the best wetting ability was achieved by the modified (i.e. activated) samples (q=10). The q-values of the acid-etched and SLA surfaces were the highest, with values of 121 and 134, respectively. This indicates very low surface wetting characteristics. The results of the polished and stained surfaces ranged between 92° and 93°.

Biofilm formation

The results of the microbial biofilm analysis are summarized in Tables 2 and 3.

Figure 3A shows a SLA surface after incubation. A strong microbial colonization was evident after 16.5 h of incubation.

The mean number of microorganisms adhering to the discs after 20 min showed only minor differences between the groups and ranged between 4.5 to 4.8 $\log_{10}$ CFU. The highest initial adherence was observed on modified SLA discs ($P \leq 0.01$).

After the first feeding period (4 h of incubation), there was no growth on all surfaces observed. In contrast, all test groups showed a colonization decrease of 0.2 to 0.7 log steps, with the exception of the modified SLA, as compared to the initial adherence phase.

After 8 h, the particular pattern of microbial colonization on the different titanium surfaces persisted, however, at this time point there was significantly increased growth observed on all test surfaces. In the following time period up to 16.5 h, growth continued further with a low but material specific rate (Table 2).

Regarding the number of initially adhering microorganisms (Table 3), *V. dispar* showed by far the strongest adherence to these titanium surfaces (≈log 4 steps). *S. oralis* and *C. albicans* exhibited a one log step lower adherence.

After 4 h incubation, the microbial composition of the biofilms had started to shift. *V. dispar* remained at the level of initial adherence. The numbers of *S. oralis* and *C. albicans* decreased dramatically while *S. sobrinus,* started to increase. *F. nucleatum* fell, with one exception (SLA), below detection level.

After 8 h incubation, biofilm growth increased again: *V. dispar* increased by approx. 1 log step reaching a level of log 5; *S. sobrinus* continued to grow, almost attaining the level of *V. dispar*; and *S. oralis* continued to grow, albeit at a lower growth rate. The colonization density of *C. albicans* remained unchanged.

From the 8 h reading to the end of the experiment at 16.5 h, microbial density continued to increase, but at a slower rate. It was evident that *V. dispar* was primarily responsible for this increase, and seems to have profited from the lactic acid produced by the streptococci as an energy source. Both streptococcal species only showed minute changes. The other species remained stagnant. *F. nucleatum* colonized during the entire experimental period near or below detection level.

DISCUSSION

The results of this study support the hypothesis that regardless of the titanium's surface roughness and despite highly significant differences in wetting properties, bacterial colonization was quite similar on all implant materials over time. The differences between the different colonization mass was within the range of one log step. That rough and smooth machined surface values showed quite comparable colonization in this study is in contrast to the existing opinion of a threshold value of 0.2 µm[23].

The so-called "Zurich biofilm model" was applied, which has been validated in several studies[10,11]. Although this model represents a supragingival plaque model, its relative ease to cultivate, as well as its basic biofilm characteristics and role in the ensuing development of subgingival plaque/peri-implantitis, allow for a realistic and reproducible laboratory simulation of the oral condition.

With the exception of *C. albicans,* all selected bacteria are found in high numbers in supra-gingival plaque responsible for gingivitis. Colonization of implants preceding pocket formation is dependent on initial inflammation occurring in the absence of sufficient dental hygiene measures. The subgingival microbiota responsible for peri-implantitis, including in addition high numbers of anaerobic Gram-negative bacteria, can only establish gradually after inflammation and pocket formation have progressed. As Gram-positive bacteria form the first layer of firmly adherent microorganisms on the root surface, selection of the present biofilm consortium cannot be considered too far-fetched[26].

The spatial arrangement and the associative behavior are well documented and the model has been used to assess different aspects in microbiology as well as clinically based and oriented research in the dental field[10]. Whereas the experiments described above were predominantly

performed on sterile hydroxyapatite discs, this is the first study assessing the biofilm growth on titanium samples. In another laboratory study, the applicability of our model was assessed on other dental materials[6]. Similar to the present study, the influence of surface roughness and contact time on the formation of a multi-species biofilm on different materials was tested. This earlier study showed that surface roughness may influence initial biofilm adherence after 15 min, but differences vanished following growth and maturation phases after 15 h, irrespective of whether rough or smooth surfaces were tested.

Within the limitations of the employed study design, the results are in line with implants of different surface characteristics, although moderately rough surfaces (Sa between 1 and 2 µm) tended towards higher plaque accumulation[1]. A recently published *in situ* study revealed a significantly lower biofilm adherence after 2 h on smooth turned titanium surfaces, however, after 14 h, the biofilm volume on all surfaces was similar again, suggesting that the influence of surface characteristics on adhesion was compensated for by biofilm development, which is again in line with our findings[7]. Another *in vitro* evaluation used the same implant surface types and a slightly modified Zurich biofilm model incorporating 9 bacterial strains, which were assessed after a 24 h incubation using either culture medium or human saliva, with checkerboard analysis[2]. The saliva incubation technique corresponds to our set-up and showed lower counts of bacteria as compared to culture medium. This study also showed that SLA and modified SLA had a significant increase in bacterial adhesion when using human saliva. The findings of the study showed by micro-topography that biofilm formation and composition was affected by hydrophilicity of the surface. This observation is not supported by our results. This difference may be explained, only in part, by the different bacterial strains used but not by the microbiological evaluation techniques (culture versus checkerboard analysis).

The investigation of the initial adherence and growth of the 6 microbial species on these differently crafted titanium surfaces provided other interesting insights. The strongest initial adherence among the 6 microorganisms used for biofilm formation was exhibited by *V. dispar* and *C. albicans*. Among the differently crafted titanium surfaces, adherence differences were very similar to those described for total CFU. Differences in affinity of *A. oris* for these surfaces were more distinct. The lowest adherence was observed on stained titanium and on surfaces with a roughness <0.2 µm. A higher but still low affinity for this species was observed on the acid etched, modified acid etched and titanium surfaces with a roughness of >0.2 µm. Significantly stronger adherence, in comparison with the acid etched and modified etched surfaces, were evident for the SLA ($Pf<0.5$) and even more for the modified SLA surfaces ($Pf<0.01$). *F. nucleatum* remained, with one exception, below detection level. That is not surprising. In all biofilm experiments within the last ten years, this particular bacterial strain hibernated during the first 16.5 h and only started to grow on saliva-preconditioned hydroxyapatite disks in the period between 16.5 h and 64.5 h. It appears that the accompanying microbiota has to first prime the conditions in the biofilm, to allow growth of this fastidious species. The proportion of *F. nucleatum* in a study by Almaguer-Flores and co-workers was also low after 24 h, which supports our observation[2]. Astonishingly low was also the adherence of the streptococci on all titanium surfaces. This is in contrast to biofilm experiments with natural tooth or hydroxyapatite surfaces. Guggenheim and co-workers showed an initial adherence for *S. mutans* and *S. oralis* in the order of magnitude of log 4 on pellicle-coated hydroxyapatite discs of similar size[10]. The influence of structural, physical and chemical micro-surface characteristics should not be neglected, as it has been demonstrated in some studies that these factors, including the microenvironment, may influence the attachment and growth of individual microorganisms[2,3,9].

After the first feeding period and during the 4 h of incubation, detachment of all microorganisms was observed. The intensity of this shedding was primarily dependent upon the nature of the titanium surface structure and showed substantial differences between species. Consistent at this time point was the highly significant difference ($Pf<0.01$) between the modified SLA surface, showing the highest number of microorganisms, and the titanium surfaces with a surface roughness < and >0.2. However, after 8 h, all species on all surfaces showed re-growth, with the exception of *C. albicans* and *S. sobrinus* on the titanium surfaces with surface roughness <0.2 and >0.2 µm. Up to 16.5 h growth continued and the colonization level of *A. oris* and *V. dispar* exceeded in numbers the initial adherence counts. Both streptococcal species showed growth reaching the density of the initial adherence phase while the numbers of *C. albicans* remained almost unchanged. This failure of C. albicans to grow, however, may be explained by the anaerobic incubation of the biofilms.

In summary, it was found that titanium disks with moderately rough surfaces tended to exhibit more biofilm formation patterns. However, most differences in colonization density between the tested titanium surfaces did not reach statistical significance. Biofilm formation on implant surfaces appeared to be controlled not only by growth

conditions, but also, albeit to a minor extent, by the nature of the colonized surface. Although the adherence pattern of the total biofilm microbiota on the different titanium surfaces remained remarkably constant through out the experiment, the minute differences visible after the initial adherence phase became more prominent over time but ranged within one log step. The surface modification had no effect on biofilm formation.

CONCLUSION

Surface roughness moderately influenced biofilm formation under the experimental conditions described, whereas wettability was less influential. From a clinical point of view, the implant surface needs daily meticulous oral hygiene. If this condition is met, then the choice of implant surface characteristics may be salient to the long-term health of any implant placed. However, if the biofilm is allowed to grow uncontrolled, the influences of different titanium surfaces become irrelevant.

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