Dentistry
ISSN: 2161-1122

Pereira et al., Dentistry 2015, 5:5
DOI: 10.4172/2161-1122.1000296

Research Article Open Access

Relation between Dental Implant Joint Surfaces and Biofilm Formation

Pereira J[1,2], Tavares FP[3], Lima KC[3], Carreiro AFP[3], Henriques B[4], Silva FS[4], Nascimento RM[3], Lopez-Lopez J[1] and Souza JCM[4,5]*

[1]*Faculty of Dentistry, University of Barcelona, Barcelona, Spain*
[2]*School of Dentistry, School of Health Sciences (FCS), University Fernando Pessoa (UFP), Portugal*
[3]*Department of Dentistry, Rio Grande do Norte Federal University, Natal/RN, Brazil*
[4]*Department of Mechanical Engineering, University of Minho, Guimaraes, Portugal*
[5]*Post-Graduation Program in Dentistry, School of Dentistry, Federal University of Santa Catarina, Brazil*

Abstract

Objective: The main aim of this work was to evaluate the multi-species biofilm formation in vitro on surfaces of dental abutment and implants.

Methods: Five commercial implant-abutment assemblies (Titamax CM; Neodent®, Curitiba; Brazil) were assessed in this study. Also, commercially pure (cp) titanium grade IV square samples (10×10×1 mm) were used to prepare surfaces similar to those of titanium implant and abutments (n=10). Titanium square samples and implant-abutment assemblies were placed into 24 well-plates containing diluted human saliva at 37°C under microaerophilic conditions (5% CO_2). After 24, 48, 72 and 96 hours of incubation, biofilms were analyzed by scanning electron microscopy (SEM) and microbiological analyses.

Results: The multi-species biofilm formed at retentive areas of commercial abutments and implants like scratches, micro-gaps and defects revealed a high biofilm agglomeration, as shown by SEM analysis. The biofilm density and the colony-forming unit number were significant higher ($p<0.05$) on titanium rough surfaces than that of polished titanium surfaces along the growth time.

Conclusions: Biofilm analyses revealed a higher biomass density and cell viability on SLA rough surfaces than on polished ones. Abutment and implants revealed the presence of several rough areas promoted by the surface treatment that increase the biofilm accumulation at peri-implant areas.

Keywords: Oral biofilm; Titanium; Abutment; Dental implant; Topography

Introduction

Microorganisms in the oral cavity are often associated with biofilms, which are complex microbial communities embedded in an extracellular matrix composed of polysaccharides, proteins, nucleic acids, and water [1,2]. The cell growth and division in complex biofilm structures follow nutritional and environmental conditions in the oral cavity [3-5]. The biofilm structure comprising inner canals can accumulate external nutrients and acidic substances from dietary as well as acidic substances produced by oral microorganism metabolism. As a result, the pH in the oral cavity is frequently altered reaching low values after the intake of acidic substances and/or acids release from microbial metabolism [1,5]. Thus, the oral cavity habitat must notbe considered as uniform since there are different micro areas depending on the saliva composition, oxygen content, pH, temperature, nutrient accumulation, tissue and restorative surfaces, and resident microorganisms [1].

In the oral cavity, microbial adhesion can take place in both soft tissues and hard structures represented by tooth and restorative structures. These surfaces are usually coated with a conditioning film (0.1-10 μm) that is composed of glycoproteins, ions (e.g. Ca^{2+}, Mg^{2+}), and water [1,6,7]. The conditioning film or enamel acquired pellicle, determines the adherence of microorganisms [1,4,6,7]. However, the primary microorganism colonizers present protein macromolecules on their surfaces named adhesins that bind to receptors present on glycoproteins (e.g. mucin) in the conditioning film at oral surfaces [1,8-10]. *Streptococcus* species such as *S. sanguinis, S. oralis, S. gordonii, S. mitis, S. mutans,* and *S.sobrinus* represent 60 to 80% of all primary colonizers, which also include 5-30% species of *Actinomyces naselundii, Fusobacterium nucleatum, Capnocytophaga ochraceae*. Moreover these bacteria are able to produce hydrated extracellular polysaccharides composed of proteoglycans and signaling molecules that control the homeostatic dynamic state of the entire extracellular matrix and perform the biofilm agglomeration [1,11]. Since there is a modification of the environment associated to the presence of early colonizers, secondary or late colonizers can co-aggregate with previous species forming multi-species biofilms [3,4]. For instance, latepathogenic colonizers such as *Prevotella intermedia* and *Porphyromonas gingivalis* can co-aggregate with filamentous (*Actynomices naeslundii*) and fusi form (*Fusobacterium nucleatum*) bacteria.

The topography of oral rehabilitation systems supported by implants is of major importance for microbial colonization taking into account that rough surfaces are more susceptible to be colonized by microorganisms than smooth ones [1,5,12-16]. In a dental implant-supported fixed prosthesis, the microbial colonization begins at prosthetic areas exposed to the oral environment taking into account that biofilm formation depends on the prosthetic design, surface conditions, and on the microbiota oral [14,17]. After implantation, a part of the margin area of implant fixture is in contact with connective and epithelial tissues while another part is in contact with abutment and oral fluids. The localization of the implant-abutment connection at or below the original bone margin level has been reported as responsible for an increase of microbial colonization [5,12,14,18-20]. In literature, a mean interfacial discrepancy of about 1-60 μm in implant fixture-abutment gaps was reported [21,23]. This consists in the passage of

***Corresponding author:** Julio CM Souza, PhD, DDS, MSc School of Dentistry, Federal University of Santa Catarina, Campus Trindade, 88040-900, Florianopolis, Brazil, Tel: +55 48 3721-9077; E-mail: julio.c.m.souza@ufsc.br

Received January 07, 2015; **Accepted** April 13, 2015; **Published** April 17, 2015

Citation: Pereira J, Tavares FP, Lima KC, Carreiro AFP, Henriques B, et al. (2015) Relation between Dental Implant Joint Surfaces and Biofilm Formation. Dentistry 5: 296. doi:10.4172/2161-1122.1000296

fluids, microorganisms and small debris along the micron-sized gaps at the connection between the implant fixture and the abutment [22,23]. Thus, inflammatory cells such as macrophages and neutrophils are recruited when microbial antigens are present at periodontal tissues [18,19]. As a result, the chronic inflammatory process established around peri-implant tissues contributes to crestal and apical bone loss and increased possibilities for higher amplitude micro-movements [14,18,23,24]. In fact, the use of dental implants with novel surface treatments and abutment connections has increased. The presence of microorganisms on different surfaces of implant and abutments must be studied once peri-implant inflammations or corrosion of materials are associated with biofilm accumulation.

Concerning such biological considerations on dental implants, the main aim of this in vitro studywas to evaluate the formation of multi-species biofilm on surfaces of titanium dental abutment and implants.

Material and Methods

Commercial implant and titanium test samples

Five commercial Morse tapper abutments (Titamax CM; Neodent®, Curitiba; Brazil) were tightened to the respective dental implants on 32 Ncm by using a digital toquemeter (Lutron TQ8800, Lutron, Taiwan) coupled to a metallic holding device [25,26]. Commercial abutment surfaces were machined while the outer implant surfaces were grit-blasted and etched (SLA treatment) by the manufacturer.

On the other hand, twenty commercially pure (CP) titanium grade IV square samples (10×10×1 mm) were used in this study to produce surfaces similar to those of titanium abutment and implants in order to evaluate the influence of the surface roughness on biofilm adhesion.A group of ten square titanium samples was polished onto SiC papers down to 2400 mesh reaching a Ra roughness of 0.1 µm mimicking abutment surfaces. After grinding, the samples were cleaned in propanol for 10 min and 5 min in distilled water using an ultrasonic bath. Another group of ten square samples was prepared by SLA treatment in order to synthesize rough surfaces of dental implants. For SLA treatment, the surfaces were grit-blasted by alumina ($Al2O_3$) particles (250 µm diameter) at 5.5 bar (0.551 MPa) and at a distance of 10 mm for 15 s. After cleaning in propanol for 10 min and 5 min in distilled water, the titanium samples were immersed in Kroll´s solution for 10 min. Finally, the samples were cleaned again by using the same protocol. The samples were kept in a desiccator for 24 h and sterilized by autoclaving at 121°C for 15 min before contact with biofilms.

Biofilm formation and microbiological analysis

Human saliva was collected from four participants ranging from 20 to 31 years of age for biofilm formation. Each participant was in good dental and oral health, with no history of antibiotic treatment during the previous 6 months. None of the participants suffered from any systemic or salivary gland disease that could affect salivary secretion. A history of periodontitis or a probing depth more than 6 mm was the exclusion criteria. Saliva was stimulated by neutral chewing gum previously immersed in deionized water for 24 h. The saliva was swallowed during the first minut, then 10 ml of saliva was collected from each participant and diluted (1:5) in Phosphate Buffered Solution (PBS) every day over a period of 4 days. The optical density of the initial solution was measured by using an ELISA spectrophotometer (BIOTEK) and then adjusted to an optical density (OD) at 0.5. An OD at 0.5 corresponded to approximattely 1×109 colony-forming unit per mL (CFU/mL). Then, 5 µL of the initial suspension was inoculated in brain heart infusion (BHI, Sigma-Aldricht, USA) medium enriched with 5% sucrose for biofilms growth. Titanium square and commercial implant-abutment samples were placed into 24 well-plates containing 2 ml of BHI medium with cell suspensions and incubated for 96 h at 37°C under micro aerophilic conditions (5% CO_2). The growth medium was renewed every day.

For Ti square samples, after 24, 48, 72 and 96 hours of incubation, the samples were transferred for new well-plates and washed twice with PBS for biofilm detachment. Well-plates containing the samples were incubated at 37°C for 50 min for biofilm detachment by 1% protease treatment [27,28].The suspension was aspirated at 200 µl and placed in 96-well plates to determine the OD at 630 nm. Also, aliquots of 50 µL of that suspension were diluted in PBS and plated in BHI agar for CFU enumeration. The experiments were run in triplicate and carried out in three independent assays.

Surface analysis

Values of Ra roughness of the titanium square samples were obtained before biofilm formation by using a Profile meter (Mahr S5P, Germany). For Scanning Electron Microscope (SEM) analyses, Ti square and abutment-implant surfaces covered with biofilms were washed twice in PBS and fixed in glutaraldehyde 2% for 5 min. The surfaces were then washed three times in PBS and dehydrated through a series of graded ethanol solutions (50, 70, 80, 90, and 100%). Then the samples were sputter-coated with gold, and analyzed by SEM (S360 Leica Cambridge) by Secondary electron (SE) mode at 10-20 kV.

Statistical analysis

The results were statistically analyzed via one-way analysis of variance (ANOVA), using a significance level of $p<0.05$ by using the SPSS 17.0 software for Windows (Chicago, IL, USA).

Results

Biofilm formation on commercial abutment-implant assemblies

The topography of Morse taper dental abutments covered with a multi-specie biofilm grown for 72 hours is shown in Figure 1.

Abutment polished surfaces shaped to perform contact with soft tissues revealed scratches probably resultant from a mishandled industrial polishing process. The multi-specie biofilm grew as agglomerates (Figure 1). Moreover, retentive areas such as scratches and defects revealed a high agglomeration of biofilms.

The topography of Morse taper dental implants covered with multi-species biofilms is shown in figure 2. A mixed surface shaped by SLA and polishing treatments is noticed on the upper area of the implant platform (Figures 2A and B). The topography of the SLA surfaces of the dental implants covered with multi-species biofilms grown for 72 h is revealed in figure 2C. Additionally, the microgap existing between abutment and implant was measured as shown in figure 2D.

Biofilm formation on titanium square samples

Polished titanium square surfaces covered or not with biofilms are shown in figure 3A.

After 24 hours of growth, the biofilms were composed basically of streptococcus covering almost the entire surface of the polished titanium square samples (Figure 3B). The **Ra** roughness of the polished surfaces was 0.2 ± 0.1 µm. After 48 hours, other species such as fungal filamentous species in co-aggregation with bacteria can be noticed on the Ti surfaces (Figure 3C). Also, the extracellular matrix surrounding

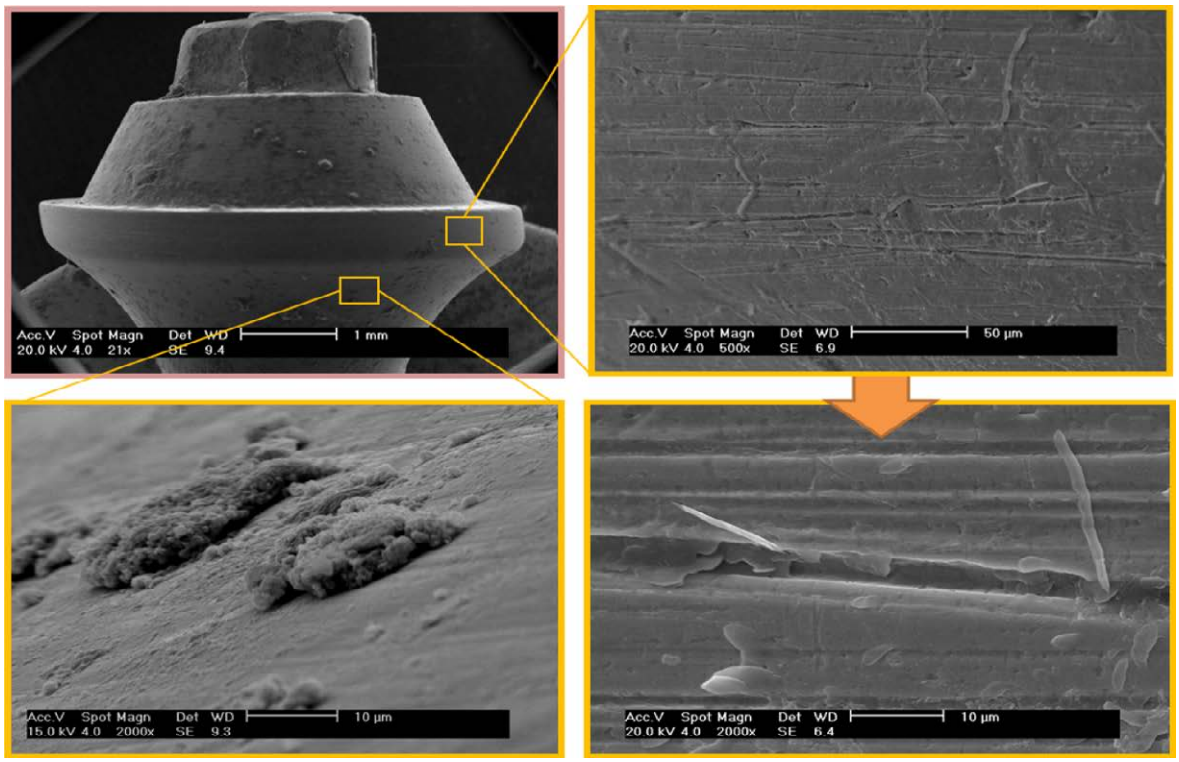

Figure 1: SEM micrograph of (A-D) commercial abutment surfaces covered with a multi-specie biofilm grown for (B-D) 72 h in BHI medium enriched with 5%s sucrose, obtained by secondary electron (SE) mode at 15 kV.

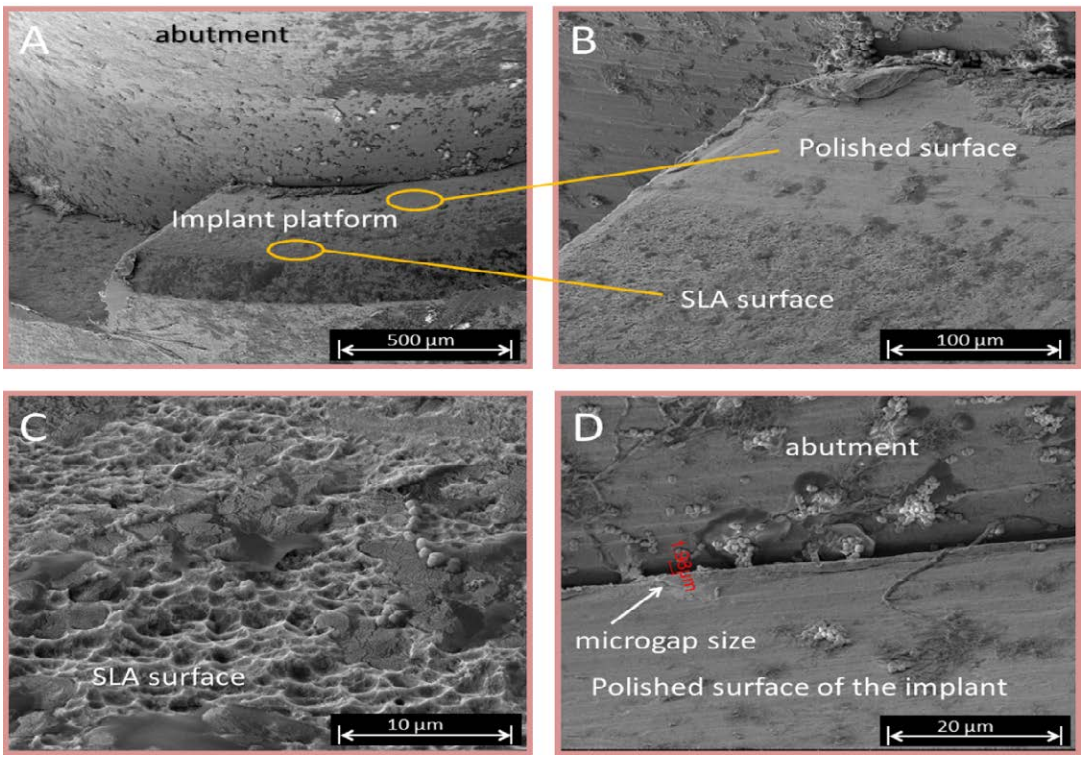

Figure 2: (A) SEM micrograph of commercial abutment-implant assembly. (B) Upper view of the platform revealing rough (SLA) and polished surfaces. (C) Topography of the commercial SLA surface covered with a multi-specie biofilm grown for 72 h in BHI medium with 5% sucrose. (D) Microgap size at the implant-abutment connection. SEM micrographs obtained by secondary electron (SE) mode at 15-20 kV.

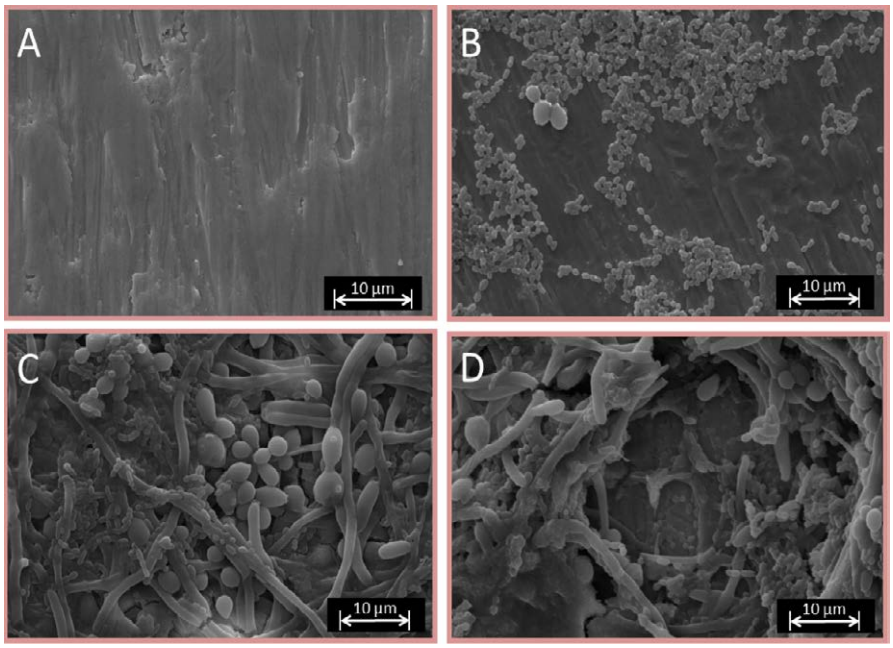

Figure 3: SEM micrograph of polished titanium surface (A) free of biofilms and covered with a multi-species biofilm grown for (B) 24, (C) 72 and (D) 96 h in BHI medium enriched with 5%s sucrose, obtained by secondary electron (SE) mode at 15 kV.

microbial species establish a biofilm agglomeration that can increase the density of the biomass, as noticed in figures 3C and 3D. After 96 hours, polished titanium surfaces were entirely covered with biofilms revealing a higher thickness (Figure 3D).

On SLA titanium square surfaces (Figure 4), the biofilm agglomeration seems to be similar to those noticed on the polished titanium square surfaces. However, the increase of **Ra** roughness (1.4 ± 0.2 µm) caused by the SLA surface treatment and resulting in several retentive areas can perform a higher density of the biofilm.

The optical density of the multi-species biofilm formed on titanium samples recorded after 24, 48, 72 and96 h revealed the evolution of the biofilm growth (Figure 5A).

The biomass density was significantly higher ($p<0.05$)on SLA titanium surfaces relative to polished titanium surfaces for 24 h of growth, which reveals the influence of the titanium surface roughness on the ability of multi-species biofilm formation. Also, the number of colony-forming unit (CFU) of multi-species biofilm biomass grown on SLA surfaces was higher than that recorded on polished Ti square surfaces (Figure 5B).

Discussion

The accumulation of multi-species biofilms was noticed on both polished or rough surfaces of abutment and implants surfaces in this study. On commercial dental abutment and implants, multi-species biofilms were formed and agglomerate on scratches, irregularities or smooth surfaces (Figures 1 and 2). In our study, the surface roughness showed to have a noticeable influence on the multi-species biofilm growth (Figures 3-5), which is in agreement with previous studies that reveal a decrease in biofilms formation associated with low values of surface roughness [5,12,15,16]. The surface roughness can assist the initial adhesion of the biofilms providing a mechanical protection against shear stresses from forces acting in the surrounding environment [5]. Additionally, the production of extracellular matrix composed of polysaccharides (e.g. glucan and frutan) and glycoproteins promotes an increase in the biofilm formation onto the surfaces and consequently microbial agglomeration (Figures 3-5) [1,5,27,28]. Thus, retentive areas like scratches or surface irregularities can establish a perfect micro-environment to the initial microbial adhesion [1,5,16]. Li et al. [16] recommends a surface roughness of Ra<0.4 µm and Rz<3.4 in order to decrease the biofilm colonization on titanium abutments. Also, previous studies have reported on a decrease of biofilm colonization on restorative surfaces with Ra roughness values below a threshold value of 0.2 µm [5]. However, smooth surfaces can become rough or retentive due to the mechanical sliding contact of abrasive particles from food and toothpastes or due to the contact of dental explorer instruments [27-30].

The microgaps existing between abutment and implant connections (Figure 2D) also become retentive and susceptible areas for accumulation of microorganisms and consequent acidic substances produced from the microbial metabolism [18-20].The presence of acidic microbial metabolites accumulated at the retentive areas decreases the pH (Figure 5A), which can promote the corrosion of dental materials including titanium abutment and implant joints [27,28]. However, Mabilleau et al. [31] revealed a significant increase of the Ra nano-roughness of titanium surfaces by atomic force microscopic (AFM) analyses after colonization by Streptococcus mitis for 21 days. The increase of the roughness could probably be promoted due to a release of Ti ions from the titanium oxide film on the surface. Guindy et al. [32] detected localized corrosion and areas of oxidation at implant-abutment microgaps. Also, a higher release of metallic ions was noticed from bone surrounding implants in comparison to physiologic baseline values detected in healthy bone [32]. Therefore, surfaces of abutment-implant joints are often in relative sliding contact under micro movements from mastication loads that could increase the surfaces' roughness and, consequently, the microgap size in the joints [27,28,33]. As a result, the accumulation of biofilms at implant-

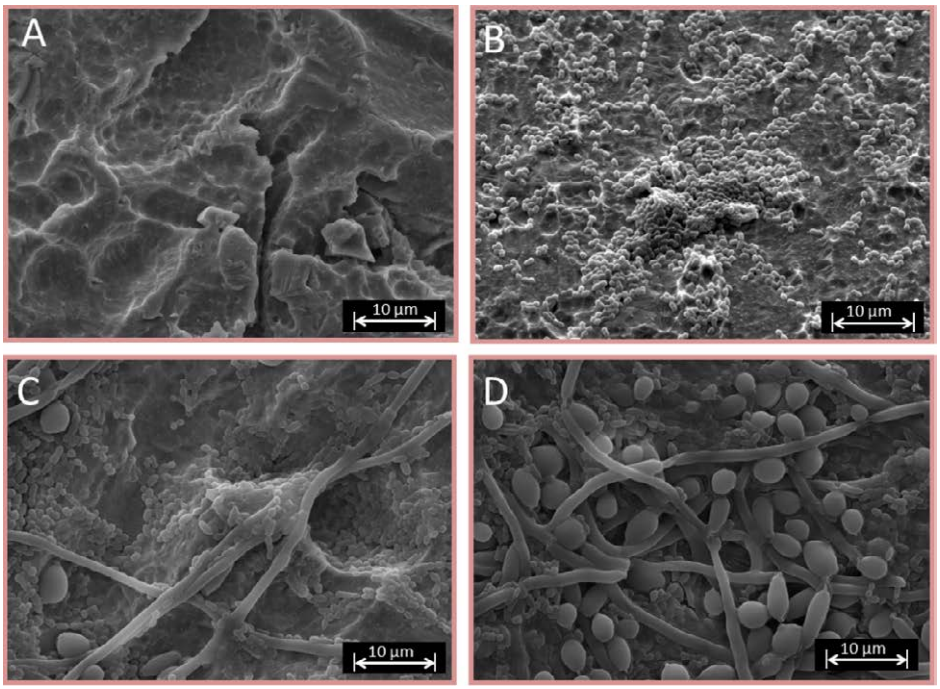

Figure 4: SEM micrograph of SLA titanium surface (A) free of biofilms and covered with a multi-specie biofilm grown for (B) 24, (C) 72 and (D) 96 h, obtained by secondary electrons (SE) mode at 15 kV.

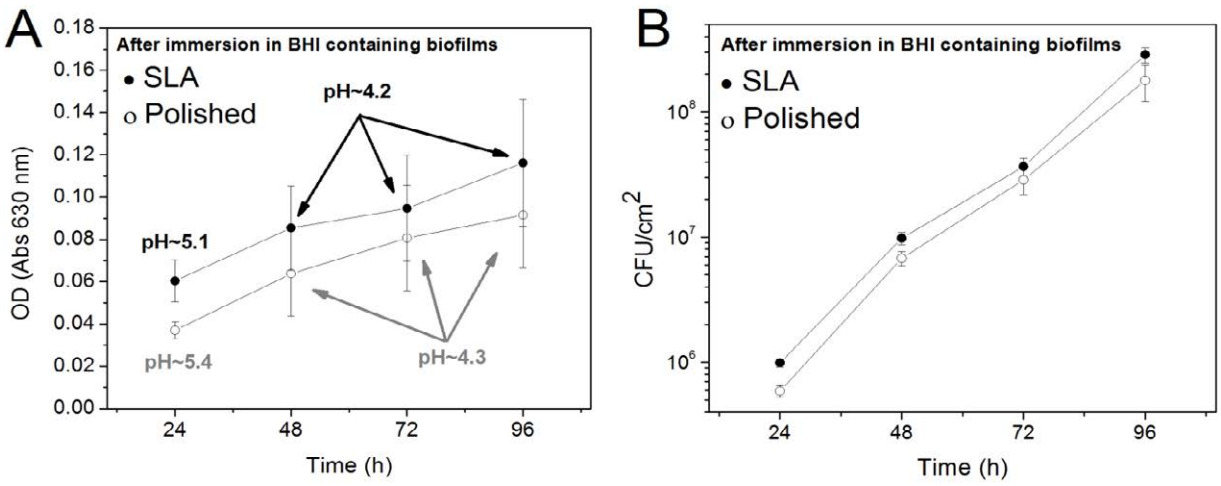

Figure 5: (A) Optical absorbance (Abs) expressing the development of multi-species biofilm biomass on polished and SLA surfaces. Also, the pH of the growth medium after 96 h in BHI medium enriched with 5% sucrose (37°C, 150 rpm). (B) Colony-forming unit (CFU) of multi-species biofilm biomass grown on polished and SLA surfaces for 96 h in BHI medium enriched with 5% sucrose (37°C, 150 rpm).

abutment microgaps and subsequently close to periodontal tissues can establish peri-implant inflammatory reactions [5,12,18,19].

Different materials are involved in a dental implant-prosthesis assembly such as ceramic crowns, metallic frameworks and resin composite luting agents that can form micro-gaps with different surface compositions as well as promote surfaces with different roughness values Thus, it is important to compare the biofilms adhesion on different materials in order to distinguish where the preferential microbial agglomeration takes place. The roughness affects the biofilm growth as shown in our study; however, the biofilm can be also found on polished surfaces that are dependent on chemical interactions of pathways biofilm adhesion. The adhesion of early colonizers on different kind of surfaces and materials has been showed in previous studies [12,27,28,31,34]. The study of Rosentritt et al. [34] revealed a higher S.mutans adhesion on polished composite surfaces (Ra<0.08 µm) than that on alloys or ceramic surfaces on the same values of Ra roughness. A correlation between substrate hydrophobicity and bacterial adhesion is reported in the literature whereas S.mutans has been classified as hydrophobic and readily adherent to hydrophobic surfaces [34,35]. That is an important aspect considering that a large number of metallic hydrophobic materials can be used to synthesize

metal-ceramic fixed prostheses such as Au-Pd-In, Ni-Cr, Pd-Ag-In, Au-Pt-Pd [35]. Also resin composites have hydrophobic nature [34]. On the other side, hydrophilic interactions take place on commercially pure titanium surfaces [36]. Then, the initial microbial adhesion on titanium surfaces are supported by other bonding agents in the oral environment such as glycoprotein (e.g. mucin) and polyssacharides (e.g. glucans) [1]. Electrostatic interactions in the adsorption of mucin onto titanium [36] as well as between mucin and bacteria [1,5,36] are responsible for the initial adhesion of multi-specie biofilms.

Conclusion

The growth of multi-species biofilms formed in-vitro on titanium surfaces of abutment and implants could be characterized by scanning electron microscopy associated with microbiologic analyses. Biofilm analyses revealed a higher biomass density and cell viability on SLA rough surfaces than on polished ones. That reveals the influence of the surface morphology on the microbial accumulation onto titanium surfaces. Moreover, the presence of a high biofilm density increased the concentration of acidic substances released by microbial metabolism leading to a decrease of the surrounding pH. That can contribute to ions release from titanium surface and subsequent material loss during long period of acidic substances accumulation. Also, commercial abutment and implant revealed the presence of several rough areas promoted by the surface treatment that increases the biofilm accumulation at peri-implant areas.

Acknowledgments

The authors acknowledge the financial support provided by CAPES/ FCT (proj. 317/11) as well as the materials provided by Neodent® (Curitiba, Brazil).

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