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RESEARCH AND EDUCATION

Effect of titanium and zirconia dental implant abutments on a cultivable polymicrobial saliva community

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ABSTRACT

Statement of problem. Peri-implantitis is considered the most important biological complication responsible for late implant failure. The physical chemical properties intrinsic to each material can affect the first step to biofilm development and is an important precursor to the adaptive behavior of pathogenic bacteria species.

Purpose. The purpose of this in vitro study was to evaluate the effect of 2 commercially available implant abutment materials on the adhesion phase and biofilm formation.

Material and methods. Disks (8 mm in diameter, 2 mm thick) of machined pure titanium (Ti) and yttrium-stabilized zirconia (ZrO_2) materials were used to mimic implant abutments. The physical chemical surface properties were investigated using different approaches. Initial adherent bacteria and biofilm formation were evaluated after 16 and 48 hours by incubating the disks in a rich medium containing representative saliva-derived oral microbial community. Unpaired *t* test, 2 tailed, was used to compare the groups.

Results. Ti presented lower hydrophobicity and surface free energy values than the ZrO_2, and 6.1-fold fewer bacteria adhered to the Ti. After 48 hours, detailed quantitative analysis showed that biofilm biomass and biofilm density were lower on the Ti disks than on ZrO_2. The quantity of phylotypes on the Ti and ZrO_2 surfaces was relatively similar during the attachment and early biofilm formation periods.

Conclusions. Although no difference in the bacteria profile was observed between both materials independent of the time point, the highest level of colonization was on ZrO_2. (J Prosthet Dent 2017;118:481-487)

The biologic complications of peri-implantitis may present a problem during dental implant therapy.[1-3] Peri-implantitis is associated with about 17% of installed implants after 10 to 16 years of follow-up.[4] In a recent review, Mombelli et al[5] revealed that the prevalence of peri-implantitis seems to be 20% between 5 and 10 years after implantation. Although peri-implant disease is immunologically stimulated by the host, the inflammatory process is bacteria dependent. A similar pattern of colonization is present in periodontal and peri-implant disease,[6-10] although with some differences in patients with partial and complete edentulism.[11] This disease pattern has direct clinical implications, especially with regard to choosing implant surface materials.

Surface attachment is the first step to biofilm development and is an important precursor to the adaptive behavior of bacteria species as it is the base for the colonization and invasion of pathogens.[12] The hydrophobic attractive forces and electrostatic charge interactions between cells and material surfaces[13-16] are

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Clinical Implications

Zirconia promoted a significant microbial saliva community growth compared with titanium. Because esthetic implications are considered an indicator of success and implant abutment surfaces are in intimate contact with the peri-implant tissue, the findings of this study could offer a starting point for clinical studies with patients who are periodontally compromised.

considered the key properties of dental implant surfaces for biofilm formation. The chemical composition and surface characteristics of the different substrates[17-25] used for abutment components may directly affect microorganism adhesion and oral biofilm maturity.[20-28]

The abutment components seem to be of decisive importance for biofilm formation because of their supragingival and subgingival location. Thus, materials used to manufacture implant abutments should inhibit bacteria colonization on their surface. However, the authors are unaware of any antimicrobial coating of implant abutment surfaces, and therefore, uncoated titanium (Ti) and zirconia (ZrO_2) are the most common commercially available dental implant abutment materials.

While several investigations have demonstrated differences in terms of biofilm formation on Ti and ZrO_2 materials,[28-33] others have reported no differences in biofilm formation between the material surfaces.[34,35] Therefore, the effect on the adhesion phase and biofilm formation using human polymicrobial oral communities of 2 different materials was evaluated. The hypothesis was that different material surfaces interfere with the quality and quantity of adhered bacteria, the biofilm formed, and the bacteria profile.

MATERIAL AND METHODS

Machined pure titanium (grade 2) and yttrium-stabilized zirconia disks (8 mm in diameter, 2 mm thick) (Conexão Sistemas de Próteses Ltda) were used in this study. The surface roughness of all disks was determined with a portable roughness analyzer (Surftest SJ-401; Mitutoyo Corp).[19,20] For each material, each side was measured twice, the mean values were calculated, and the morphology of the disks was examined with a scanning electron microscope (SEM) (JSM-JEOL 7500F; JEOL Ltd). The specimens were directly mounted on aluminum stubs, and the SEM images were obtained in high resolution with the microscope working between 2 and 15 kV. Three disks of each material and 5 areas were analyzed. The surface free energy (SFE) was determined by the contact angle formed between different wet agents and materials to analyze the physicochemical characteristics of Ti and ZrO_2 surfaces. The contact angles for liquid drops (aqueous phase with varying pH, water, ethylene glycol, polyethylene glycol, and diiodomethane) were measured by using the sessile drop technique, as described previously.[19,20] Five disks of each material were used, and the procedure was repeated 3 times to assess the reproducibility of the experiment. The average of each surface and each wetting agent was entered into specialized drop-shaped analysis software (SCA-Software/OCA-20; DataPhysics Instruments GmbH), and the SFE was calculated from the concept of polar and dispersion components as described by Owens and Wendt.[36] Before measurement, each disk was cleaned with acetone to remove any organic material, followed by a 15-minute rinse with ultrapure water in ultrasonic baths. In sequence, the disks were sterilized with gamma irradiation at a dose of 25 kGy from an artificial cobalt 60 source (ISO-11137-1:2006, Sterilization of health care products—radiation—part 1: requirements for development, validation and routine control of a sterilization process for medical devices; this standard was last reviewed and confirmed in 2016).

To simulate clinical conditions and increase the relevance of this in vitro study, a cultivable microbial saliva community representative of the complex oral microbiome was used as an in vitro model biofilm.[37,38] The bacterial community was grown overnight anaerobically (85% N_2, 10% H_2, and 5% CO_2)[38,39] at 37°C in a modified rich medium (SHI-FSMS) developed to support the high number of oral bacteria from human saliva samples (50% SHI medium, 25% filtered saliva [filter pore size 0.22 μm], 0.5% mannose, 0.5% sucrose).[39] Initially the optical density at 600 nm was adjusted to 0.1 in fresh medium to decrease bacterial concentration, and then 800 μL of the oral microbial community was placed onto disks in a sterilized 24-well polystyrene culture plate (Corning Costar cell culture plates; Fisher Scientific). As positive control, the oral microbial suspension was cultured directly on the polystyrene surface of the plate. Additionally, sterile medium was incubated with and without Ti and ZrO_2 disks to act as a negative control. The adhesion phase and biofilm formation were evaluated after 16 and 48 hours' incubation, respectively. After each time point, the disks were transferred to a new 12-well polystyrene culture plate and rinsed 3 times with 1 mL sterilized phosphate-buffered saline (PBS) before further processing.

The surface property described above was examined as to whether it had any influence on the ability of a complex oral community to adhere and develop biofilms on 2 abutment surfaces. The quantity and quality of bacteria adhered to Ti and ZrO_2 materials were evaluated after 16 and 48 hours' incubation using different approaches. The disks were placed in a 24-well plate with 1 mL of SHI medium, and the surface was scraped with a

pipette tip to detach the biofilm. One milliliter of culture was transferred to a 1.5 sterilized Eppendorf tube (tube 1). Subsequently, tube 1 was vortexed, and 100 μL from tube 1 was transferred to a new tube containing 900 μL of SHI medium (tube 2). The serial dilution procedure was repeated until tube 8. Twenty-five microliters of each tube was spread on fresh SHI medium agar and the plates kept at 37°C under anaerobic conditions. The colonies were counted after 3 days. The overall methodology applied in this experiment followed the detailed sequence as previously described.[39]

To determine the biomass accumulation on Ti and ZrO_2 disk surfaces, the PBS-washed disks were transferred to a new 24-well plate and incubated with 800 μL of 0.5% crystal violet solution for 20 minutes. A similar procedure was conducted for control wells (no disks). After that, the disks were rinsed with PBS to remove excess crystal violet and incubated with 800 μL of 95% ethanol for 15 minutes. The ethanol solution was transferred into cuvettes (polystyrene spectrophotometer cuvette; USA Scientific), and the optical density of the specimens was determined for total biomass assessment at 595 nm. Each data point represented 3 well experiments, and 3 independently repeated tests were performed to ensure reproducibility. To evaluate the viability and density of the complex oral bacteria on both surfaces, the disks were stained with a bacterial viability kit (LIVE/DEAD BacLight; Invitrogen) according to the manufacturer's instructions. The bacteria adhered, and the biofilm formed was observed through ×10 dry and ×40 oil immersion objectives from a confocal laser scanning microscopy module (LSM 510 v4.2; Carl Zeiss MicroImaging Co Ltd) using an excitation wavelength of 488 nm and a fluorescence emission range above 505 nm.

Next, confocal microscopy was used to optically slice comparably thick specimens. The biofilm images were obtained from 3 arbitrary positions and vertical and horizontal optical sections with a set slice thickness at 1 μm generated *Z* series. Confocal images from each disk were exported to a freeware program (ImageJ 1.48 for Macintosh v10.2; http://imagej.nih.gov/ij/download.html) and converted into red, green, and blue. The area occupied and the density of biomass by live bacteria within each section was calculated by using the integrated density of pixels. The analyses were performed in duplicate with 2 repetitions.

Last, to evaluate the effect of both materials on the bacteria profile, each surface was scraped with a sterile pipette tip in a well containing 150 μL of PBS, and the total genomic DNA solution was transferred to a 1.5 mL Eppendorf tube for DNA extraction (MasterPure DNA Purification Kit; Epicentre). The DNA amplification was performed as described by de Avila et al.[39] Denaturing gradient gel electrophoresis (DGGE) of the polymerase chain reaction (PCR) products was performed using the DCode System (Bio-Rad Laboratories Inc) at 60 V and 58°C for 17 hours, according to the detailed experimental steps previously published.[39] A 45 μL aliquot of the PCR product was loaded into each well, and electrophoresis through 8% polyacrylamide gels was completed to separate, identify, and purify nucleic acids.

In sequence, gel images were acquired with the Molecular Imager Gel Documentation system (Bio-Rad Laboratories Inc). Two samples of each material were used for this experiment, and the method was repeated 3 times for each time point. An overnight oral saliva community culture (S) was used as a positive control. The intense DGGE bands were excised, and 5 μL of the DNA sample was used as a template for reamplification. Briefly, reamplification was performed with the same universal primers used for PCR amplification, and the product was sent for sequencing using the universal primer.[37] The 16S DNA sequences were compared with the GenBank sequences by using the BLAST program in the Human Oral Microbiome Database (HOMD) to identify the phenotypic from the oral bacteria.

The results were evaluated as means ±SD, except for qualitative analysis using confocal microscopy. All statistical analyses were performed by statistical software (Prism v5.0c; GraphPad Software Inc). The unpaired 2-tailed *t* test was used to compare the Ti and ZrO_2 disks (α=.05).

RESULTS

For Ti disks used in this study, the mean roughness average (Ra) was 0.21 ±0.06 μm and for ZrO_2 was 0.22 ±0.03 μm. To complement the roughness analysis, the morphology of Ti and ZrO_2 materials was examined at high (×1000) magnification. SEM revealed a rough homogeneous surface for Ti and a smooth surface for ZrO_2. Ti surfaces showed a circular configuration of the irregular plane, smooth, and rough surface areas (Fig. 1A), whereas for ZrO_2, a spherically shaped characteristic was detected in granules (Fig. 1B).

According to Table 1, although both materials were hydrophobic with contact angles higher than 50 degrees, a significant difference was noted between them, with ZrO_2 presenting less wetting with water. Conversely, no significant difference was noted between the contact angle of diiodomethane and materials, revealing a similar nonpolar characteristic of Ti and ZrO_2. The variability of the data collected for the contact angle of each liquid to each material was evaluated by descriptive analysis. Low SD values were assumed, and the final averages were inserted into the software (SCA-Software/OCA-20) to obtain the SFE values. ZrO_2 specimens presented higher surface tension (6.1 mJ/m^2) than Ti (5.75 mJ/m^2).

Colony-forming unit per milliliter disclosed a statistically significant difference (*P*=.019) in the overall bacteria adhered, with 6.1-fold more colonies evident on the

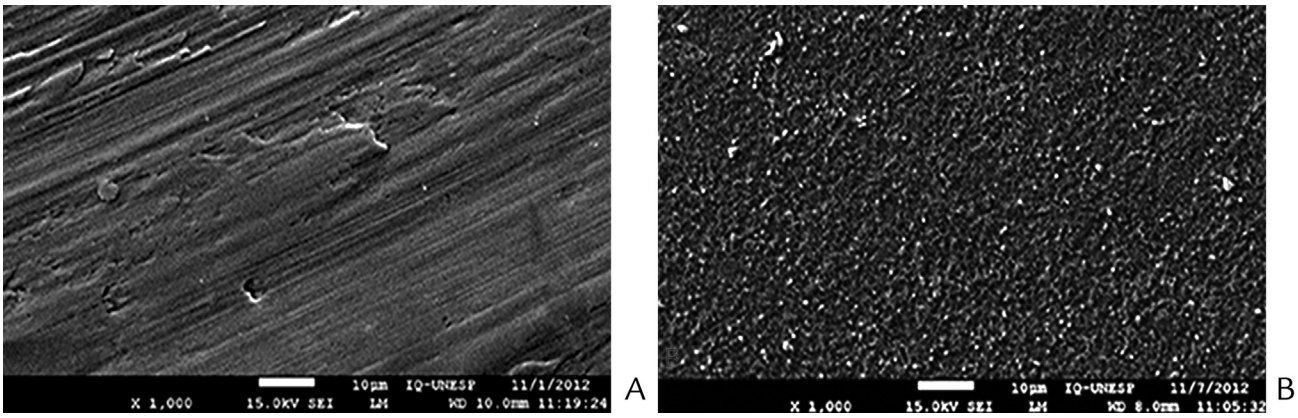

Figure 1. Scanning electron microscope images showing surface topography. A, Ti disk. B, ZrO_2 disk. (Original magnification, ×1000.)

Table 1. Means ±SD of contact angle (θ) of liquid for each specimen

	Specimen	
Wetting Agent	**Ti**	**ZrO_2**
Water	67.73 ±3.32	73.14 ±1.82
Ethylene	43.83 ±1.27	39.45 ±3.80
Polyethylene	25.90 ±3.46	23.39 ±1.06
Diiodomethane	24.09 ±1.82	24.77 ±1.56

ZrO_2 than Ti surfaces. Because the attached phase showed fewer salivary bacteria adhered to Ti disks, this outcome was evaluated to see whether it would persist during the biofilm formation and growth process. Colony-forming unit per milliliter also disclosed a statistically significant difference (P=.046) in overall biofilm formed after 48 hours with 1.6-fold more bacteria adhered on ZrO_2 than on Ti surfaces (Fig. 2).

The confocal microscopy images of the surface attached biomass confirmed that Ti disks accumulated significantly fewer bacteria compared with their counterparts (Fig. 3A, B). Further, the biofilm viability of both materials was similar, as revealed by fluorescent stain. The difference in biofilm formation between Ti and ZrO_2 disks was also confirmed by confocal microscopy analysis (Fig. 3C, D). The observation under confocal laser microscopy showed that the biofilm thickness on ZrO_2 was deeper than that of the Ti group (Fig. 3E, F). Quantitative measurements from images acquired by confocal analysis revealed that the density of adherent bacteria was 1.6-fold higher on the ZrO_2 than on the Ti disks (Fig. 4) after 16 hours and that this difference remained after 48 hours.

Consistent with confocal analysis, crystal violet staining showed substantially higher biomass at both time points, with 2.7-fold more bacteria adhered on ZrO_2 than on Ti disks and 2.2-fold more biofilm developed on ZrO_2 than on Ti disks (Fig. 5). The next goal was to observe whether the different abutment material surfaces affected the biofilm bacterial community. DGGE analyses demonstrated that the quantity of phylotypes on the Ti and ZrO_2 surfaces was relatively similar during the

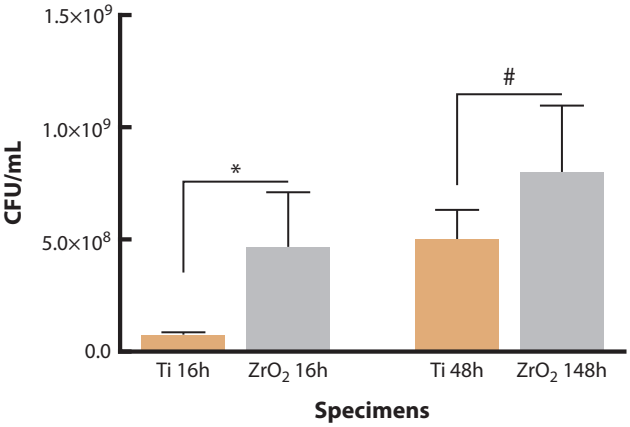

Figure 2. Effect of Ti and ZrO_2 material surfaces on bacterial attachment and biofilm formation evaluated after 16 and 48 hours' incubation using quantitative culture counts (in CFU); CFU/mL was significantly reduced on Ti disks. Data shown as means ±SD (n=9). $^{*,\#}P$<.05. CFU, colony-forming units.

attachment and early biofilm formation periods (Fig. 6). However, some differences on weakly and strongly adherent biofilm bacteria were observed for both time points. The intensity of bands 2 and 3 were highest during the adhesion phase (16 hours of incubation). The similarity of bacteria composition persisted after 48 hours, but the time affected the dominance of some bacteria species. Bands 1, 5, and 6, identified as *Fusobacterium periodonticum, Neisseria subflava,* and *Alloprevotella* sp, respectively, became more intense on Ti and ZrO_2 after 48 hours, indicating that biofilm formation on those materials is time dependent.

DISCUSSION

Studies have shown that individuals treated for periodontitis may experience more implant failure, so a better understanding of the interface between prosthetic superstructure and implants is necessary. The authors are unaware of previous studies analyzing a cultivable

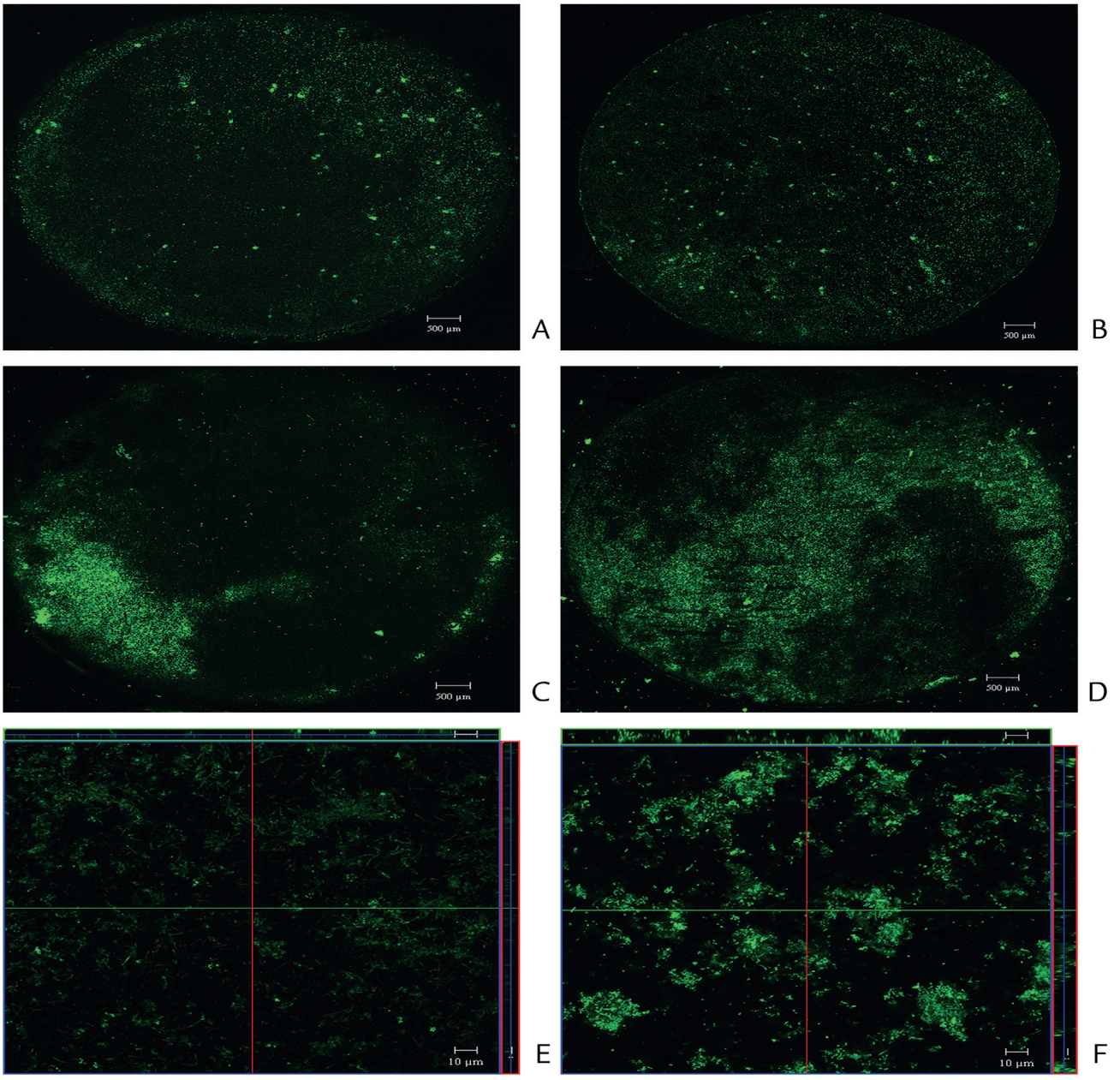

Figure 3. Effect of Ti and ZrO_2 material surfaces on bacterial attachment and biofilm formation evaluated after 16 and 48 hours' incubation using confocal microscopy. (Original magnification, ×10.) A, Ti disks after 16 hours' incubation. B, ZrO_2 disks after 16 hours' incubation. C, Ti disks surfaces after 48 hours' incubation. D, ZrO_2 disks after 48 hours' incubation. E, Images in *Z* stacks revealed biofilm thickness formed on Ti disk surfaces. F, Images in *Z* stacks revealed biofilm thickness formed ZrO_2 disk surfaces.

polymicrobial community representative of the oral cavity[38] on 2 commercial types of implant abutments. The data led to acceptance of one of the hypotheses: that more biofilm accumulated on ZrO_2 than on Ti materials. However, the other hypothesis was rejected because the type of material did not affect the bacterial profile.

The surface properties of materials have a marked influence on the early phases of biofilm development in that smooth surfaces and those with the low SFE feature may have less bacterial adherence than rough substrates.[21,23] For the purpose of this study, a standard roughness was chosen for the experimental Ti and ZrO_2 abutments that approached the optimal roughness as previously described[10,18] for permucosal implant abutments. The goal in this study was to eliminate the roughness variable, keep the homogeneity of groups, and focus on the effect of abutment materials on bacterial attachment and biofilm formation.

Differences in bacterial colonization and biofilm formation probably resulted from differences in the chemical

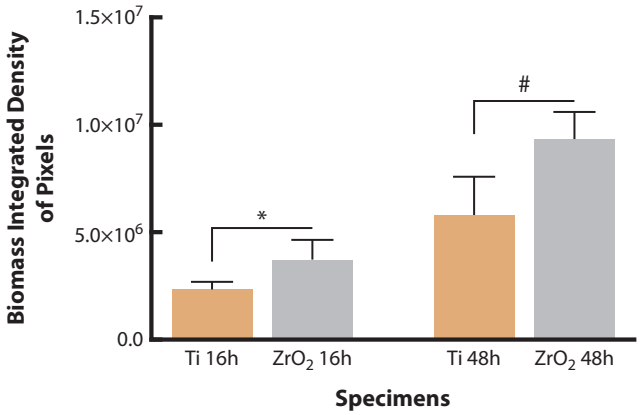

Figure 4. Integrated density significantly reduced on Ti disks (brown bar, Ti). Data shown as means ±SD. $^{*,\#}P<.05$ indicates statistically significant difference between Ti and ZrO_2 disks.

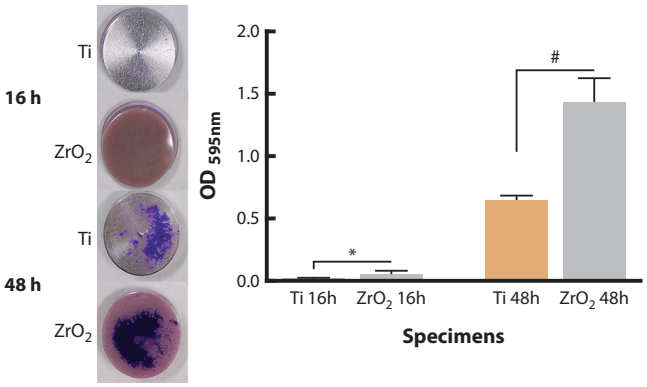

Figure 5. Effect of Ti and ZrO_2 material surfaces on biomass of bacterial attachment and biofilm formed evaluated after 16 and 48 hours' incubation using crystal violet staining. Statistically significantly higher biomass of bacterial attachment on ZrO_2 (gray bar, ZrO_2) than Ti disks (brown bar, Ti). Data are shown as means ±SD (n=9). $^{*,\#}P<.05$ indicates statistically significant difference between Ti and ZrO_2 disks.

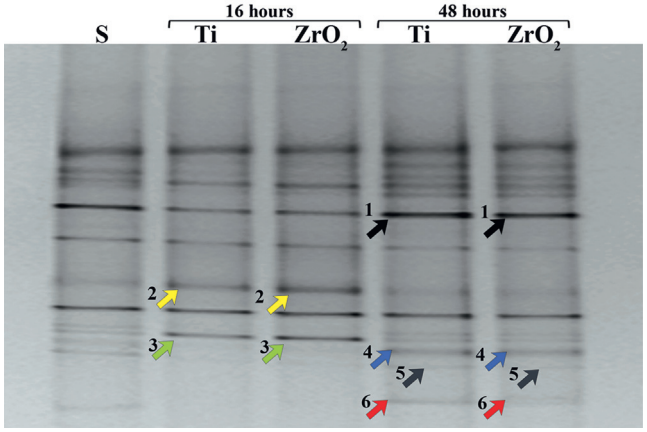

Figure 6. Denaturing gradient gel electrophoresis (DGGE) analysis of oral microbial communities formed on Ti and ZrO_2 disk surfaces after 16 and 48 hours' incubation. Bands indicated by arrows were excised and DNA sequenced. Microbial identities: (1) *Fusobacterium periodonticum,* (2) *Streptococcus* sp, (3) *Gemella sanguinis,* (4) *Veillonella parvula,* (5) *Neisseria subflava,* and (6) *Alloprevotella* sp.

composition and consequently differences in SFE (electrical conductivity). On the basis of the analysis of the total number of bacteria adhered in the initial culture stage and in a mature biofilm to different surfaces, more bacterial accumulation was observed on the ZrO_2 surfaces, the most hydrophobic material with the highest value of surface tension. The positive correlation between SFE and bacterial adhesion depended on the similar chemical nature of both surfaces and the prokaryotic cells.[13,16]

Fewer bacteria (6.1-fold) adhered to Ti than to ZrO_2. This reduction was also reflected by reduced biomass and biofilm density. The reduction in biofilm formation was sustained most notably after 48 hours. These data are in contrast with most studies that reported no difference between biofilm formations on these materials, or fewer bacteria adhered to ZrO_2 than the Ti substrate.[33,35] However, the chemical and physical properties of surfaces (surface wettability of materials) combined with one or a few species of bacteria do not reflect the actual effect of the material in the oral cavity.[25,35] A previous investigation[22] also found no difference in biofilm formation between Ti and ZrO_2 substrates for implant abutment fabrication. However, the authors kept the specimens immersed in saliva for 2 hours before the biofilm development, which may have affected the SFE, making it similar between the materials.[22] Consequently, the interactions between the hydrophobic and hydrophilic regions of the outer cell wall[24] and the material surface may also have been similar.

An intriguing observation in this study was the number of microorganisms determined in multispecies biofilm exposing the specimens to a suspension of *Streptococcus gordonii, Streptococcus mutans, Actinomyces naeslundii,* and *Candida albicans.*[22] Because bacteria express a wide variety of complex molecules that can contribute to the overall tendencies of microorganisms interacting with other cells, the selected microorganism species can create a final energy and change the interaction to their environment.[15] Because interaction among microorganisms is fundamental for initial colonization and subsequent biofilm formation on dental surfaces, the limitation of the in vitro biofilm models described in the literature so far may explain the inconsistent results.

Overall, comparable microorganisms are found around newly placed implants and the remaining dentition. This can also include periodontopathogenic species, which might even be considered a risk for future peri-implant infections. Because Ti accumulated fewer bacteria than ZrO_2 onto the surface, whether material features would also affect the profile of bacteria colonization was investigated. No differences were observed in bacterial composition in the attachment phase. The effect

of Ti and ZrO_2 beyond the early stages continued over time. After 48 hours of incubation, the bacterial profile, measured by the intensity of the bands, showed no differences between materials, but the intensity was different compared with the first time point. Ti and ZrO_2 are considered highly hydrophobic materials because of their chemical composition; the low polarity of their surfaces will attract molecules with the same chemical composition. These concepts may explain the similarity of the bacterial profile at both time points.

CONCLUSIONS

On the basis of the results of this in vitro study, the following conclusion was drawn:

The initial attachment phase and biofilm formation are affected by substrate type, with ZrO_2 accumulating significantly more bacteria and biofilm on the material surfaces than Ti.

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