Impact of Physical Chemical Characteristics of Abutment Implant Surfaces on Bacteria Adhesion

Erica Dorigatti de Avila, DDS PhD[1,2]*
Rafael Scaf de Molon, DDS, MSc[3,4]
Bruno P. Lima, DDS, PhD[2]
Renate Lux, PhD[2,5]
Wenyuan Shi, PhD[2]
Miguel Jafelicci Junior, MSc, PhD[6]
Denise Madalena Palomari Spolidorio, MSc, PhD[7]
Carlos Eduardo Vergani, DDS, MSc, PhD[1]
Francisco de Assis Mollo Junior, DDS, MSc, PhD[1]

Surface attachment is the first step in biofilm formation, and the ability of bacteria to adhere to surfaces and develop a biofilm is directly influenced by electrostatic interactions between the bacteria and the chemical composition of material surfaces. Here, we investigated the influence of physical and chemical characteristics of titanium (Ti) and zirconia (ZrO_2) as implant abutment surfaces on the bacterial adhesion phase and compared the results to bovine enamel (BE) simulating a human tooth. To achieve this goal, we used 2 common pathogens of the oral cavity, *Streptococcus mutans UA140* and *Porphyromonas gingivalis 33277*. To investigate the influence of material surfaces on bacterial adhesion, we studied the surface free energy as well as the topography by atomic force microscopy, and the chemical elements composition by scanning electron microscopy equipped with an energy dispersive X-ray spectroscope. Our results indicated a hydrophobic characteristic for all of the materials; however, the presence of polar and nonpolar components could aid in understanding why greater numbers of bacteria had adhered to BE compared to the other surfaces. Our confocal microscopy data support the proposition that electrostatic interactions, indeed, affected the initial adhesion phase. Within the limitations of a laboratory study, the results revealed bacterial adhered on BE and no bacteria could be observed by confocal images on Ti and ZrO_2 implant abutment surfaces.

Key Words: abutment implants, titanium, zirconia, bacteria adhesion

INTRODUCTION

The long-term success of dental implants is thought to be strongly dependent on the presence of healthy peri-implant tissue.[1] Although implant failure is a multifactorial process,[2] peri-implantitis has been proposed to be one of the most critical factors implicated in the loss of implants.[3,4] In this context, the physical and chemical characteristics of implant abutment surfaces have been shown to directly affect bacterial adhesion.[5]

To date, titanium has been considered the gold standard due to its excellent properties, providing it with a dominant position among the available abutment and implant materials.[6] Esthetic features favored the introduction of ceramic implant abutment materials.[7] Similarly to titanium, zirconia is also biocompatible, highly resistant to corrosion, and provides great strength and toughness.[7,8] Although, the effect of implant and abutment surfaces on bacterial adhesion and biofilm formation has been reported in both in vivo and in vitro studies,[9–11] there is no unified consensus regarding how different materials affect bacterial colonization.[5,12,13] Titanium and zirconia are hydrophobic materials. Hydrophobic attractive forces and electrostatic charge interactions between surfaces and bacteria have been proposed to play a key role in biofilm formation.[14,15] Most bacteria have many ionizable groups on their surfaces,[16–18] which confer a net negative charge, particularly during the early stationary phase of cell growth.[19] However, the charge that is present on the cell surface of some types of bacteria can create a hydrophobic effect via nonpolar molecules and result in an affinity for other hydrophobic surfaces.[20] This characteristic explains why some bacterial species preferentially interact with certain materials and why the findings reported in the literature are inconsistent depending on the type of bacterial species assessed. Given the complex relationship between

[1] Department of Dental Materials and Prosthodontics, School of Dentistry at Araraquara, Univ Estadual Paulista - Araraquara, Brazil.
[2] Division of Oral Biology and Medicine, University of California School of Dentistry, Los Angeles, Calif.
[3] Department of Diagnostic and Surgery, School of Dentistry at Araraquara, São Paulo State University, Araraquara, Brazil.
[4] Division of Diagnostic Surgical Science, University of California School of Dentistry, Los Angeles, Calif.
[5] Division of Constitutive and Regenerative Sciences, University of California School of Dentistry, Los Angeles, Calif.
[6] Department of Physical Chemical, Chemical Institute, São Paulo State University, Araraquara, Brazil.
[7] Department of Physiology and Pathology, School of Dentistry at Araraquara, São Paulo State University, Araraquara, Brazil.
* *Corresponding author,* e-mail: erica.fobusp@yahoo.com.br
DOI: 10.1563/aaid-joi-D-14-00318

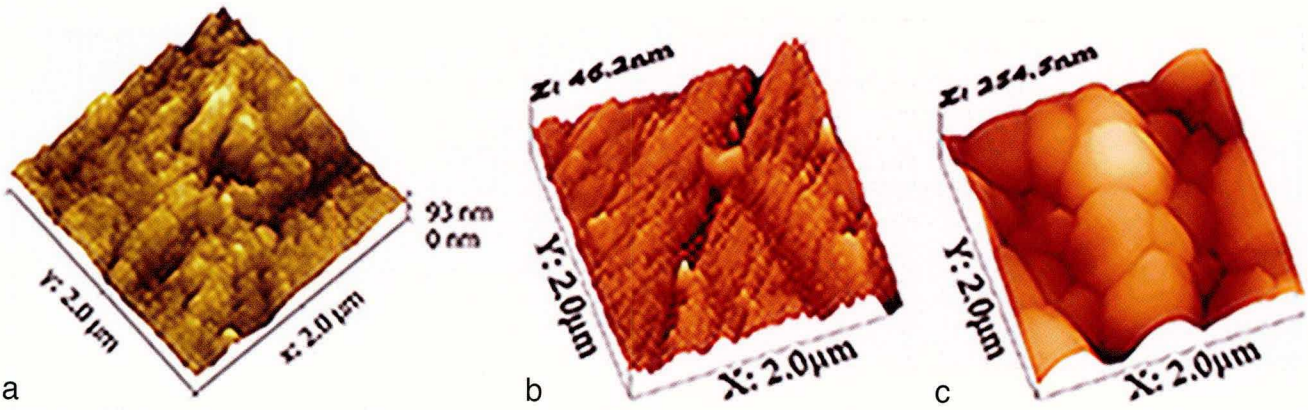


Figure 1. Micrographs taken by an atomic force microscopy to describe the surfaces: (a) bovine enamel, (b) Ti, (c) ZrO_2.

bacteria and material surfaces and the clinical relevance of this topic, this study was conducted to investigate the influence of the physical and chemical properties of 2 different implant abutment materials, titanium and zirconia, on bacterial adhesion. In addition, bovine enamel (BE) was used as a control, simulating the human cervical tooth surface.

Materials and Methods

Disc preparation and surface analysis

Standardized disc-shaped specimens with a diameter and thickness of 8 mm and 2 mm were obtained from machined pure titanium and yttrium-stabilized zirconia (Conexão Sistemas de Próteses Ltda, São Paulo, SP, Brazil). Enamel discs of the same dimensions were prepared from bovine incisors and used as a control. The enamel was cut with a diamond drill for glass thread (Dinser Diamond Tools Ltda, São Paulo, Brazil) coupled to the drill bench vise (Schulz - FSB model 16 - chuck taper DIN 238-B18, São Paulo, SP, Brazil) and then subjected to surface polishing utilizing a procedure with waterproof paper grains of 800, 2500, and 4000 (T469-SF-Noton, Saint-Gobam Abrasives Ltd, Jundiaí, São Paulo, SP, Brazil).

The surface roughness of all of the discs was quantitatively analyzed using a portable roughness analyzer (Mitutoyosurftest SJ-401, Mitutoyo Corporation, Kawasaki, Japan). To standardize the surface roughness measurements, we developed a device with 2 parallel slots to define the central area of the discs and a circular space to stabilize the samples during the readings. For each disc, 2 measurements were performed on each side, and to verify the reliability of the results, the analyses were repeated twice. The reproducibility of the readings was assessed by calculating the intraclass correlation coefficient (ICC) with a confidence interval of 95%.

To analyze the surface topography, we used 3 samples of each material and 3 randomized sites per sample (TopScan 3D), measuring an area of 2×2 µm and 10×10 µm (Figure 1). A quantitative characterization of the nanoscale surface topography and roughness was conducted using atomic force microscopy (Agilent AFM system, Model 5500, Agilent Technologies, Chandler, Ariz). Contact-mode topographic images were obtained with an applied force constant of 42 N/m, resonance frequency of 320 kHz and silicon tips with a nominal apical radius of less than 8 nm. Next, we examined the chemical element composition of 3 discs from each material by scanning electron microscopy (FEG-MEV; JEOL 7500F model) equipped with an energy dispersive X-ray spectroscope (EDX) (JSM-5900LV, Joel Ltd, Tokyo, Japan).

To evaluate the wetting and surface tension, we used 5 discs of each material, and the measurement was repeated 3 times for each sample. The contact angle value determined for each wet agent was calculated using the Young–Laplace equation and then inserted into specialized drop-shape analysis software to perform the measurements (SCA-SOFTWARE / OCA-20, DataPhysics Instruments GmbH, Filderstadt, Baden-Württemberg, Germany). The SFE was obtained using the concept of polar and dispersion components according to the method described by Owen.[21]

Bacterial conditions and qualitative assessment of bacterial adhesion

Porphyromonas gingivalis 33277 was cultured in blood agar supplemented with 0.1% hemin and menadione, for 48 hours at 37°C, under anaerobic condition (85% N_2, 10% H_2, 5% CO_2). *P. gingivalis* colonies were transferred to a new tube containing fresh BHI broth medium supplemented by hemin and menadione and maintained for 24 hours (a period previously defined by the growth curve analysis) inside anaerobic chamber, and then the cultures were adjusted to OD_{600nm}1.4 before the experiment. *Streptococcus mutans* UA140 was cultured in TH medium plates prepared by adding 1.5% (wt/vol) agar to the medium and incubated at 37°C, under anaerobic condition. One colony of *S. mutans* was diluted in TH broth medium and overnight cultures were diluted to an $OD_{600nm} = 0.02$ in fresh TH medium containing 0.5% (wt/vol) sucrose. Eight-hundred microliters of each culture was transferred to a 24-well plate containing the discs of each material. The discs were completely immersed in the prepared bacterial suspensions and incubated at 37°C under anaerobic conditions. *S. mutans* were analyzed after 16 hours incubation, while the discs that were immersed in the *P. gingivalis* culture were analyzed after 48 hours.

In order to evaluate the density of the bacteria adhered on Ti and ZrO_2 disc surfaces, after 16 and 48 hours incubation, the

TABLE

Average value, surface roughness (Ra), peak to valley height (Maximum), median and average peak spacing (Rms) measured for the different surfaces: Ti, ZrO_2, bovine enamel (BE) in nanometer (nm)

Statistical Quantities	Ti	ZrO_2	BE
Average Value	60	294	206
Maximum	121	594	378
Median	60	297	203
Ra	10	72	18
Rms	13	88	25

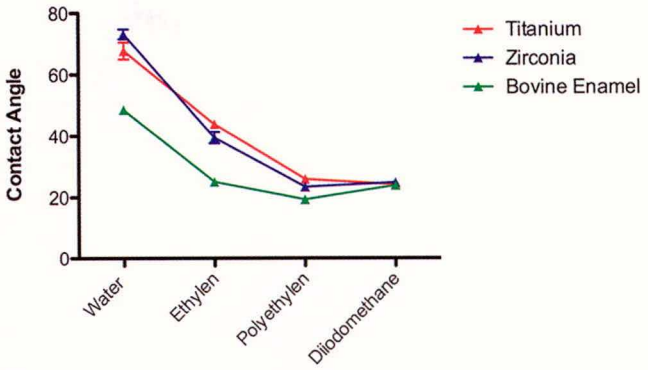


FIGURE 2. Effect of material surface on the wettability. In each material, 4 liquids were used: water, ethylene glycol, polyethylene glycol, and diiodomethane. Red indicates Ti; blue, ZrO_2; green, BE.

discs were transferred to a new 12-well plate and washed 3 times with 1 mL of sterile phosphate-buffered saline (PBS) to remove unattached bacteria. The bacteria were then labeled with 0.01 mM of Syto-9 and 0.06 mM of propidium iodide (PI), LIVE/DEAD stain BacLight Bacterial Viability Kit (Invitrogen Corporation, Grand Island, NY), for 15 minutes, according to the manufacturer's instructions. For visualization, the discs were placed on a glass cover slip, and the adhesion of the bacteria was visualized with a 40x oil-immersion (Plan NeoFluar NA 1.3 oil) objective using an LSM 510 confocal laser-scanning microscope (Version 4.2, Carl Zeiss MicroImaging Co, Ltd, Jena, Germany).

RESULTS

Physical and chemical characteristics

The roughness average (Ra) was used as a physical parameter, and the values for the Ti and ZrO_2 discs, respectively, were as follows: 0.21 μm (±0.06) and 0.22 μm (±0.03). For the BE discs, only discs with a roughness range between 0.05 and 0.1 μm were included in the study. Table 1 shows the average surface roughness (Ra) and maximum surface roughness (Rmax) as determined using AFM and an optical profilometer.

The presence of Ti, as unique element contained in Ti discs, was confirmed by elemental analysis using an energy dispersive spectroscopy. For ZrO_2, we detected zirconium (Zr), oxygen (O), and a small amount of carbon (C) in its chemical composition. And, to the BE, hydroxyapatite constituents were found: calcium (Ca), phosphor (P), and O were found. To analyze the physical and chemical characteristics of Ti, ZrO_2 and BE, we dropped 4 liquids of different polarities on each material and measured the contact angle between them. Among the surfaces studied, BE presented hydrophilic and lipophilic characteristics, in contrast, Ti and ZrO_2 presented the highest contact angle values with water and the lowest with hydrophobic wet agents (Figure 2). The SFE was defined by the intersection between a straight line and the *y*-axis (Figure 3a, b, c). ZrO_2 materials presented the highest SFE value, and BE showed the lowest.

Effect of surface hydrophobicity on bacterial adhesion

To identify the bacterial adhesion structure and viability of the cells that had adhered onto the different surfaces, we performed high-resolution analyses using CLSM. A wetting behavior effect of the 3 different materials on bacterial adhesion was confirmed by confocal microscopy analysis. Higher magnification confocal imaging (×40) revealed that bacterial colonization on BE was clearly characterized by larger, taller, and more widespread microcolonies in comparison to the Ti and ZrO_2 surfaces for both *S. mutans* (Figure 4) and *P. gingivalis* (Figure 5). In the case of *S. mutans*, a small number of bacterial cells was observed on the ZrO_2 discs, but no bacteria were detected on the Ti discs.

DISCUSSION

The physical and chemical characteristics of material surfaces have been shown to have a direct impact on the adhesion phase in bacteria.[22] Numerous studies have reported a relationship between the types of substrate and the bacterial species,[12,13,23,24] and some of them have shown that the initial phase is highly influenced by a surface roughness above a threshold of 0.2 μm.[25] Hence, to minimize the effect of roughness in our study, we only used discs with mean roughness values equal to or less than 0.22 μm. Furthermore, we introduced BE simulating the cervical of a human tooth, as a control to investigate the effect of the chemical composition on bacterial adhesion and to improve our understanding of the opposite effect on Ti and ZrO_2 materials. For control group, we selected only the discs with a superficial roughness ranging between 0.05 and 0.1 um, close to a human tooth.[26] Both *S. mutans* and *P. gingivalis*[27] adhered better on BE than on Ti or ZrO_2 surfaces. This preference in adhesion was confirmed by confocal images and could be explained by the presence of intermolecular interactions between chemical groups on the cell walls of bacteria and polar and nonpolar regions of BE surfaces. Different intermolecular forces can dictate how bacteria interact with different surfaces.[28] Depending on the characteristics of these forces, the interaction can be stronger or weaker. The cell wall of *S. mutans* consists of a thick layer of peptidoglycan that is covered mainly by neutral and acidic polysaccharides, several proteins and teichoic acid.[29] Surface proteins on *S. mutans* confer the charge to these Gram-positive bacteria. The

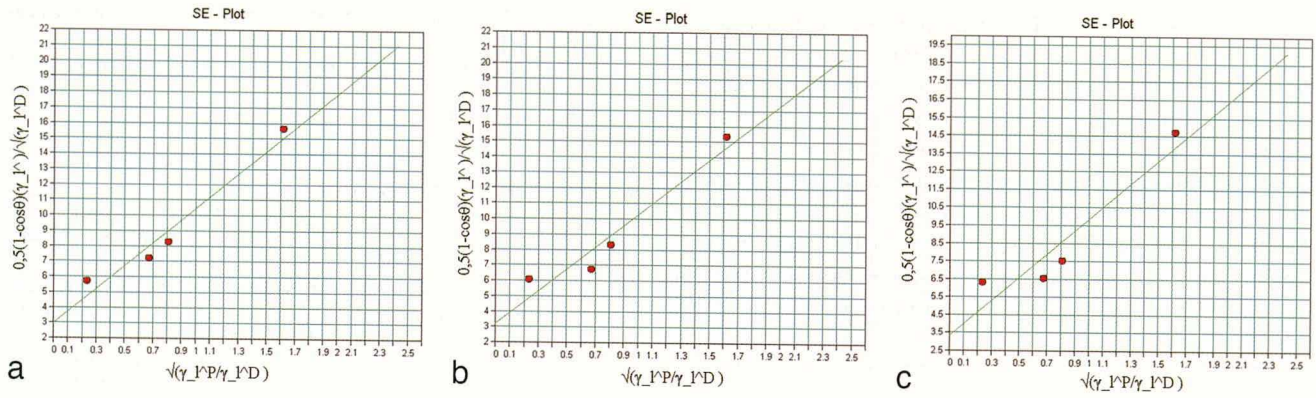
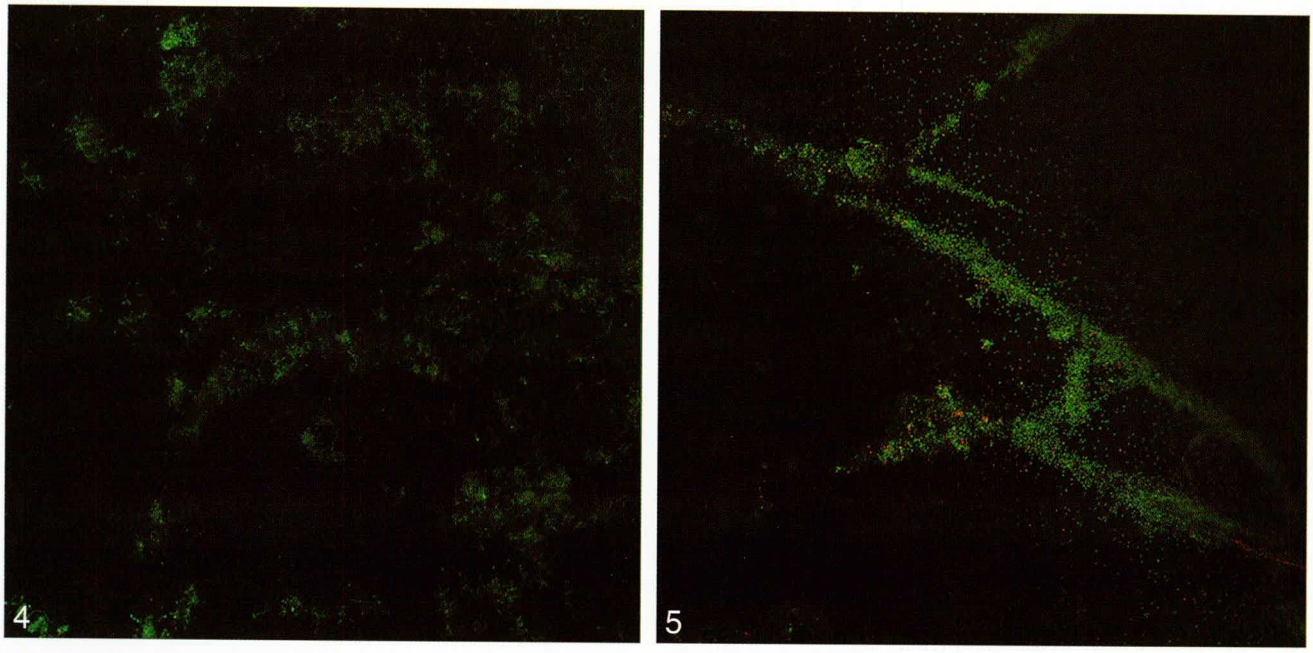


Figure 3. Energy-free surface (mN/m) analysis in surfaces: (a) BE; (b) Ti, (c) ZrO_2.

wetting behavior of BE with liquids of different polarities revealed that this type of material has polar and nonpolar characteristics, and consequently, it provides improved electrostatic interactions between the material and the bacteria.[30] The hydrophobic regions of *S. mutans* can also interact with Ti and ZrO_2; however, the forces exhibited by the nonpolar molecules are weak. This weak interaction could have allowed the easy detachment of the bacteria from the Ti and ZrO_2 discs during the rinsing step.

In the case of Gram-negative bacteria, these species possess a thinner peptidoglycan layer and an outer membrane that consists of proteins, phospholipids and lipopolysaccharides (LPS).[31] Although the presence of LPS confers a negative net charge, which has been found to contribute hydrophilic characteristics,[32] the degree to which LPS plays a role in these hydrophilic features is largely dependent on the structural components, which vary between different species.[33,34] According to our results, the high hydrophobic and lipophilic characteristics of Ti and ZrO_2 surfaces, which were evaluated based on the contact angle, could have influenced the ability of *P. gingivalis* to adhere to these materials. However, the charges present on the BE surfaces could have enhanced the electrostatic interaction with the bacterial cells.

Interactions between bacteria and materials are also influenced by salivary proteins. Although the salivary pellicle has a definitive impact on bacterial adhesion,[35,36] in the current study, a salivary pellicle was not used to more distinctively identify the effects of the chemical characteristics of the



Figures 4 and 5. Figure 4. Biofilm of *S. mutans* UA159 were grown on different surfaces in TH medium, stained with LIVE/DEAD BacLight fluorescent dye and analyzed with CLSM. The figure shows cross-section images of biofilms after 16 hours developed on bovine enamel (BE) surfaces. Dead cells were stained red, and live cells were stained green. **Figure 5.** Biofilms of *P. gingivalis* 33277 were grown on different surfaces in BHI medium, stained with LIVE/DEAD BacLight fluorescent dye and analyzed with CLSM. The figure shows cross-section images of biofilms after 48 hours developed on BE surfaces. Dead cells were stained red, and live cells were stained green.

material surfaces on bacterial adhesion. The composition of the materials and their physicochemical properties may modulate initial bacterial adhesion[37] and, consequently, affect the microbial quality. However, our results cannot be generalized because we examined the impact of the material surfaces using 2 different species of bacteria and must consider the various contributions affecting the final results. These contributing factors include the intermolecular interactions between different species of bacteria, the salivary pellicle and the pH of the oral cavity, which can cause imbalances in the microbial community.

We believe that the results and concepts presented here will improve understanding regarding the inherent characteristics of materials that affect bacterial adhesion and can be helpful in the design of implant abutment material surfaces to prevent plaque accumulation.

Conclusion

Within the limitations of a laboratory study, the present results suggest that bacterial adhesion on Ti was lower than that on ZrO_2, independent of the bacterial species or Gram-positive or Gram-negative status, but it was consistently higher on BE than on abutment materials.

Abbreviations

BE: bovine enamel
EDS: energy-dispersive X-ray spectroscopy
LPS: lipopolysaccharides
SFE: surface free energy

Acknowledgments

Erica Dorigatti de Avila received funds from São Paulo Research Foundation (FAPESP), Grant number: 2011/05106-6, Coordination for the Improvement of Higher Education Personnel (CAPES) and Lemann Foundation. The authors thank Conexao Sistema de Protese for donating the pure titanium and zirconia stabilized with yttrium discs used in this study.

References

1. Quirynen M, De Soete M, van Steenberghe D. Infectious risks for oral implants: a review of the literature. *Clin Oral Implants Res*. 2002;13:1–19.

2. Paquette DW, Brodala N, Williams RC. Risk factors for endosseous dental implant failure. *Dent Clin North Am*. 2006;50:361–374.

3. Safii SH, Palmer RM, Wilson RF. Risk of implant failure and marginal bone loss in subjects with a history of periodontitis: a systematic review and meta-analysis. *Clin Implant Dent Relat Res*. 2010;12:165–174.

4. Oh TJ, Yoon J, Misch CE, Wang HL. The causes of early implant bone loss: myth or science? *J Periodontol*. 2002;73:322–333.

5. Al-Ahmad A, Wiedmann-Al-Ahmad M, Faust J, et al. Biofilm formation and composition on different implant materials in vivo. *J Biomed Mater Res B Appl Biomater*. 2010;95:101–109.

6. Andersson B, Odman P, Lindvall AM, Lithner B. Single-tooth restorations supported by osseointegrated implants: results and experiences from a prospective study after 2 to 3 years. *Int J Oral Maxillofac Implants*. 1995;10:702–711.

7. Piconi C, Maccauro G. Zirconia as a ceramic biomaterial. *Biomaterials*. 1999;20:1–25.

8. Bachle M, Butz F, Hubner U, Bakalinis E, Kohal RJ. Behavior of CAL72 osteoblast-like cells cultured on zirconia ceramics with different surface topographies. *Clin Oral Implants Res*. 2007;18:53–59.

9. do Nascimento C, da Rocha Aguiar C, Pita MS, Pedrazzi V, de Albuquerque RF, Jr, Ribeiro RF. Oral biofilm formation on the titanium and zirconia substrates. *Microsc Res Tech*. 2013;76:126–132.

10. Nascimento C, Pita MS, Fernandes FH, Pedrazzi V, de Albuquerque Junior RF, Ribeiro RF. Bacterial adhesion on the titanium and zirconia abutment surfaces. *Clin Oral Implants Res*. 2014;25:337–343.

11. Burgers R, Gerlach T, Hahnel S, Schwarz F, Handel G, Gosau M. In vivo and in vitro biofilm formation on two different titanium implant surfaces. *Clin Oral Implants Res*. 2010;21:156–164.

12. Rimondini L, Cerroni L, Carrassi A, Torricelli P. Bacterial colonization of zirconia ceramic surfaces: an in vitro and in vivo study. *Int J Oral Maxillofac Implants*. 2002;17:793–798.

13. van Brakel R, Cune MS, van Winkelhoff AJ, de Putter C, Verhoeven JW, van der Reijden W. Early bacterial colonization and soft tissue health around zirconia and titanium abutments: an in vivo study in man. *Clin Oral Implants Res*. 2011;22:571–577.

14. van Loosdrecht MC, Norde W, Zehnder AJ. Physical chemical description of bacterial adhesion. *J Biomater Appl*. 1990;5:91–106.

15. Ener B, Douglas LJ. Correlation between cell-surface hydrophobicity of *Candida albicans* and adhesion to buccal epithelial cells. *FEMS Microbiol Lett*. 1992;78:37–42.

16. Walker SL, Hill JE, Redman JA, Elimelech M. Influence of growth phase on adhesion kinetics of *Escherichia coli* D21g. *Appl Environ Microbiol*. 2005;71:3093–3099.

17. Husmark U, Ronner U. Forces involved in adhesion of *Bacillus cereus* spores to solid surfaces under different environmental conditions. *J Appl Bacteriol*. 1990;69:557–562.

18. Dan N. The effect of charge regulation on cell adhesion to substrates: salt-induced repulsion. *Colloids Surf B Biointerfaces*. 2003;27:41–47.

19. Hayashi H, Seiki H, Tsuneda S, Hirata A, Sasaki H. Influence of growth phase on bacterial cell electrokinetic characteristics examined by soft particle electrophoresis theory. *J Colloid Interface Sci*. 15 2003;264:565–568.

20. Hazen KC, Lay JG, Hazen BW, Fu RC, Murthy S. Partial biochemical characterization of cell surface hydrophobicity and hydrophilicity of *Candida albicans*. *Infect Immun*. 1990;58:3469–3476.

21. Owens DK, Wendt RC. Estimation of the surface free energy of polymers. *J Appl Polym Sci*. 1969;13:1741–1747.

22. Strevett KA, Chen G. Microbial surface thermodynamics and applications. *Res Microbiol*. 2003;154:329–335.

23. de Avila ED, de Molon RS, Spolidorio DMP, Mollo FD. Implications of surface and bulk properties of abutment implants and their degradation in the health of periodontal tissue. *Materials*. 2013;6:5951–5966.

24. de Avila ED, de Molon RS, Vergani CE, Mollo FD, Salih V. The relationship between biofilm and physical-chemical properties of implant abutment materials for successful dental implants. *Materials*. 2014;7:3651–3662.

25. Quirynen M, Bollen CM. The influence of surface roughness and surface-free energy on supra- and subgingival plaque formation in man. A review of the literature. *J Clin Periodontol*. 1995;22:1–14.

26. Anaraki SN, Shahabi S, Chiniforush N, Nokhbatolfoghahaei H, Assadian H, Yousefi B. Evaluation of the effects of conventional versus laser bleaching techniques on enamel microroughness. *Lasers Med Sci*. 2014.

27. de Avila ED, Avila-Campos MJ, Vergani CE, Palomari Spolidório DM, de Assis Mollo Junior F. Structural and quantitative analysis of a mature anaerobic biofilm on differnet implant abutment surfaces. *J Prosthet Dent*. In press.

28. Tuson HH, Weibel DB. Bacteria-surface interactions. *Soft Matter*. 2013;9:4368–4380.

29. Jefferson KK. What drives bacteria to produce a biofilm? *FEMS Microbiol Lett*. 2004;236:163–173.

30. Vanoss CJ, Giese RF. The hydrophilicity and hydrophobicity of clay-minerals. *Clay Clay Miner*. 1995;43:474–477.

31. Beveridge TJ. Structures of gram-negative cell walls and their derived membrane vesicles. *J Bacteriol*. 1999;181:4725–4733.

32. Ferris FG, Beveridge TJ. Binding of a paramagnetic metal cation to

Escherichia coli K-12 outer-membrane vesicles. *FEMS Microbiol Lett.* 1984;24: 43–46.

33. Popovici J, White CP, Hoelle J, Kinkle BK, Lytle DA. Characterization of the cell surface properties of drinking water pathogens by microbial adhesion to hydrocarbon and electrophoretic mobility measurements. *Colloids Surf B Biointerfaces.* 2014;118:126–132.

34. Langley S, Beveridge TJ. Effect of O-side-chain-lipopolysaccharide chemistry on metal binding. *Appl Environ Microbiol.* 1999;65:489–498.

35. Busscher HJ, Rinastiti M, Siswomihardjo W, van der Mei HC. Biofilm formation on dental restorative and implant materials. *J Dent Res.* 2010;89: 657–665.

36. Hahnel S, Rosentritt M, Handel G, Burgers R. Influence of saliva substitute films on initial *Streptococcus mutans* adhesion to enamel and dental substrata. *J Dent.* 2008;36:977–983.

37. Teughels W, Van Assche N, Sliepen I, Quirynen M. Effect of material characteristics and/or surface topography on biofilm development. *Clin Oral Implants Res.* 2006;17 Suppl 2:68–81.