Bacterial adhesion and biofilm formation on yttria-stabilized, tetragonal zirconia and titanium oral implant materials with low surface roughness - an in situ study

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Bacterially-driven mucosal inflammation and the development of periimplantitis can lead to oral implant failure. In this study, initial bacterial adhesion after 2 h, and biofilm formation after 1 day and 3 days, were analysed in situ on novel 3 mol % yttria-stabilized tetragonal zirconia polycrystal samples, as well as on alumina and niobium co-doped yttria-stabilized tetragonal zirconia samples. Pure titanium implant material and bovine enamel slabs served as controls. The initially adherent oral bacteria were determined by 4',6-diamidino-2-phenylindole-staining. Biofilm thickness, surface covering grade and content of oral streptococci within the biofilm were measured by fluorescence in situ hybridization. No significant differences between the ceramic and titanium surfaces were detectable for either initial bacterial adhesion or the oral streptococci content of the in situ biofilm. The oral biofilm thickness on the implant surfaces were almost doubled after three days compared to the first day of oral exposure. Nevertheless, the biofilm thickness values among the different implant surfaces and controls did not differ significantly for any time point of measurement after 1 day or 3 days of biofilm formation. Significant differences in the covering grade were only detected between day 1 and day 3 for each tested implant material group. The content of oral streptococci increased significantly in parallel with the increase in biofilm age from day 1 to day 3. In conclusion, oral implant zirconia surfaces with low surface roughness are comparable to titanium surfaces with respect to initial bacterial adhesion and biofilm formation.

INTRODUCTION

Endosseous implants are used as a standard treatment in prosthetic dentistry for partially edentulous individuals (Esposito et al., 2014; Wen et al., 2014), as well as for patients with loss of a single tooth (Benic et al., 2014; Chen & Buser, 2014). To date, pure titanium has been the most widespread and successfully used material for oral implants

Abbreviations: DAPI, 4',6-Diamidino-2-phenylindole; Zr, 3Y-TZP; AFM, atomic force microscopy; BES, bovine enamel slabs; Rν, average surface roughness; Rr, root mean square surface roughness; Rz, ten-point average roughness; FISH, fluorescence in situ hybridization; Ti, titanium implant surfaces; Zr, 3 mol% yttria-stabilized tetragonal zirconia polycrystal surfaces (3Y-TZP); Al-Zr, Alumina and niobium co-doped yttria-stabilized tetragonal zirconia polycrystal surfaces (Al2O3/Y(Nb)-TZP).
due to its biocompatibility, high corrosion resistance, durability and ease of processing (Amoroso et al., 2006). However, allergies against titanium have been reported in some individuals (Jacobi-Gresser et al., 2013; Sicilia et al., 2008). With respect to edentulous patients, the success rates of titanium implants have been reported to range between 73.4 and 100 % for the upper and lower maxilla over 10–15 years (Moraschini et al., 2015). Until now the vast majority of oral implants have been manufactured using titanium (Andreiotelli et al., 2009). However, zirconia oral implants with different designs and surface modifications have also been proposed as clinically relevant alternatives (Kohal et al., 2013). Due to the proven biocompatibility of zirconia, its resistance to corrosion, its high-quality aesthetics, its high level of toughness and its strength (Bachle et al., 2004; Miyazaki et al., 2013; Scarano et al., 2004), research on oral zirconia implants has increased (Andreiotelli et al., 2009; Wenz et al., 2008). Indeed, pre-clinical investigations have shown that zirconia can withstand masticatory forces over an extended period of time (Kohal et al., 2002). Furthermore, no allergic reactions have been reported for dental implant materials based on zirconium oxide, a fact which makes them suitable for patients with severe allergic reactions to titanium (Jacobi-Gresser et al., 2013; Sicilia et al., 2008).

In a recent review on zirconia oral implants, Depprich et al. (2014) collected the available clinical data from all relevant case reports, as well as from prospective, retrospective and randomized multicentre studies in this field. Despite the fact that the reported survival rates for zirconia oral implants ranged from 74 to 98 % after observation periods of 12–56 months, the results need to be interpreted with caution due to short observation periods, small numbers of participants, minimal information provided on study methodology and the relatively little evidence extracted from these reports.

In addition to the mechanical properties of the implant materials selected, microbial adhesion and subsequent biofilm formation on dental implants could also substantially influence their long-term survival in the oral cavity. Low debris accumulation and biofilm formation on implant surfaces are vital prerequisites for healthy implant osseointegration (Bahat & Sullivan, 2010; Ozkurt & Kazazoglu, 2011). Otherwise, bacterially-driven inflammation of the adjacent mucosa and development of peri-implantitis can lead to implant treatment failure (Lima et al., 2008). The prevalence of peri-implantitis has been reported to be in the range 28–56 % in patients with dental implants (Renvert et al., 2012; Smeets et al., 2014; Zitzmann & Berglundh, 2008).

The process of initial bacterial adhesion and biofilm formation on a biomaterial surface is dependent on the surface itself and may be influenced by its chemical structure, surface roughness, surface energy and various other surface characteristics, such as skewness, summit density and texture aspect ratio (Barbour et al., 2007; Crawford et al., 2012; Karygianni et al., 2013; Teughels et al., 2006). Additionally, bacterial cell surface properties, such as hydrophobicity, affect bacterial adhesion and subsequent biofilm formation (Vadillo-Rodriguez et al., 2005). To date, it has primarily been in vitro studies that have characterized oral implants with respect to initial bacterial adhesion and biofilm formation, as highlighted earlier in our own studies (Al-Ahmad et al., 2010, 2013; Scarano et al., 2004; Zhao et al., 2014). Due to the considerable differences in composition between the pellicles formed by human sterilized saliva in vitro and those formed in the oral cavity in situ, results of in vitro studies could differ significantly from those of in situ investigations (Hannig & Hannig, 2009; Yao et al., 2003). Furthermore, the oral microbiota consists of 700–1000 different species (Dewhirst et al., 2010; Griffen et al., 2012), and contributes to the formation of microbial biofilms with high microbial diversity within the oral cavity (McLean, 2014). As a result, the characterization of the complex process of oral biofilm formation on implant materials can only be simulated to a limited extent in vitro, although a high diversity of adherent oral bacteria on implant materials has been demonstrated in vitro (Nascimento et al., 2015). Nevertheless, realistic in situ biofilm models, such as splint systems worn by healthy volunteers, have been successfully introduced to study the interaction between dental implant materials and oral bacteria, as described in previous studies (Al-Ahmad et al., 2010).

4',6-Diamidino-2-phenylindole (DAPI)-staining has been shown to be an appropriate method for the visualization and quantification of oral microorganisms, which are initially adherent on a variety of implant materials (Al-Ahmad et al., 2013; Jung et al., 2010). Fluorescence in situ hybridization (FISH) has been shown to be a useful staining technique for the detection and visualization of bacteria in initial and mature oral biofilms, and has the advantage of functioning without disrupting the natural biofilm structure (Keijser et al., 2008). For example, oral streptococci have been shown to be representative oral biofilm colonizers. This in turn makes them a bacterial target group particularly suitable for the visualization and quantification of the oral biofilm formed in situ (Al-Ahmad et al., 2010, 2013).

In the present study, initial bacterial adhesion and biofilm formation were analysed in situ on novel 3-mol% yttria-stabilized tetragonal zirconia polycrystal samples (Zr; 3Y-TZP), as well as alumina and niobium co-doped yttria-stabilized tetragonal zirconia samples (Al-Zr; Al2O3/Y (Nb)-TZP). Standard titanium implant surfaces (Ti; grade 4) and bovine enamel slabs (BES) served as controls (Table 1). The surface roughness parameters of all three implant materials and BES were characterized using atomic force microscopy (AFM), whereas surface energy was measured by the determination of water contact angle. Adherent oral bacteria on all tested surfaces were investigated by both DAPI-staining and by FISH. The null hypothesis was that oral implant zirconia surfaces with low average surface roughness (Ra<200 nm) are not significantly different to titanium surfaces with respect to the initial adhesion of oral bacteria and biofilm formation in situ.
Surface roughness parameters such as the average surface roughness \( R_a \) and bovine enamel specimens were used: 3 mol% yttria-stabilized tetragonal zirconia polycrystal surfaces (3Y-TZP). The control surfaces, BES, were made and disinfected as previously described (Al-Ahmad et al., 2013). The control surfaces, BES, were gained from the freshly extracted anterior teeth from the upper jaw of 2-year-old, bovine spongiform encephalopathy (BSE)-free, cattle from the central slaughterhouse in Freiburg, Germany. BES were made and disinfected as previously described (Al-Ahmad et al., 2013). In brief, cylindrical enamel specimens (diameter, 5 mm; 19.63 mm² surface area; height, 1.5 mm) were first obtained and then wet-polished with abrasive paper (400–4000 grit) in order to yield plain and parallel BES surfaces. BES disinfection included double ultrasonication in NaOCl (3 %) for 3 min, and subsequent rinsing (in double-distilled water for 10 min. For hydration, BES slabs were placed in distilled water for 24 h.

**METHODS**

**Implant materials and control surfaces**

For the examination of initial adhesion of bacteria in situ, the following different ceramic oral implant materials (diameter, 5 mm; 19.63 mm² surface area; height, 1.5 mm) were used: 3 mol% yttria-stabilized tetragonal zirconia (Zr; 3Y-TZP, Acucera) and alumina and niobium co-doped yttria-stabilized tetragonal zirconia (Al-Zr; Al₂O₃/Y(Nb)-TZP, Acucera), Titanium (Warrantec) (Ti; grade 4, diameter, 5 mm; 19.63 mm² surface area; height, 1.5 mm) was taken as the standard implant material; the preparation of the implant surfaces has been described in detail previously (Kim et al., 2013). The control surfaces, BES, were gained from the freshly extracted anterior teeth from the upper jaw of 2-year-old, bovine spongiform encephalopathy (BSE)-free, cattle from the central slaughterhouse in Freiburg, Germany. BES were made and disinfected as previously described (Al-Ahmad et al., 2013). In brief, cylindrical enamel specimens (diameter, 5 mm; 19.63 mm² surface area; height, 1.5 mm) were first obtained and then wet-polished with abrasive paper (400–4000 grit) in order to yield plain and parallel BES surfaces. BES disinfection included double ultrasonication in NaOCl (3 %) for 3 min, and subsequent rinsing (in double-distilled water for 10 min. For hydration, BES slabs were placed in distilled water for 24 h.

**Surface characterization of the titanium, ceramic and bovine enamel specimens**

Surface roughness parameters such as the average surface roughness \( R_a \), the root mean square surface roughness \( R_q \), the ten-point average roughness \( R_{10} \) and the peak-to-valley height \( R_{p} \), as well as the surface topography were measured in three specimens of each material using atomic force microscopy (AFM, XE-100; Park Systems) in a contact mode of 50 × 50 µm². For the characterization of surface energy the sessile drop method was used, as has been described in detail (de Oliveira, et al., 2012). The contact angles were measured using a video contact angle analyzer [Phoenix 150; Surface Electro Optics (SEO)]. Three samples from each material group were used to determine the contact angle of deionized water. The calculation of the degree of the contact angle was determined using the software provided with the equipment. All values had already been determined in an earlier in vitro study into bacterial adhesion to surfaces of the same materials (Kim, et al., 2013).

**Patient collective and preparation of the splint systems**

Six healthy volunteers (two female and four male) aged between 23 and 57 years, were selected for the clinical experimental trials. All volunteers signed their consent to be included in the study, which was approved by the ethics commission (EK-63-07, University of Freiburg, Germany), before the beginning of the study. Volunteers with diseases of the salivary glands or other general disorders were excluded from the study. In addition, the oral health status of all volunteers was recorded and no caries or inflammation of the marginal periodontium could be detected. Full dentition with sufficient space for the stable placement of the splint containing the implant and BES was required. Exclusion criteria for participation in the study were drug-, nicotine-, or alcohol-consumption and/or the use of antibacterial mouth rinses or antibiotics within the 6-month period prior to the study. The implementation of oral hygiene activities in the 2 h period before and during the investigations was strictly forbidden.

For each of the volunteers an individual, intraoral splint system for the upper jaw was manufactured (Fig. S1, available in the online Supplementery Material), as previously described (Karrigiani et al., 2014). This splint system contained three different implant materials and one control BES surface. Biofilm formation was not disturbed by the tongue or the cheek because the materials were held in place by red wax and inserted facing the interdental area between two adjacent teeth. Before use, the splint system was disinfected with 70 % (v/v) ethanol. All volunteers wore the splint for 120 min to investigate initial bacterial adhesion (Fig. 1), whereas for the examination of oral biofilm formation the splint was worn for 1 day and 3 days. The splint was allowed to be taken out of the oral cavity and stored in 0.9 % (w/v) NaCl solution only during daily oral hygiene procedures and at meal times. After the test periods the splints were washed with 0.9 % (w/v) NaCl for 30 s to dislodge non-adherent bacteria. The samples of material were subsequently carefully removed from the splints and stored until further investigation in physiological saline solution.

**Microbiological examinations**

**DAPI-staining.** The implant materials and the BES control surfaces were examined with DAPI after the 120 min test period, allowing for the visualization of all adherent microorganisms under the epifluorescence microscope. Staining procedures and quantitative analysis of the microbes were performed as previously described (Al-Ahmad et al., 2013). In brief, all implant and control samples were covered with 1 ml distilled water (Braun Melsungen) containing a final concentration of 1 µg ml⁻¹ DAPI. After staining for 10 min in a dark chamber, the DAPI solution was rinsed from specimens with distilled water. The samples were then dried at room temperature, placed on a slide and covered with Citifluor (Citifluor). To quantify the total bacterial counts, bacteria were analysed by epifluorescence microscopy (Axioskop II, Carl Zeiss Jena) at 1000× magnification using a DAPI filter set (BP 365, FT 395, LP 397). Since salivary bacteria are semi-planktonic rather than planktonic, single cells as well as flocs (agglomerates) were counted as single units. Counting all cells within the flocs required a confocal laser scanning microscope and was not necessary for quantifying initial oral microbial adhesion. The number of bacterial cells counted in 10 randomized microscopic ocular grid fields per sample was determined. From the area of the ocular grid (0.0156 mm²) the numbers of cells per cm² could be estimated.
Bacterial adhesion on oral implant materials

Fig. 1. Boxplots showing the bacterial count of oral microorganisms, which were initially adherent on different implant material surfaces and control BES after an in situ incubation of 120 min in the oral cavity. BES, bovine enamel surfaces; Ti, titanium implant surfaces; Al-Zr, alumina and niobium; Zr, 3 % yttria-stabilized zirconia polycrystal surfaces.

FISH and confocal laser scanning microscopy

FISH was conducted according to Amann et al. (1995) with modifications as previously described (Al-Ahmad et al., 2013). In brief, the biofilms from the different materials were fixed for 12 h at 4 °C with 4 % paraformaldehyde in phosphate-buffered saline (PBS: 1.7 mM KH₂PO₄, 5 mM Na₂HPO₄ with 0.15 M NaCl, pH 7.2). All specimens were washed after fixation with PBS and fixed again in an ethanol containing solution (50 % in PBS, v/v) for 12 h. To permeabilize adherent bacterial cells, the probes were then washed twice with PBS followed by incubation in a solution containing 7 mg of lysozyme per ml of 0.1 M Tris-HCl-5 mM EDTA, pH 7.2, for 10 min at 37 °C. Dehydration was carried out through a series of ethanol washes containing 50, 80 and 100 % (v/v) ethanol for 3 min each. The specimens were then incubated with oligonucleotide probes at a concentration of 50 ng each per 20 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 25 % formamide (v/v) and 0.01 % sodium dodecyl sulphate (w/v)). The two oligonucleotide probes used in the study were 5'-end labelled with different fluorochromes and HPLC purified (Thermo Electron). The EUB 338 probe (GCTGCTCCCGTAGCAGGT) was 5'-end labelled with fluorescein and targeted eubacteria (Amann et al., 1990). The Strep 405 probe (TAGCGTCCCTTTTCTGGT) was 5'-end labelled with Cy3 and targeted Streptococcus spp. Hybridization was conducted in 96-well plates (Greiner bio-one) at 46 °C for 2 h. Following probe hybridization, specimens were incubated for 15 min at 48 °C in wash buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 159 mM NaCl and 0.01 % sodium dodecyl sulphate (w/v). After washing, the labelled bacteria were analysed in a chambered coverglass (µ Slide 8 well; ibidi) by confocal laser scanning microscopy (CLSM: Leica TCS SP2 AOBS) using a 63× water immersion objective (HCX PL APO lbd.BL 63.0x1.2W, Leica). The excitation of the FISH probes was carried out using the following wavelengths: 488 nm (fluorescein) and 543 nm (Cy3). The areas measured were from three separate and representative locations on the surface exposed to bacteria for each of the tested samples, and a mean of the three measurements was taken for further statistical analysis. To avoid overlaps, the in situ biofilms formed were scanned from these three starting points generating optical sections of approximate thickness 0.5 µm each at 2 µm intervals throughout the thickness of the biofilm. Standard images were made with a zoom setting of 1.7 corresponding to physical dimensions of 140×140 µm for each image and 1024×1024 pixels.

In order to quantify the biomass of the different targets within the oral biofilm, total fluorescent staining of the confocal micrographs was analysed using the image analysis program MetaMorph 6.3r7 (Molecular Devices Corporation). The fluorescent volume corresponding to the EUB 338 probe was set at 100 % for the bacterial biomass in the biofilm, and was also used to calculate the covering grade of the different implant surfaces and the control material. The corresponding fluorescent volume of Strep 405 was calculated as a percentage of this total biomass. Fluorescence intensity thresholds were manually set for each of the fluorescent colours. Finally, the resulting covering grade and biofilm contents (as % streptococci) were analysed for statistical significance.

Statistical analysis

Mixed models were fitted with the subject as a random effect. Each continuous response variable was modelled as a linear function of time (1 day and 3 days), group (implant material), and with the time-group interaction as explanatory variables. In those cases where an interaction was significant, group comparison was then carried out separately for each time point. Variance components were used for the covariance structure. To determine the degrees of freedom the between–within method was chosen. The P-values for the pairwise comparison were adjusted by the method of Tukey–Kramer with a significance level of P<0.05. All calculations were done using the PROC MIXED procedure from the statistical software SAS 9.2 (SAS Institute, Cary).
RESULTS

Surface roughness and contact angles

Table 1 shows the surface roughness values, as well as the contact angles of the implant materials and the control BES. The average surface roughness varied between 1.8 nm (Ti) and 3.3 nm (Zr). The surface roughness of Al-Zr was 2.5 nm. The controls showed a surface roughness of 14.3 nm. All implant materials as well as BES showed contact angles of less than 90° in water and were therefore characterized as hydrophilic.

Total bacterial count

DAPI-staining was used to determine the total amount of adherent bacteria initially on implant and control BES surfaces after 120 min of oral exposure. Fluorescence microscopy showed that primarily cocci and more specifically single cocci, diplococci, chain cocci and rods were detected (Figs 2 & S2). The total bacterial count after 120 min of initial colonization in situ on the materials investigated is shown in Fig. 1 & Table S1. Interestingly, the highest bacterial count was detected on BES. No significant differences between the ceramic implant materials and titanium were detectable. The total bacterial count of initially adherent oral bacteria was significantly lower on alumina/niobium-toughened zirconia (Al-Zr; \( P = 0.0281 \)) and zirconia surfaces (Zr; \( P = 0.0011 \)) compared to BES. No significant differences between the bacterial count on Ti and BES were detected (\( P > 0.05 \)).

Biofilm thickness

Fig. 3 and Table S2 illustrate the distribution of biofilm thickness values after 1 day and 3 days, respectively, of oral exposure of the tested implant surfaces in situ. The predicted means for biofilm thickness on the implants were between 14.8 and 17.1 µm (median 16 µm) after the first day of in situ application of the splints, whereas biofilm thickness values were considerably higher after 3 days of in situ oral biofilm formation (\( P < 0.008 \)), in the range 27.8–31.7 µm (median 26 µm). Interestingly, the values of oral biofilm thickness on implant surfaces were almost doubled after 3 days compared to the first day of their oral exposure in situ (\( P < 0.008 \)). Nevertheless, values of biofilm thickness among the different implant materials and controls did not differ significantly at any time point of measurement after 1 day or 3 days of biofilm formation in the oral cavity (\( P > 0.05 \)).

Covering grade

Fig. 4 & Table S3 depict the grade of the oral biofilm covering the tested implants and control BES surfaces calculated as percentages (%) after 1 and 3 days of oral exposure, respectively. Significant differences in the covering grade were only detected between day 1 and day 3 for each implant material group tested. As far as the controls are concerned, the predicted means of the covering grade for BES (3.3 %, \( P = 0.0001 \)) as well as Ti (3.4 %, \( P = 0.0147 \)) surfaces decreased significantly from day 1 to day 3 of oral exposure in situ. However, the covering grade of Al-Zr (3.7 %, \( P = 0.0003 \)) and Zr (2.2 %, \( P = 0.0311 \)) increased significantly along with increasing age of the oral biofilm from 1 to 3 days.

Content of streptococci in the oral biofilm

Using FISH and specific oligonucleotide probes, the content of oral streptococci within the in situ biofilm on each implant material and BES surface was determined after 1 and 3 days of oral exposure (Fig. 5 & Table S4). The percentages of oral streptococci on each of the tested surfaces ranged from 19.7 to 26.8 % in the 1-day old oral biofilm, and from 31.8 to 41.8 % in the 3-day old oral biofilm. As depicted for all tested materials, the content of oral streptococci increased significantly (BES: 22.1 %, \( P = 0.0001 \); Ti: 8.3 %, \( P = 0.008 \); Al-Zr: 5.9 %, \( P = 0.005 \); Zr: 9.1 %, \( P = 0.0001 \)) along with the increase in biofilm age from day 1 to day 3. No significant differences (\( P \geq 0.05 \)) were detected among all of the tested materials with respect to oral streptococci content in the in situ biofilm, independently of age. As shown in Fig. 6 & Fig. S3), eubacteria are visualized in green (fluorescein-marked specific gene probe), whereas streptococci were depicted in magenta (Cy3-marked specific gene probe; Fig. S4). The confocal images confirm the fact that streptococci represent a major part of the bacterial colonizers within the oral biofilm.
DISCUSSION

In the present study initial microbial adhesion and biofilm formations were studied in situ on novel zirconia implant materials with low surface roughness. Standard titanium implant material and bovine enamel, also with low surface roughness, served as controls. Initial microbial adhesion, biofilm thickness, covering grade of the biofilm as well as the content of oral streptococci within the biofilm were comparable on all implant materials. Furthermore, no significant differences between all implant materials tested and BES were detected.

The outcomes of the present study confirm that microbial colonization of implant materials with low average surface roughness ($R_a$), ranging from 1.8 nm (Ti) to 2.5 nm (Al-Zr) or 3.3 nm (Zr), is independent of their chemical structure, surface energy and hydrophobicity (Kim et al., 2013). Despite the fact that titanium has a higher total surface energy (47.4 mJ m$^{-2}$) compared to Al-Zr (32.0 mJ m$^{-2}$) and Zr (37.5 mJ m$^{-2}$), no significant differences with respect to initial bacterial adhesion or biofilm formation were detected between the material surfaces tested. In addition to the low surface roughness (<200 nm), the lack of discrepancies among the different materials depicted in the present study can be attributed to the neutralizing effects of the oral pellicle on physico chemical surface properties of all pellicle-coated surfaces (Hannig & Hannig, 2009). Unlike the implant materials used in the present study, Scarano et al. (2004) investigated titanium and zirconium oxide disks with a high surface roughness of 730 nm and 760 nm, respectively. Using scanning electron microscopy, the study indicated a higher covering grade of the implant materials with 1-day old biofilm as a result of the aforementioned high surface roughness. Concerning the similar biofilm thickness and oral streptococci content in the 1-day and 3-day old biofilms, the findings of this report are in agreement with our previous results with respect to initial microbial adhesion and biofilm formation on different implant materials in situ (Al-Ahmad et al., 2010, 2013). In the studies mentioned above we were able to show that the physico chemical surface properties of the implant materials affected the quantity of the adherent bacteria, especially in the initial phase of adhesion, 30–120 min after exposure of the materials in the oral cavity. Using the FISH technique, no differences were found for key oral microorganisms such as Streptococcus spp., Fusobacterium nucleatum, Veillonella spp. and Actinomyces naeslundii. Surprisingly, implant surface parameters affected the quantity of in situ oral biofilm formation only within the first 3 days of oral exposure. These effects were then neutralized in mature 5-day old biofilms (Al-Ahmad et al., 2010). However, bacterial composition and diversity of the oral biofilm were similar on all implant materials tested, and were independent of biofilm age. Similar findings for in situ oral biofilm formation on various biomaterials have been also reported by other authors using molecular microbiological analytical methods to depict a more representative and diverse bacterial population within the oral biofilm (Frojd et al., 2011; Grössner-Schreiber et al., 2009). The present study only examined the proportion of oral streptococci within the mature oral biofilm, making it difficult to conclude whether or not all of the tested implant materials had a similar bacterial composition. However, the percentages for Streptococcus spp. demonstrated are in agreement with earlier results with respect to the oral biofilm on control BES and other implant materials.

![Fig. 4. Boxplot demonstrating the covering grade as a percentage of the investigated oral biofilm on the tested implant and control BES surfaces after oral exposure for 1 and 3 days in situ, respectively. BES, bovine enamel surfaces; Ti, titanium implant surfaces; Al-Zr, alumina and niobium co-doped yttria-stabilized tetragonal zirconia ($\text{Al}_2\text{O}_3$($\text{Y}$($\text{Nb}$)-TZP); Zr, 3% yttria-stabilized tetragonal zirconia polycrystal surfaces (3Y-TZP).](http://jmm.microbiologyresearch.org)

![Fig. 5. Boxplot demonstrating the content of Streptococcus spp. (as a percentage) within the oral biofilm formed on the implant material and control BES surfaces in situ after 3 and 5 days of oral exposure. BES, bovine enamel surfaces; Ti, titanium implant surfaces; Al-Zr, alumina and niobium co-doped yttria-stabilized tetragonal zirconia ($\text{Al}_2\text{O}_3$($\text{Y}$($\text{Nb}$)-TZP); Zr, 3% yttria-stabilized tetragonal zirconia polycrystal surfaces (3Y-TZP).](http://jmm.microbiologyresearch.org)
The splint system described in the present study has been successfully applied in several in situ studies aimed at examining oral microbial adhesion and biofilm formation (Al-Ahmad et al., 2010; Karygianni et al., 2012). As discussed in these reports, bovine enamel was indicated to be a useful control surface due to it having similar structural and chemical properties to human enamel (Nakamichi et al., 1983). BES are also easier to obtain and can be prepared in large numbers, in contrast to hard-to-find slabs of human enamel.

DAPI-staining has been successfully used to visualize and quantify initially adherent oral microorganisms in situ (Jung et al., 2010), while FISH is an established fluorescence-based staining method allowing the qualitative and quantitative determination of in situ oral biofilm formation without destroying the natural environment of the adherent oral microorganisms (Zijing et al., 2010). The efficient exploration of the oral biofilm on bovine enamel and various other biomaterial surfaces is only possible with the aid of such non-destructive visualizing techniques (Karygianni et al., 2012; Schaudinn et al., 2009).

Though human saliva is extremely rich in proteins forming the salivary pellicle, it should be kept in mind that dental implants are actually intended to be placed into the bone. Therefore, they will primarily be in contact with gingival fluid, which has a completely different bacterial composition to human saliva, in situ. In future studies, to evaluate the effects of gingival fluid on microbial adhesion, pre-treatment of the implant materials with fetal bovine serum, which mimics gingival fluid (Jorand et al., 2015), should be conducted prior to the main microbiological assays.

Consequently, initial microbial adhesion and biofilm formation on both materials seem to be comparable. The present study concentrated solely on supragingival biofilm formation on implant materials. In addition to supragingival biofilm, implant materials can be colonized by subgingival biofilm, which actually represents the natural late process of oral biofilm formation in situ and is considered to be an aetiological factor for implant failures. The use of healing abutments has been shown to be an effective model to study supra- as well as subgingival biofilms and their microbial diversity on implant materials (Heuer et al., 2011). Such an in situ model has been shown to be valuable and realistic in studying the influence of surface modification of implant materials on supra- and subgingival biofilm formation in situ (Elter et al., 2008). Therefore, subgingival biofilm formation on implant materials used in the present study should also be investigated using healing abutments to amend the present results. In future studies a thorough microbiological analysis involving pathogenic oral bacteria should be conducted to characterize the influence of

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**Fig. 6.** Confocal image stacks of in situ oral biofilms formed on the implant material and control BES surfaces. Images were generated with FISH and CLSM following hybridization with the oligonucleotide probes, EUB 338 and Strep 405, after 3 days of oral exposure. Eubacteria are depicted in green and Streptococcus spp. marked in magenta. BES, bovine enamel surfaces; Ti, titanium implant surfaces; Al-Zr, alumina and niobium co-doped yttria-stabilized tetragonal zirconia (Al₂O₃/Y(Nb)-TZP); Zr, 3% yttria-stabilized tetragonal zirconia polycrystal surfaces (3Y-TZP). Bar, 20 µm.
implant material surfaces on the bacterial composition of in situ oral biofilms.

In conclusion, within the limitations discussed above this study showed that oral implant zirconia surfaces with low surface average roughness ($R_a$) are comparable with titanium surfaces with respect to initial oral bacterial adhesion and biofilm formation. The null hypothesis was, therefore, rejected. In addition to the promising mechanical properties and high biocompatibility of zirconia, the findings of the present report encourage the further clinical application of zirconia implant materials.

REFERENCES


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