UNESP - Universidade Estadual Paulista

"Júlio de Mesquita Filho"

Faculdade de Odontologia de Araraquara

ERICA DORIGATTI DE AVILA

ADESÃO BACTERIANA E FORMAÇÃO DE BIOFILME EM MATERIAIS PARA IMPLANTES DENTÁRIOS

Araraquara
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Tese apresentada ao programa de Pós-Graduação em Reabilitação Oral Área de Prótese, da Faculdade de Odontologia de Araraquara, da Universidade Estadual Paulista para titulo de Doutor em Reabilitação Oral.

Orientador: Prof. Dr. Francisco de Assis Mollo Junior

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Araraquara, 24 de março de 2015.

DADOS CURRICULARES

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Salmo 21

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“A vontade de se preparar precisa

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Bob Knight

de Avila ED. Adesão bacteriana e formação de biofilme em materiais para implantes dentários [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2015.

RESUMO

O sucesso clínico da terapia com implantes depende da osseointegração primária e da ausência de inflamação no tecido peri-implantar. Um importante fator contribuinte para o desenvolvimento da inflamação é a colonização de microrganismos e subsequente formação de biofilme tanto nos materiais para implante quanto para componentes protéticos. Neste estudo, nós focamos no efeito de um tratamento de superfície para implante de titânio (Ti) e em materiais para *abutments* na adesão de bactérias e formação de biofilme. O tratamento em questão, consiste na conversão das propriedades hidrofóbicas do Ti para hidrofílicas por meio da foto funcionalização da luz UV. Biofilme foi desenvolvido pela incubação dos discos com e sem tratamento, em rico meio de cultura contendo uma complexa comunidade oral representativa da saliva humana. A biomassa e o perfil das bactérias aderidas em ambas as superfícies foram analisadas após 3 e 16 horas. Para os demais experimentos, foram utilizados discos de Ti e zircônia (ZrO_2) simulando as superfícies dos *abutments* de implantes e esmalte bovino (EB) como controle positivo. Biofilmes simples, com *Streptococcus mutans* (*S. mutans*), *Porphyromonas gingivalis* (*P. gingivalis*) e *Fusobacterium nucleatum* (*F. nucleatum*), e em multiespécie (*P. gingivalis* e *F. nucleatum)* foram desenvolvidos sobre as amostras e os resultados foram analisados quantitativamente e qualitativamente. O perfil das bactérias, também, foi analisado por meio do método de eletroforese em gel com gradiente desnaturante (DGGE). Em relação ao primeiro trabalho, embora o perfil das bactérias aderidas apresentou similaridade após 16 horas de incubação, a

biomassa do biofilme formado sobre as superfícies de Ti tratado com a luz UV, foi significativamente menor. Ti e ZrO_2 apresentaram fortes características hidrofóbicas enquanto o EB, dada a estrutura molecular da hidroxiapatita, revelou ambas as propriedades, hidrofóbicas e hidrofílicas. Contudo, a imersão dos discos em saliva humana, alterou as características físico-químicas de todos os materiais, aumentando a hidrofilicidade do Ti e da ZrO_2. Imagens qualitativas revelaram um evidente aumento na densidade das células aderidas sobre as superfícies de EB em comparação com os demais materiais. Na formação de um biofilme anaeróbico maduro, as superfícies de Ti e ZrO_2 mostraram menor número de células depositadas em caso de biofilmes simples com *P. gingivalis* e em multiespécie, respectivamente. Imagens qualitativas mostraram maior densidade de pixels representativos da biomassa do biofilme formado sobre o controle positivo; enquanto uma menor densidade, sugerindo um biofilme pouco espesso, foi observada para as superfícies de ZrO_2. Em contraste com estes resultados, quando utilizamos uma complexa comunidade de bactérias advindas da saliva humana, observamos que embora a composição dos microrganismos se apresentaram semelhantes em ambas as superfícies para o mesmo tempo de incubação, a biomassa do biofilme sobre o material de ZrO_2 foi significativamente maior em comparação com o Ti. Estas informações podem direcionar a estratégias para criação de superfícies antimicrobianas e desta forma, potencializar melhores resultados na prática da terapia com implantes.

Palavras-chave: Placa dentária, implantes dentários, titânio e cerâmicas.

de Avila ED. Bacterial attachment and biofilm formation on dental implant materials. [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2015.

ABSTRACT

Dental implant rehabilitation has become a routine procedure to replace missing teeth. Its success depends on proper osseointegration, as well as the lack of inflammation. An important factor contributing to the development of inflammation is the attachment of microorganisms and subsequent development of biofilms on the prosthetic components. This study focused on effect of materials and treatment surfaces on the bacterial adhesion and biofilm formation. We, first, assessed the biofilm formation on machined titanium (Ti) discs treated and untreated with UV-photofunctionalization. UV-treatment was performed using a photo-device for 12 minutes. Hydrophobic-to-hydrophilic conversion was confirmed on UV-treated titanium surfaces. Biofilm was formed by incubating the discs in rich medium containing a complex representative saliva-derived oral microbial community. After 3 and 16 hours, the biomass of biofilm and the bacteria profile was analyzed. For other experiments, it was used titanium and zirconia (ZrO_2) discs as abutment implants and bovine enamel (BE) as a positive control. Roughness means that topography and energy-free surface analysis were performed prior to applying microbiology biofilms onto materials. Biofilms in single species with *Streptococcus mutans* (*S. mutans*), *Porphyromonas gingivalis* (*P. gingivalis*) and *Fusobacterium nucleatum* (*F. nucleatum*) and multi species with *P. gingivalis* and *F. nucleatum* were developed on each samples and quantitatively evaluated by counting forming units (CFU) and qualitatively by confocal microscopy. Also, we analyzed the development of a complex oral biofilm on two different abutments implants surfaces. Quantitatively and qualitatively analysis were performed after 16 and 48 hours of incubation under anaerobic conditions. In relation to first study, the biomass of biofilm formed on UV-treated surfaces was significantly lower than untreated surfaces. Among Ti, ZrO_2 and

BE surfaces, the mean roughness in all discs was ≤ 0.21 µm and it did not affect the bacterial adhesion. Our results indicated a strong hydrophobic characteristic for Ti and ZrO_2 whereas BE, due to its molecular structure, revealed both properties; however, after immersion of the samples in human saliva, these properties were changed. Qualitative image revealed higher density of cells onto BE surfaces than other materials. *P. gingivalis* single biofilm on titanium, and *P. gingivalis* + *F. nucleatum* multi-specie on zirconia, showed small amount of cells on disc surfaces. Qualitative images with confocal revealed a thin layer of multispecies biofilm on ZrO_2 materials. In contrast to these outcomes, the results with a complex oral community indicated that although we found similarity on bacteria profile in both materials surfaces, the biomass of a complex oral biofilm was higher on ZrO_2 than Ti materials. In conclusion, the UV-photofunctionalization treatment of titanium reduces saliva-originated biofilm formation. Our findings suggest that the material composition and the bacteria community species used for experiments have an important impact on the type of bacteria that adhered onto abutment surfaces. This information can lead to important development in strategies to create anti-microbial surfaces, and thus potentially improve outcome of implant placement.

Keywords: Dental plaque, dental implants, titanium and ceramic.

SUMÁRIO

1 Introdução

1 INTRODUÇÃO

A cavidade bucal apresenta uma das mais complexas comunidades bacterianas associadas com o corpo humano. Mais de 700 diferentes espécies de bactérias tem sido identificadas[2, 29, 30] e parte destas, estão associadas com a placa dental. Esta diversidade microbiana residente interage com o meio e com outras espécies e formam estruturas altamente organizadas conhecidas como biofilme. A matriz extracelular produzida pelos microrganismos durante a formação do biofilme, atua como uma proteção, tornando-o resistentes tanto a danos mecânicos[11, 37] quanto a químicos[10]. Consequentemente, este novo ambiente favorece o surgimento de bactéria patogênese que por sua vez, age causando doença em hospedeiros susceptíveis[22, 23].

A adesão bacteriana a superfícies é a primeira etapa para o desenvolvimento do biofilme. Os microrganismos tendem a se aproximar de todo tipo de substrato[4], tanto hidrofóbicos quanto hidrofílicos, seja estes naturais (dentes, mucosa) ou artificiais[12, 15]. Contudo, são as características inerentes a cada material que determinará o perfil das bactérias aderidas, tornando estas, base para colonização e invasão das espécies patogênicas. Com a odontologia moderna, uma série de diferentes tipos de superfícies, tais como implantes e próteses dentárias, foram introduzidas na cavidade bucal com o objetivo de reabilitar a estética e função do paciente[9, 28, 43]. Concomitantemente a este novo cenário, surge a preocupação com a longevidade e sucesso destes substratos artificiais, principalmente em pacientes periodontalmente comprometidos[3, 27, 34].

O sucesso dos implantes dentais depende da osseointegração primária e da manutenção desta entre o implante e o osso subjacente, assim como da ausência de processo inflamatório entre o abutment (pilar intermediário) e o tecido peri-implantar[8,

[13]. Embora os implantes e componentes protéticos são necessários, estas novas superfícies também são capazes de atrair microrganismos[7] e desenvolver biofilmes. Não obstante, alguns problemas tais como perda precoce do implante por deficiência no processo de osseointegração[19, 33] ou perda tardia, como resultado da doença peri-implantar[24, 26], podem surgir. Uma vez que o biofilme é a chave das infecções peri-implantares[1, 36], conhecer os mecanismos de adesão bacteriana `as superfícies e os materiais que alteram a quantidade do biofilme aderido e o perfil das bactérias presentes pode direcionar tanto as futuras pesquisas quanto o clínico na escolha do material a ser utilizado.

A adesão de células bacterianas a superfícies e a qualidade do biofilme formado sobre estas dependem das propriedades físico-químicas dos materiais, tais como: hidrofilicidade, energia livre de superfície e rugosidade superficial[17, 18, 39]. O primeiro é definido pelo ângulo, formado entre um plano tangente a uma gota de água e um plano contendo a superfície onde o líquido se encontra depositado, inferior a 50 graus[41]. Isto significa que os grupos iônicos ou polares do material possuem afinidade com o dipolo da água, e o líquido se espalha. Interações hidrofóbicas e hidrofílicas desempenham um importante papel na adsorção de proteínas sobre superfícies de biomateriais e influenciam na relação destes com o tecido humano e na adesão de microrganismos[35, 38]. A energia livre de superfície ou tensão superficial é definida como a soma de todas as energias em excesso dos átomos de um substrato e é determinada a partir das mensurações de ângulos de contato com líquidos de diferentes polaridades. A alta energia disponível reflete a presença de grupos iônicos expostos sobre o material, e esta carga resultante determinara as interações intermoleculares com a interface bacteriana[21]. Assim como a superfície de um material, os microrganismos também expressam uma

grande variedade de moléculas na camada externa[25]. Os grupos químicos presentes nestas moléculas interagem entre si, por meio das interações intramoleculares, e também geram uma carga final, individual a cada organismo[31, 40]. As regiões hidrofóbicas da interface celular reflete a tendência da bactéria a ser atraída `a regiões com a mesma natureza química; o mesmo ocorre em caso de superfícies hidrofílicas. Contudo, as interações que conduzem a adesão das bactérias `a superfície de um material ainda são pouco compreendidas. É preciso considerar a magnitude das cargas que variam de espécie para espécie as quais também são influenciadas pelas condições do meio, como disponibilidade de nutrientes e alteração do pH[14, 20, 42]. Em relação a rugosidade, a topografia do substrato também pode interferir na resposta biológica. Em caso de implantes dentários, as micro ranhuras na superfície do material pode ter um maior efeito na remodelação óssea aumentando a adesão dos osteoblastos[44]. Ao contrário dos componentes protéticos, por exemplo. Dada a localização dos abutments e o íntimo contato destes com o tecido peri-implantar, um valor de 0,2 μm tem sido aceito como o limiar de rugosidade média, abaixo do qual nenhuma redução adicional de acúmulo de bactérias pode ser esperada e ao mesmo tempo em que a lisura da superfície não traumatizaria o tecido mole ao redor[6, 32].

O conceito pioneiro de "a corrida para a superfície", lançado pelo cirurgião ortopédico Anthony G. Gristina em 1987, descreveu o destino de um biomaterial de implante infectado e a corrida para encontrar uma superfície que interferisse na formação do biofilme e ao mesmo tempo se conectasse ao tecido vivo[16]. Para este fim, uma variedade de abordagens tem sido desenvolvida, incluindo diferentes revestimentos antimicrobianos e sistemas de liberação de antibiótico. Este conceito expressa a importância de conhecer a relação entre material – biofilme para, em um

segundo momento, individualizar as superfícies conferindo a estas características que iniba o surgimento de bactérias patogénicas. Mecanismos que regem a formação do biofilme têm gerado considerável interesse na área da saúde em geral, mas especificamente na odontologia[5]; E, diante deste contexto, neste projeto, nós primeiro focamos em superfícies de implantes e investigamos se a alteração das propriedades físico-químicas de um tipo de tratamento afetaria a adesão e consequente formação de um complexo biofilme oral em superfícies de titânio. Em um segundo momento, nós analisamos o efeito de duas superfícies para abutments de implantes na fase de adesão e na formação de um biofilme anaeróbico maduro. Por conseguinte, nós analisamos o perfil de um complexo biofilme oral e a quantidade de bactérias aderidas sobre dois diferentes materiais para abutments de implantes.

2 Capítulo 1

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Review

The Relationship between Biofilm and Physical-Chemical Properties of Implant Abutment Materials for Successful Dental Implants

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Abstract: The aim of this review was to investigate the relationship between biofilm and peri-implant disease, with an emphasis on the types of implant abutment surfaces. Individuals with periodontal disease typically have a large amount of pathogenic microorganisms in the periodontal pocket. If the individuals lose their teeth, these microorganisms remain viable inside the mouth and can directly influence peri-implant microbiota. Metal implants offer a suitable solution, but similarly, these remaining bacteria can adhere on abutment implant surfaces, induce peri-implantitis causing potential destruction of the alveolar bone near to the implant threads and cause the subsequent loss of the implant. Studies have demonstrated differences in biofilm formation on dental materials and these variations can be associated with both physical

and chemical characteristics of the surfaces. In the case of partially edentulous patients affected by periodontal disease, the ideal type of implant abutments utilized should be one that adheres the least or negligible amounts of periodontopathogenic bacteria. Therefore, it is of clinically relevance to know how the bacteria behave on different types of surfaces in order to develop new materials and/or new types of treatment surfaces, which will reduce or inhibit adhesion of pathogenic microorganisms, and, thus, restrict the use of the abutments with indication propensity for bacterial adhesion.

Keywords: biofilm; dental implants; titanium; zirconia

1. Introduction

The success of dental implants depends on the maintenance of osseointegration that is defined as a direct bone-to-implant contact without interposition of any other tissue [1]. Simultaneously, in order to preserve osseointegration around dental implants it is desirable to have no relationship between the maxillary and mandibular or parafunctional forces, mal-aligned forces of stress, peri-implantitis [2,3], absence of systemic diseases, e.g., diabetes mellitus (24), and to consider the host immune-inflammatory response to the bacterial challenge [5]. Despite the relatively high success rates of dental implant survival, reported to be higher than 90% for both partially or completely edentulous patients in longitudinal studies, some groups have demonstrated the role of putative periodontal pathogens in the etiology of peri-implantitis and their deleterious effects on hard and soft peri-implant tissues [6–10].

Late implant failure could be due to a disruption between implant and the mineralized tissues after osseointegration has been established due to overloading or microbial infection [11–13]. Whereas the main problem of osseointegration is solved by the use of high quality implants, with appropriate surface treatment and adequate surgical technique, the peri-implant tissue inflammation as a consequence of biofilms on abutments in the subgingival region is currently considered a major contributor to implant loss [14,15]. The presence of biofilms near to the implant abutments is characterized clinically by inflammation of the peri-implant mucosa progressing to subsequent destruction of the alveolar bone in contact with the implants threads. The teeth are unique structures, unlike the implants, which have the prosthetic restorations that bind to the implant body, e.g., crowns, metal structures or simple metal rods, which can lead to cracks or gaps forming between the implants and connectors. When compared to its natural non-implanted counterpart, peri-implant tissue comprises fewer fibroblasts, an increased amount of collagen fibers, blood supply, and the periosteal vascular plexus and parallel orientation of the gingival fibers [16].

In addition to these inherent factors in histopathology of peri-implant tissue, there are several differences in the designs of implants or macrostructure (screw *versus* cemented; one or two surgical stages), the type of surface or microstructure (commercially pure titanium,

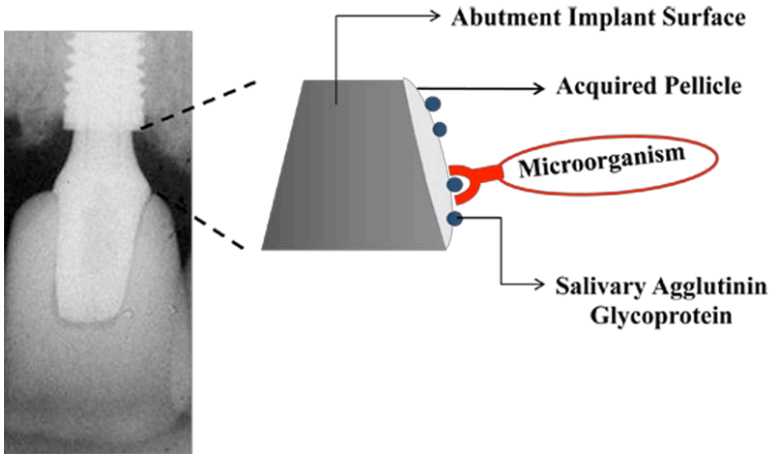
titanium alloys, titanium plasma sprayed, hydroxyapatite surfaces blasted with oxides, treated with acids, or a combination thereof) and the degree of smoothness or roughness or ultrastructure (crystallinity of the hydroxyapatite coating the implant, or nitrous acid type used), as well as different shapes and abutment materials [17]. Thus, these parameters existing between tooth and implant materials profoundly and directly influence the local microbiota.

2. Surface Characteristics of Abutments Implants

The scientific literature shows that bacterial plaque may play a prominent role as an etiologic factor responsible for implant loss after osseointegration, due to the presence of high levels of bacteria in the peri-implant sites [2,18–22]. As observed for teeth, the microorganisms need to interact with the implant abutment surface for the formation and growth of biofilm. Several studies suggest that some restorative materials have antibacterial activity, while others induce bacterial growth.

The physical and chemical characteristics of the materials will determine the type and quantity of the microbiota around these surfaces [23,24]. The non-specific physicochemical mechanisms of bacterial adhesion involve the superficial free energies and interaction surfaces theory in which adhesion is regarded as the interaction of Van der Waals forces and electrostatic phenomena [25]. Surface chemical composition, surface energy, surface water contact angle [26], and roughness are important parameters that may have a critical and fundamental influence on the interaction of biomaterial surfaces with proteins and cells. Once biomaterial surfaces have contact with biological molecules either *in vitro* or *in vivo*, the proteins present in the biological medium immediately coat the surfaces. Thereafter, salivary acquired pellicle formation takes place as the first step to biofilm formation (Figure 1).

Figure 1. Image showing salivary acquired pellicle formation upon an implant surface as the first step in biofilm formation.



With regard to the influence of surface roughness on biofilm formation, previous reports showed that protein adsorption and bacterial adhesion *in vivo* might be determined by a threshold surface roughness of 0.2 μm [27,28]. Burgers *et al.* [29] evaluated the initial biofilm formation, *in vitro* and *in vivo*, on different titanium surfaces and correlated these findings with different surface properties. Before biofilm formation, the authors determined the surface roughness and the surface free energy of samples and observed that the initial bacterial adhesion to differently textured titanium surfaces was primarily influenced by roughness surfaces values. This can be explained because the rough surfaces tend to entrap bacteria into micropits, protecting them from washing forces [28]. The difference in results from *in vivo*, *in situ*, and *in vitro* experiments is clear and the interfering factors involved are inclusion criterions established to select the patients, in relation to *in vivo* and *in situ* studies, and the number and types of bacteria used to biofilm formation, in case of *in vitro* study. Freitas *et al.* [30] in 2005 showed that a rougher surface causes an exponential increase in the number of bacterial cells, when just one kind of bacterium, *Streptococcus sanguis*, was utilized. However, when the study was performed upon the same type of surface, titanium, changing only the roughness value, and using a large number of bacteria species, the roughness does not act as an influential factor. In this case, no difference on bacteria adhesion can be justified by the same physical characteristic. The hydrophobicity and hydrophilic characteristic surfaces are other crucial elements that can directly influence bacterial adhesion [31]. In the case of implant surfaces, it is known that bone cells are attracted to a hydrophilic surface [32]. Recent studies have focused on the mechanism of chemical alterations within the dioxide titanium coating to enhance osteoconductivity and improve early osseointegration [33–35]. The increase in surface wettability may also have an influence on the amount of adsorbed proteins, since a very hydrophobic surface may prevent water from wetting the available surface, and, thus, further protein interaction with it. Alternatively, an increase in surface hydrophilicity may reduce the hydrophobic interaction between proteins and the surface, causing a lower adsorption affinity. Moreover, bacteria also have biomolecules in their cell wall that determine the surface properties and the adhesion dynamics [36]. In the case of gram-negative bacteria, the presence of lipopolysaccharide (LPS) in the outer membrane, tends to become more hydrophilic bacterial cell, and increase the attraction to hydrophilic surfaces too [37]. According to Husmark and Ronner, surface charge can also be influenced by the pH of the medium and consequently, change the bacteria adhered to it [38]. The relationship between surface and bacterial cell is mediated by a complex array of chemical and physical interactions, which add to the complexity of identifying the ideal surface with respect to abutment implants.

2.1. Types of Implant Abutments

In relation to the implant material types, titanium is the most commonly used material in dentistry due to its excellent physical and chemical characteristics, *i.e.*, biocompatibility,

stability and corrosion resistance [39]. To date, titanium is considered the “gold standard” and has maintained a dominant position as an abutment and implants material in long-term dental implant treatments. However, the high demand for aesthetic restorations has led to the introduction of ceramic implant abutments made from zirconium oxide stabilized with yttrium [40]. The microstructural and mechanical properties of the zirconia, as well as its excellent biocompatibility, have been well documented [41,42]. In dentistry, zirconia has been used for clinical applications in ceramic crowns, fixed partial dentures, orthodontic treatment supports, implants as well as abutments [43]. In addition, it has been shown that zirconia accumulates less plaque than titanium [42]. Despite the ceramic being used as abutment material for several years, only a limited number of related articles have been published concerning biofilm and abutment implants surfaces [44,45].

2.1.1. Microbiology of Periodontal Disease

Periodontitis is a chronic inflammatory disease, initiated by the accumulation of plaque on enamel surfaces in close proximity with the gingival tissue, in which disease expression involves intricate interactions of the biofilm with the host immune inflammatory response and subsequent alterations in bone and connective tissue homeostases [46]. With the permanence of dental plaque on the tooth surface, the population dynamics of the microbiota is changed, favoring the development of biofilm with anaerobic bacteria, in particular microorganisms of the red complex (a group of bacteria that are grouped together based on their association with severe forms of periodontal disease) [47], which are responsible for alveolar bone loss and ultimately the tooth. Among periodontopathogenic bacteria, (*Porphyromonas gingivalis*), a gram-negative anaerobe and one of the most important pathogens in chronic periodontitis, has the ability for co-aggregation not only with (*Fusobacterium nucleatum*), but also with early colonizers (such as *Streptococcus gordonii*) [48], which could help explain its early appearance in the development of dental plaque biofilms [49,50]. However, it is important to mention that the virulence of *P. gingivalis* has been attributed to a variety of potential factors associated with its cell surface: fimbriae, lipopolysaccharides, capsules, proteases, hemagglutinins, and major outer membrane proteins [51]. On the tooth surfaces, these microorganisms are detected in dental plaque samples within six hours after professional tooth cleaning [52], and their numbers increase in compromised sites. Moreover, these structures can bind with receptors of epithelial cells, invade them and initiate an inflammatory process. The increase of cytokines released by the host defense cells can cause bone resorption and, consequently, loss of teeth or even implants. Attention has also been given to *F. nucleatum*, *a* gram-negative anaerobic bacteria, commonly found in the subgingival biofilm in periodontal pockets. This organism also has an important role in biofilm maturation, acting as a bridge between the early and late colonizers, guiding biofilm architecture and, consequently, enhancing the adherence of more periodontitis-associated bacteria [53]. As well as *P. gingivalis*, *F. nucleatum* is also capable of adherence to and

invasion of host epithelial cells and stimulates the host immune inflammatory response. Since the presence of these microorganisms increases and/or decreases in the presence of other primary and intermediate colonizers, the successful treatment of periodontal disease would suggest an increase of the *Actinomyces spp*, and simultaneously, a reduction of pathogens of the orange and red complex [54].

2.1.2. Periodontal Disease—Peri-Implant Disease

There is a philosophy that patients with periodontal disease should be considered a risk factor for peri-implantitis [55]. After partial alveolar bone loss as a consequence of periodontal disease, the periodontopathogenic microorganisms remain within periodontal pockets, and these microorganisms have the ability to colonize various implants even after osseointegration has been successfully achieved [47]. The remaining microorganisms adhere to the teeth, as well as on crowns and implants, and directly influence the peri-implant microbiota to promote the plaque development for a more subgingival microbiota [56–58]. The history of periodontitis has been associated with peri-implant disease. Marrone *et al.* [59] showed the prevalence of peri-implantitis in patients with active periodontitis was 57.1%. Thus, if a patient is not stable with respect to periodontitis they could have more chances to present peri-implantitis on one of their implants after >5 years duration. This finding is in agreement with a study regarding prevalence and risk variables for peri-implant disease in Brazilian subjects where those with periodontitis were more prone to develop peri-implantitis [60]. In addition, other studies have also associated a history of periodontitis with peri-implant disease [57,58,61]. Karoussis *et al.* [62] compared the survival rate of implants in patients with and without a history of periodontitis. They concluded that in 10 years, the implants survival rate for the group with a past history of chronic periodontitis was 90.5% while for the group with no past history of periodontitis was 96.5% [62]. Roos-Jansaker *et al.* [63] evaluated the long-term result of implant therapy, using implant loss as an outcome variable. The patients were called in for a complete clinical and radiographic examination, 9–14 years after implant placements. A significant relationship was observed between implant loss and periodontal bone loss of the remaining teeth at implant placement. Other authors associate the microbiota with unsuccessful healing of the implants [10,59,64–66]. What perhaps make such conclusions more difficult to interpret are the conflicting definitions of peri-implantitis found in the literature [60,67,68]. Depending on how peri-implantitis is defined, the frequencies of occurrence will considerably vary and it may become difficult for comparison between studies. Berglundh *et al.* [16] in a systematic review, reported frequencies of peri-implantitis ranging of 0% to 14.4%, with a weighted mean on fixed partial dentures of 6.4%. The authors observed that late implant loss (5–10 years) occurs in the range of 2.1% to 11.3%. This suggestion may be partly explain the controversial range of peri-implantitis and posterior implants loss. The bacterial colonization upon implant surfaces and in the gingival tissues may occur only minutes after implantation [69] and, after 10 days, the bacterial microbiota

composition around these new implants becomes similar to microbiota around periodontally compromised teeth [70].

2.2. Biofilm Formation on Abutment Implant Surfaces

Strategies to reduce bacterial adhesion and biofilm formation on implant abutment surfaces play an important role on clinical practice and may be used to maintain soft tissue integrity or improve peri-implantitis treatment [71]. However, conflicting opinions exist on biofilm formation among different types of materials [15,72–75]. Titanium and zirconia are hydrophobic materials. Since gram-positive bacteria present hydrophobic characteristics due to a thick peptidoglycan layer, they will be attracted immediately to these materials. In contrast to gram-negative bacteria, those in direct contact will be repelled. Hydrophobic/hydrophilic interactions may explain why some reports do not show differences between biofilm formation when utilizing material surfaces of a similar chemical nature. Brakel *et al.* [76] compared the early bacterial colonization and the health of soft tissues adjacent to the mucosal surfaces of the titanium and zirconia abutments. Microbiological sampling and measurement of clinical parameters were performed two weeks and three months after abutment implantation. The authors concluded that there was no significant difference in bacterial adhesion in both abutments, titanium and zirconia [76]. Although titanium and zirconia are hydrophobic materials, titanium exhibits semi conductor features due to its bioactive dioxide layer [77] and this may explain controversial results in the scientific literature. Scarano *et al.* [15] showed that zirconia discs fixed on a device worn intraorally showed less plaque accumulation than Titanium discs, even with similar surface roughness. This was attributed to lower electrical conductivity of zirconia in comparison to titanium. Al-Ahmad *et al.* [75] also evaluated biofilm formation in various types of titanium and zirconia abutment surfaces *in vivo* and found that the oral biofilm accumulation was lower on the zirconia surface compared to the titanium surface.

It is also important to discuss, that when the implants are in contact with plasma or saliva, proteins can direct the attraction or repulsion of bacteria present on external layers since proteins have different degrees of hydrophobic to hydrophilic regions. The main salivary protein adsorbed to titanium *in vivo* and *in vitro* is albumin [78,79], and albumin adsorption to titanium occurs through calcium (Ca^{+2} bridges [80]. The negative charge from titanium dioxide may attract positive ions, such as Ca^{+2} and its presence thus increases the adhesion of some bacteria species. Hauslich *et al.* [81] 2012, demonstrated that pretreatment of titannium surfaces with Ca^{++} ions increased the adhesion of *S. mutans* and *F. nucleatum* to the Ti surfaces, but did not influenced the *P. gingivalis* adhesion. *F. nucleatum* possesses Ca^{+2}-dependent binding proteins on the cell surface similar to those of *S. mutans* [82]. These findings indicate that the divalent ion Ca^{+2} may serve as a bridging agent in the adhesion of bacteria to Ti surfaces.

Bacteria can detect the non-biological substrate and express different genes, probably as part of the adaptation to a new microenvironment. The differences in the depth and viability

of the biofilms on the different materials are a result of physical and chemical properties that determine gene expression profiling of bacteria, regardless of film formation [75].

3. Conclusions

In the case of partially edentulous patients affected by periodontal disease, the type of abutment implant to be used requires careful consideration. In general, previous reports compare biofilm formation on different types of surfaces using few numbers of bacteria. The multiple factors involved in complex biofilm formation, such as roughness and electrostatic interactions between bacteria and surfaces and interbacterial interactions can make it difficult to characterize and determine the ideal abutment implant surface. However, understanding the influence of materials surfaces on bacterial adhesion will help future development of new materials or surface treatments, in order to reduce or inhibit adhesion of pathogenic microorganisms on them.

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Author Contributions

Erica Dorigatti de Avila and Rafael Scaf de Molon wrote the manuscript. All authors participated of study conception, acquisition of data, design, critical revision and corrections.

Conflicts of Interest

The authors declare no conflicts of interest.

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3 Capítulo 2

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Review

Implications of Surface and Bulk Properties of Abutment Implants and Their Degradation in the Health of Periodontal Tissue

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Abstract: The aim of the current review was to investigate the implications of the surface and bulk properties of abutment implants and their degradation in relation to periodontal health. The success of dental implants is no longer a challenge for dentistry. The scientific literature presents several types of implants that are specific for each case. However, in cases of prosthetics components, such as abutments, further research is needed to improve the materials used to avoid bacterial adhesion and enhance contact with epithelial cells. The implanted surfaces of the abutments are composed of chemical elements that may degrade under different temperatures or be damaged by the forces applied onto them. This study showed that the resulting release of such chemical elements could cause

inflammation in the periodontal tissue. At the same time, the surface characteristics can be altered, thus favoring biofilm development and further increasing the inflammation. Finally, if not treated, this inflammation can cause the loss of the implant.

Keywords: abutment implants; bacterial adhesion; cell adhesion; titanium; zircônia

1. Introduction

Dental implants have achieved great clinical success in the last 20 years. However, late failure due to a disruption between the implant and the mineralized tissues after osseointegration has been established can still occur due to overloading or microbial infection [1–3]. While the role of implant surfaces in achieving and maintaining osseointegration has been researched extensively, the second reason for the failure of implants, *i.e.*, the presence of bacterial biofilms on the implant surfaces, has received less attention. Specifically, the main problem of osseointegration has been solved through the use of high-quality implants with appropriate surface treatments and adequate surgical techniques to avoid peri-implant tissue inflammation. However, the biofilm on these surfaces may cause inflammation of the peri-implant mucosa, leading to subsequent destruction of the alveolar bone that is in contact with the implant threads. In addition to sustained osseointegration, good integrity of the peri-implant mucosa at the transmucosal implant surface is another vital factor in long-term implant success.

Experimental results from *in vitro* and *in vivo* studies strongly suggest that some types of surface modifications promote more rapid bacterial and epithelial cell adhesion than machined surfaces. This difference may depend on an altered surface chemistry and/or increased texture at the micrometer scale [4,5]. Studies have also shown that surface characteristics play a special role in the biological performance of abutment implants. The surface properties of interest for abutment implants can broadly be divided into structural properties and chemical properties. Thus, the aim of the current review was to investigate the implications of the surface and bulk properties of abutment implants and their degradation in relation to periodontal health.

2. Attention to Prosthetic Components—Abutments

For dental implants to be successful, direct bone-to-implant contact without interposition of any other tissue is needed [6]. At the same time, to preserve osseointegration around dental implants, biocompatible surfaces that are adherent to epithelial cells but non-adherent to bacteria are likewise needed. Patients who have lost teeth due to periodontal disease have

periodontal bacteria in their mouths. These bacteria can adhere to other surfaces present in the oral cavity, including restorations, prosthesis and abutment implants. Biofilms that develop on abutment surfaces may cause peri-implantitis. Peri-implantitis is defined as a bacterial infection characterized by inflamed, swollen, and bleeding soft tissues resulting in suppuration and crater-like destruction of the alveolar bone adjacent to a functional implant [7,8]. Because bacterial adhesion and colonization has been implicated as the main causative factor in the initiation and progression of peri-implant disease, the implant and periodontal structures need to be protected from bacterial invasion and subsequent infection [9]. To this end, surfaces that can inhibit bacterial adhesion but are also nontoxic to the periodontal tissue are needed [10]. The response of cells and tissues to foreign bodies depends on the latter's properties and behavior upon contact with body fluids. The chemical composition of the bulk material is often significantly different from the surface interfacing with organic tissues. Some materials, such as titanium, undergo surface oxidation, and the mode of preparation or sterilization may result in chemical contamination of the surface [11].

3. Structural and Chemical Properties of Surfaces

Numerous *in vitro* experiments and animal studies have shown the importance of the implant surface's characteristics in the host response [12]. It is known that abutment implant surfaces must present smooth surfaces to favor cell adhesion whereas implant surfaces must be rough to promote osteoblast proliferation [13]. However, the optimal surface topography for implant abutments has yet to be determined [14].

The manufactured surface can be considered one of the factors that will determine the formation of new tissue around the implant. The surface properties of any material will be different from the bulk of the material. The creation of a surface inevitably involves breaking of the chemical bonds that keep the material together. A freshly created surface represents an energetically unstable situation, often referred to as having a high surface energy. When the new surface is exposed to novel environment, the surface energy will rapidly be lowered by binding to and reacting with surrounding molecules. For metals such as titanium, these reactions involve oxygen in the air to form a thin surface layer of oxide. At the same time, the surface characteristics are also strongly influenced by the method of surface preparation, handling and storage. During the preparation of abutment implants, the material surface is subjected to various chemical processes that leave residues on the surface. If the preparation involves elevated temperatures, the surface oxide will grow as a result. Sterilization and storage in sterile packaging are also likely to influence the surface, for example, via the transfer of molecules from the packaging material to the implant surface. The close connection between surface preparation and the resulting surface characteristics means that all aspects of the manufacturing process and ensuing logistics need to be carefully controlled to produce consistent abutment implant surfaces.

A particularly important structural property of dental abutment implants is the surface topography or surface roughness. Figures 1 and 2 show the different topographies of two kinds of surfaces, titanium and zirconia, by means scanning electronic microscopy (SEM).

Figure 1. Scanning electron microscopy (magnification 20,000×) of the titanium microstructure.

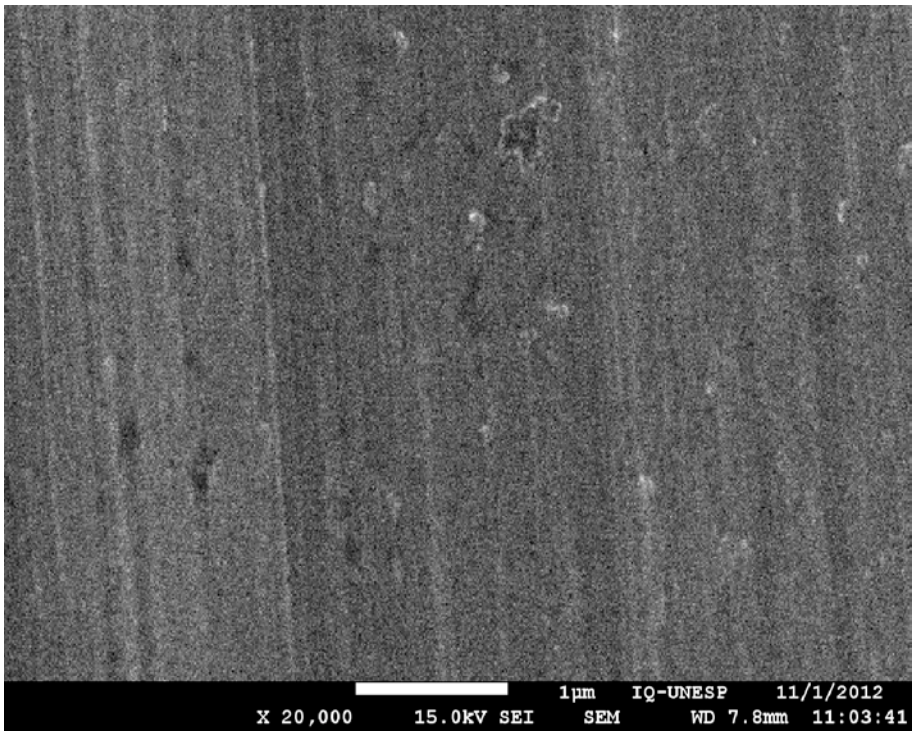
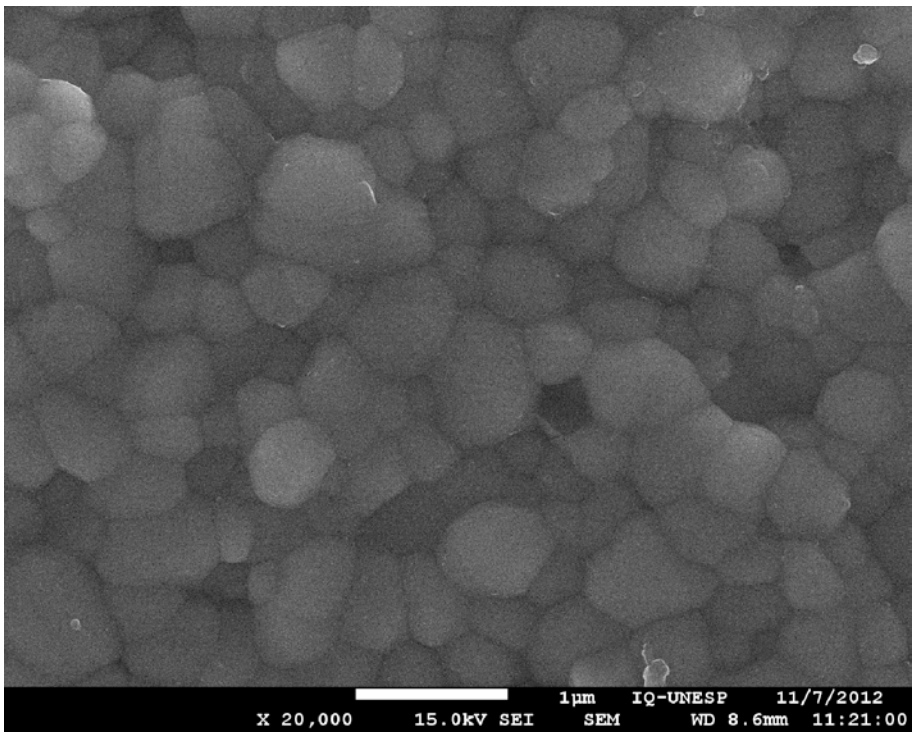


Figure 2. Scanning electron microscopy (magnification 20,000×) of the zirconia microstructure.



Depending on the scale being considered, the roughness will be determined by the surface oxide layer or by the bulk material. The surface structure may be completely dominated by the surface oxide layer if it is thick. In other cases, it is determined by a combination of a micrometer-scale rough metal surface covered by a thin oxide layer with nanometer-scale roughness. Whereas it is well established that surface roughness on the micrometer scale plays an important role in cellular reactions, tissue healing and implant fixation (24), the role of surface topography on the nanometer scale has not yet been explored in a systematic manner. The variety of surface characteristics that are possible for abutment implants opens up opportunities for modifying implant surfaces to enhance their biological performance. The clinical abutment implants currently in use display a wide variety of micro-structural and chemical properties. Different mechanical, chemical and optical methods are used to produce abutment implant surfaces with various surface topographies and oxide layers of different thicknesses, crystallinities and compositions.

There are two main kinds of dental abutment implants on the market: titanium and zirconia (or zirconium dioxide, ZrO_2). Pure titanium or titanium alloys, and to a lesser extent, zirconium, are metals that are often used in direct contact with host tissues. These metallic biomaterials are highly reactive, and on exposure to fluid media or air, quickly develop a layer of titanium dioxide or zirconium dioxide. This layer of dioxide forms a barrier at the interface between the biological medium and the metal structure, determining the degree of biocompatibility and the biological response to the implant. For titanium, the chemical composition of the material is usually the dioxide, TiO_2, which is a chemically inert, semiconducting material that also exhibits photocatalytic activity in the presence of light of energies equal to or higher than its band-gap energy. These characteristics offer an extensive

range of applications in dental implants as prosthetic components. For these reasons, titanium implants have gained widespread attention over recent decades. The surface oxide may also include varying amounts of other substances as impurities. Organic molecules originating from adsorbed molecules from the air, process residues or packaging materials also cover these surfaces. The residues formed on the surfaces may influence their wetting properties and, hence, important interactions such as protein adsorption. The thickness of the surface oxide layer on titanium can vary from a few nanometers to several micrometers depending on the method of preparation and the temperatures involved.

Abutment implants made of ceramic materials can eliminate the problems associated with metal being visible in the peri-implant area, offering important esthetic advantages. The yttria-doped tetragonal zirconia polycrystal (Y-TZP) has become an alternative to alumina as a structural bioceramic because of its significantly higher fracture toughness and strength [15,16]. Y-TZP was first used in orthopedics, allowing new implant designs that were not possible with the more brittle alumina. Biomedical grade Y-TZP exhibits the best mechanical properties of single-phase oxide ceramics, which are the results of phase-transformation toughening to increase its crack-propagation resistance. Zirconia exists in three phases (monoclinic, tetragonal and cubic) according to the temperature [17]. In zirconia, the high strain energy at a crack tip creates T-M (tetragonal-monoclinic) transitions. This crystalline modification is followed by a 4% volumetric expansion that closes the crack [18–20]. Y-TZP ceramics can exhibit toughnesses higher than 6 MPa·pm and strengths higher than 1000 MPa. On the other hand, due to its metastability, Y-TZP is prone to low-temperature degradation (LTD), also referred as aging, in the presence of water. Aging is a progressive transformation from a tetragonal phase to monoclinic, which results in surface modification and microcracking. This process occurs due to the high modulus of elasticity of zirconia, which inevitably influences the performance and reliability of zirconia devices and reduces their lifetime [18]. In orthopedics, clinical reports show that Y-TZP can exhibit progressive degradation even under well-controlled process conditions, which limits its long-term stability. Interestingly, Y-TZP is no longer used in orthopedics, and major companies in this field have switched to alumina-zirconia composites. In dentistry, zirconia is used in the monolithic phase as 3Y-TZP. Polycrystalline tetragonal zirconia stabilized by yttria (3 mol%) results in a ceramic material with high toughness and hardness [21]. In recent years, zirconia dental abutment implants have been favored over titanium implants, especially in the anterior part of the oral cavity, for implant-supported prostheses [22,23] due to their excellent strength and toughness but also due to their esthetic properties, translucency, ability to be colored, the availability of new powders with superior aging resistance and ability to be manufactured by computer-aided design and manufacturing procedures. Even though a few general papers devoted to dental zirconia have underlined the fact that some forms of zirconia are susceptible to aging and that processing conditions can play a critical role in the LTD of zirconia [21], this problem has not received sufficient attention to date.

4. Problems of Dental Abutment Implant Surfaces

Corrosion is the deterioration a metal undergoes as a result of interactions with the surrounding medium (electrochemical attack), which causes the release of ions into the environment. It is important to mention that no metal or alloy is entirely inert *in vivo*. And corrosion phenomena at the surface interface are particularly important in the evolution of both dental and orthopedic implants and are possible causes of implant failure after an initial success. The degradation of a metallic implant is undesirable because it negatively alters the structural integrity of the implant [24]. Treatments of Ti-based implants give rise to an outer rutile layer that improves corrosion resistance and reduces the friction coefficient of rubbing contact [25,26]. By definition, rutile is a mineral composed primarily of titanium dioxide, TiO_2. These surfaces improve osteoblast adhesion *in vitro* and increase the percentage of bone-to-implant contact *in vivo* [27,28]. Rutile debris are expected to arise from these modified surfaces after long-term functional loading. Valles *et al.* [29] investigated whether human osteoblasts were able to absorb rutile particles compared with their intake of titanium particles. The dry rutile and titanium particles used in the experiment were different in size (rutile of 0.9–1.6 mm in diameter and commercially pure titanium (Ti) particles of 0.20 mm in diameter). Cell treatments were performed with equivalent amounts of each type of material. In principle, the osteoblasts should have received a substantially higher number of rutile particles than titanium particles. However, examination of the particles as suspensions in the culture media before being applied to the cells revealed the formation of micrometric aggregates in both cases. Therefore, the cells were actually in contact with agglomerates of rutile or titanium particles of a similar size range rather than with individual particles, and the rutile particles induced a lower response *in vitro*, as defined by their ability to induce the secretion of inflammatory cytokines (TNF-a, IL-6 and IL-1b) in macrophage cultures of different sources. Other authors have noted that sub-micrometric dry alumina particles aggregate to the same extent as micrometric dry titanium particles and have previously detected agglomerations of other kinds of particles in culture media. Treatment with titanium or rutile particles does not result in osteoblast death. Similar doses of titanium particles are not cytotoxic for human osteoblast-like MG-63 cells [30], but they severely decreased the viability of rat osteoblasts [31], suggesting that species-specific characteristics modulate the sensitivity of osteoblasts to particles generated by wear. These works collectively show that corrosion is not a local problem because the particles produced as a result can migrate to distant sites. If these particles can reduce the viability of osteoblasts in animals, it is possible that they could also cause chronic inflammation because the macrophages that phagocytose these particles cannot digest them, so they get released in the middle of their transport. Other macrophages will phagocytose these particles again, and the cycle will repeat. Interestingly, treating human primary macrophages with Ti particles releases much higher levels of inflammatory cytokines (TNF-a, IL-6 and IL-1b) than rutile, which only stimulates marginally detectable levels of secretion. These results support the higher biocompatibility of titanium-based implants modified to create an outer layer of rutile on their surfaces.

In relation to zirconia, most of the research on zirconia dental ceramics today focuses on the mechanical properties of the devices [32], their fatigue resistance [33] and surface modifications [34] that could enhance bone in-growth and, in cases of dental implants, reduce bacterial adhesion and favor the growth of epithelial cells on abutment surfaces [35]. Recently, Chevalier *et al.* [18] evaluated the resistance of biomedical-grade yttria-stabilized zirconia samples coated with a porous zirconia layer that was processed via two slightly different routes to environmental degradation. In one group, the porous surface was coated onto a pre-sintered ceramic piece. In the other group, the porous surface was coated and sintered together with the ceramic piece. The results showed that the two groups exhibited totally different degrees of LTD resistance. With the other coating process, we would expect full transformation of the porous layer after 5 years *in vivo* in the worst-case scenario. Standard steam sterilization at 134 °C for just 1 h would lead to a significant transformation of this layer. The only change was the sequence by which the porous surface was sprayed onto the surface. This result was very important because it confirmed the strong variability of 3Y-TZP with regard to LTD resistance and the critical role of the manufacturing process. It is therefore essential to more systematically evaluate the resistance of any new dental device dedicated to clinical use to LTD to avoid critical issues such as those encountered in orthopedics some years ago. The search for aging-resistant zirconia and standardized LTD evaluations should be a priority in implant research to ensure the long-term success of zirconia as a dental material.

5. Dental Abutment Implants and Periodontal Tissue

Despite the widespread use of titanium and the substantially growing research on the development of new surfaces and/or modifications of existing surfaces, a detailed understanding of the relationship among surfaces, cells and bacteria adhesion is still lacking. The soft tissue around dental implants serves as a protective barrier between the oral environment and the underlying peri-implant bone, and one factor proposed to be of importance for the long-term success of implant therapy is the development of a good seal between the abutment and soft-tissue [36]. Modifications of abutment implants to improve esthetics should not be made at the expense of biological compatibility. Placement of an abutment is followed by a sequence of biological events: covering the surfaces with a pellicle of proteins and glycoproteins derived from saliva and gingival crevicular fluid; the adherence, migration and proliferation of cells; and the secretion of microbial products [37]. The composition, as well as the configuration and density, of the proteins in the pellicle, are largely dependent on the physical and chemical nature of the underlying surface. It follows that the properties of the surface influence bacterial adhesion through pellicle protein adsorption and the adherence, migration and proliferation of cells. Improved understanding of these sequences would aid in the selection of an optimal surface texture.

In relation to cell attachment, smooth, turned titanium, nanoporous TiO_2-coated and anodized Ca^{2+}-modified surfaces have all been shown to be suitable for soft-tissue healing [38,39]. Fröjd *et al.* [39] investigated how different implant surfaces (turned titanium, sol-gel nanoporous TiO_2-coated surfaces and anodized Ca^{2+}-modified surfaces) affect biofilm formation by two early colonizers of the oral cavity. Nano-topographical modification of smooth titanium surfaces did not cause significantly greater bacterial adhesion and biofilm formation *in vitro* than turned surfaces or surfaces treated with Ca^{2+} incorporation during anodic oxidation. In the presence of saliva, adhesion increased by more than ten-fold compared with without saliva, and yet, no differences were observed among the surfaces. These data suggest that modification with sol-gel-derived nanoporous TiO_2, which has been shown to improve soft-tissue healing *in vivo*, does not lead to greater bacterial adhesion and initial biofilm formation by the two commensal species tested compared with other surfaces [40]. However, it cannot be discounted that greater differences in biofilm formation on the different surfaces could be observed over a longer time period in the presence of other bacterial species. According to Abrahamsson *et al.* [36], abutments made of titanium or highly sintered aluminum-based ceramic (Al_2O_3) allowed the formation of a mucosal attachment that included epithelial and connective tissue that were approximately 2 and 1.5 mm thick, respectively. In contrast, with porcelain dental implants, no mucosal attachment formed at the abutment level; instead, the soft tissue margin receded, and bone resorption occurred. The mucosal barrier was thus partially established at the fixture portion of the implant. Mustafa *et al* [14]. investigated the attachment and proliferation of human oral fibroblasts in densely sintered aluminum oxide specimens. The authors concluded that the initial attachment and spreading of human gingival fibroblasts were influenced by the surface texture of the ceramic abutments. Fibroblasts spread and grew effectively on sintered surfaces that had their roughness (Sa) increased to 0.34 mm by milling. Other studies have shown statistically significant differences between peri-implant soft tissues around titanium and zirconium oxide healing caps, with an overall lower inflammatory level in tissues surrounding the latter [41]. To understand these results, it is necessary to understand the relationship between periodontal tissue and prosthetic components of implants. The biological extension around natural teeth has been reported to be approximately 2 mm, 1 mm of which corresponds to epithelial attachment mediated by the junctional epithelial (JE) and 1 mm of which corresponds to gingival connective tissue attachment [42]. Several studies have described that the peri-implant JE is approximately 2 mm long [43]. This value can usually be increased because conventional implant surfaces cannot deter the formation of a “long” epithelial attachment. However, as long as the JE stays restricted to the region of the prosthetic components and not the implants, it will not cause damage. In other studies, the peri-implant epithelium (PIE) appeared to lean on the abutment implant, but was structurally very different from the JE, showing slower cell proliferation and no evidence of direct adhesion on the implant surface [44]. Poor adhesion of the PIE may contribute to the formation of inflammatory lesions and bone loss around the implants, which has become a common clinical problem [45,46]. It is

possible that low PIE adhesion allows for apical migration of plaque biofilms and could, therefore, directly explain the inflammation and bone loss around bone-level dental implants.

6. Dental Abutments Implants and Bacteria Adhesion

It is known that bacterial plaque plays a prominent role as an etiologic factor in implant loss after osseointegration due to the presence of high levels of bacteria in peri-implant sites [47–49]. As observed for teeth, the microorganisms need to interact with the abutment implant surface for the formation and growth of a biofilm. Firstly, this interaction occurs through non-specific physicochemical mechanisms. Bacterial adhesion involves the superficial free energies and interaction surfaces theory in which adhesion is regarded as the interaction of the van der Waals forces and electrostatic phenomena. After the interactions of the biomaterial surfaces with biological systems *in vitro* or *in vivo*, the proteins present in the biological medium immediately coat the surfaces [50]. In sequence, the acquired salivary pellicle formation takes place as the first step in biofilm formation. Early colonizers create an environment that favors late colonizers. Several studies have suggested that some restorative materials may have antibacterial activity, while others may induce bacterial growth [51–54]. With regard to the influence of surface roughness on biofilm formation, previous reports have shown that protein adsorption and bacterial adhesion *in vivo* appear to require a threshold surface roughness of 0.2 μm [55,56]. Burgers *et al.* [57] evaluated the initial biofilm formation on different titanium surfaces *in vitro* and *in vivo* and correlated these findings with different surface properties. Before biofilm formation, the authors determined the surface roughness and the surface free energy of the samples. Their results showed that the initial bacterial adhesion to differently textured titanium surfaces was primarily influenced by surface roughness values. According to these authors, the parts of an implant that are exposed to the oral cavity should be polished to prevent plaque accumulation. Another crucial element that directly influences bacterial adhesion is surface hydrophobicity [58] because a very hydrophobic surface may prevent water from wetting the available surface, and thus prevent protein interaction with it. Alternatively, an increase in the surface hydrophilicity may reduce the hydrophobic interaction between proteins and the surface, causing a lower adsorption affinity.

From the literature, it is still uncertain what the ideal abutment implant surface should be to reduce bacterial adhesion [52–54,59–64] (Table 1). Some *in vitro* and *in vivo* studies have confirmed differences in biofilm formation among different types of materials. According to some authors, the biomaterial-related properties of zirconia are more advantageous than titanium. Bacterial adhesion has been shown to be satisfactorily low in zirconia restorations, which is important in maintaining periodontal health [52]. Scarano *et al.* [23] studied discs attached to a device worn intraorally and reported a degree of coverage by bacteria of 12.1% on zirconia discs compared with 19.3% on titanium discs. This difference was attributed to the fact that zirconia had a lower electrical conductivity. Rimondini *et al.* [59] confirmed

these results in an *in vivo* study that showed that zirconia surfaces accumulated fewer bacteria than titanium due to their chemical properties after correcting for the standard roughnesses of surfaces for all of the samples of the same group but with different materials. In concordance, other authors evaluated biofilm formation on various types of titanium and zirconia abutment surfaces *in vivo* and concluded that oral biofilm accumulation was lower on zirconia surfaces compared with titanium surfaces [52]. At the same time, inflammatory infiltration, microvessel densities and vascular endothelial growth factor expression were found to be higher around titanium caps than zirconia caps [65]. In addition, patients have reported allergic reactions and sensitivities to titanium [39,66]. The material composition of transgingival implant components appears to influence the formation of epithelial attachment. The shape and profile of the implants are able to guide gingival contouring and, together with the color of the material, strongly influence the final esthetic results of dental implant restorations. Zirconia can be suitable for making implant abutments, but more clinical trials and mechanical testing are necessary for a fuller understanding of the behavior of zirconia abutments over a long time period.

In recent years, some new studies have attempted to compare the adhesion of aerobic bacteria (*in vitro*) and anaerobic bacteria (*in situ*) on titanium and zirconia abutments, and many of them have found no differences in the quantity of cells adhered to different surfaces [53,54,67]. Salihoglu *et al.* [61] compared zirconium dioxide (zirconia) and titanium alloys with respect to the adhesion and colonization of two periodontal pathogens on both hard surfaces and on soft tissues *in vivo*. The results showed no statistically significant differences in probing depths, number of DNA copies of *A. actinomycetemcomitans* or *P. gingivalis*, and total bacteria counts between titanium alloys and zirconium oxide surfaces and between the biopsy specimens obtained from their buccal gingival. With respect to the surface free energy, zirconia abutments showed lower surface free energies than titanium abutments. According to these authors, zirconia surfaces have comparable properties to titanium alloy surfaces with respect to the adhesion and colonization of two periodontal pathogens on both hard surfaces and in soft tissues [61]. Therefore, future research should focus on improving epithelial attachment on implants and reducing biofilm adhesion, especially on different abutments.

Table 1. Studies presenting data on microbiology associated with abutments dental implants.

Authors	Kinds of study	Surface studied	Predominant microbes	Methods used	Results
Rimondini *et al.* [59] 2002	*In vivo*	Titanium and Zirconia	*S. mutans, S. sanguis, A. viscosus, A. naeslundii,* and *P. gingivalis*	Quantification of bacteria	Zirconia accumulates fewer bacteria than titanium.
Al-Ahmad *et al.* [52] 2010	*In vivo*	Machined Ti, modified Ti, modified Zr machined alumina-toughened Zr, sandblasted alumina-toughened Zr, machined Zr, Ti, Zr	*S. spp., V. spp., F. nucleatum,* and *A. naeslundii*	Fluorescence *in situ* hybridization and confocal laser scanning microscopy	There was no difference in bacteria adhesion between titanium and zirconia
van Brakel *et al.* [54] 2011	*In vivo*	Titanium and Zirconia	*A. actinomycetemcomitans, P. gingivalis, P. intermedia, T. forsythia, P. micros, F. nucleatum, T. denticola*	Quantification by means real-time PCR	There was no difference in bacteria adhesion between titanium and zirconia
Lee *et al.* [60] 2011	*In vitro*	Titanium and Zirconia	*S. sanguis*	Quantification by means scanning electron microscope, crystal violet staining and measurement of fluorescence intensity	There was no difference in bacteria adhesion between titanium and zirconia
Salihoglu *et al.* [61] 2011	*In vivo*	Titanium and Zirconia	*A. actinomycetemcomitans, P. gingivalis*	Bacterial detection and quantification by means real-time PCR	There was no difference in bacteria adhesion between titanium and zirconia
Al Radha *et al.* [62] 2012	*In vitro*	Titanium, Zirconia, Titanium blasted with zirconia, Titanium blasted with zirconia/acid etched	*P. nigrescens, S. mitis*	Fluorescence microscopy; the area covered by bacteria was calculated using Image-J software	Zirconia and Titanium blasted with zirconia showed superior effect reducing the adhesion of bacteria
Yamane *et al.* [63] 2013	*In situ*	Titanium, gold-platinum alloy, zirconia, alumina, and hydroxyapatite	*S. mutans*	Quantification by means PCR	There was no difference in bacteria adhered and the tested materials

Oliveira1 *et al.* [64] 2012	*In vivo*	Titanium and Zirconia	*A. actinomycetemcomitans, P. gingivalis*	Quantification by means real-time PCR	There was no difference in bacteria adhesion between titanium and zirconia
Do Nascimento *et al.* [53] 2013	*In vitro*	Machined titanium, cast titanium and zirconia abutments	*F. nucleatum, N. mucosa, P. aeruginosa, P. anaerobios, S. aureus, S. gordonii, S. parasanguinis, T. forsythia*	Biofilm percentage was calculated using the relation between biofilm area and total surface area of specimens.	Zirconia accumulates fewer bacteria than titanium

7. Conclusions

Implant surfaces are composed of chemical elements, which may degrade under different temperatures or suffer damage from the forces applied to them. The release of such chemical elements may result in inflammation of the periodontal tissue. At the same time, the surface characteristics may be altered, thus favoring biofilm development, which will further increase inflammation. If not treated, this inflammation may cause the loss of the implant. Today, it is already known that implant surfaces should be modified not just to reduce microbial adhesion but also to reduce the chemical elements released by the surfaces over time. Further research is necessary to create an abutment surface that can achieve all of these goals, which is currently the biggest challenge in oral rehabilitation with dental implants.

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Conflicts of Interest

The authors declare no conflicts of interest.

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4 Capítulo 3

Artigo Publicado
"Journal of Oral Implantology"

The impact of physical chemical characteristics of abutment implant surfaces on biofilm formation

Biofilm on abutments surfaces

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The impact of physical chemical characteristics of implant abutment surfaces on bacteria adhesion

Bacteria adhesion on implant abutments materials

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Abstract

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 two common pathogens of the oral cavity, *Streptococcus mutans (S. mutans) UA140* and *Porphyromonas gingivalis (P. gingivalis) 33277*. To investigate the influence of material surfaces on bacterial adhesion, we studied the surface free energy (SFE) as well as the topography by atomic force microscopy, and the chemical elements composition by scanning electron microscopy (SEM) equipped with an energy dispersive X-ray spectroscope (EDX). Our results indicated a hydrophobic characteristic for all of the materials; however, the presence of polar and non-polar 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 greater density of bacterial adhered to BE than to implant abutment materials.

Keywords: 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] Aesthetic 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 adhered 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 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 two different implant abutment materials, titanium and zirconia, on bacterial adhesion. In addition, 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., 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, Sao Paulo, SP, Brazil).

The surface roughness of all of the discs was quantitatively analyzed using a portable roughness analyzer (Mitutoyosurftest SJ-401, Mitutoyo Corporation, Japão). To standardize the surface roughness measurements, we developed a device with two parallel slots to define the central area of the discs and a circular space to stabilize the samples during the readings. For each disc, two 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 three samples of each material and three randomized sites per sample (TopScan 3D), measuring an area of 2x2 μm and 10x10 μm (Fig. 1). A quantitative characterization of the nanoscale surface topography and roughness was conducted using atomic force microscopy (Agilent 5500 SPM/AFM - AC Mode III, Brazil). 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 three discs from each material by SEM (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 five discs of each material, and the measurement was repeated three times for each sample. The contact angle value determined for each wet agent was calculated using the Laplace-Young 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 the method described by Owen[21].

Bacterial conditions and qualitative assessment of bacterial adhesion

Porphyromonas gingivalis (*P. 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* (*S. 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 discs were transferred to a new 12-well plate and washed three 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, NY, USA), for 15 minutes, according to 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 four 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 (Fig. 2). The SFE was defined by the intersection between a straight line and the y-axis (Fig. 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 three different materials on bacterial adhesion was confirmed by confocal microscopy analysis. Higher magnification confocal imaging (40x) 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* (Fig. 4) and *P. gingivalis* (Fig. 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* adhered better on BE than on Ti or ZrO_2 surfaces. This preference in adhesion could be explained by the presence of intermolecular interactions between chemical groups on the cell walls of bacteria and polar and non-polar regions of BE surfaces. Different intermolecular forces can dictate how bacteria interact with different surfaces.[27] 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.[28] The surface proteins on *S. mutans* confer the charge to these Gram-positive bacteria. The wetting behavior of BE with liquids of different polarities revealed that this type of material has polar and non-polar characteristics, and consequently, it provides improved electrostatic interactions between the material and the bacteria.[29] 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).[30] Although the presence of LPS confers a negative net charge, which has been found to contribute hydrophilic characteristics,[31] the degree to which LPS plays a role in these hydrophilic features is largely dependent on the structural components, which vary between different species.[32,33] 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,[34,35] in the current study, a salivary pellicle was not used to more distinctively identify the effects of the chemical characteristics of the material surfaces on bacterial adhesion. The composition of the materials and their physicochemical properties may modulate initial bacterial adhesion[36] and, consequently, affect the microbial quality. However, our results cannot be generalized because we examined the impact of the material surfaces using two 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.

Acknowledgments

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Figures Legends

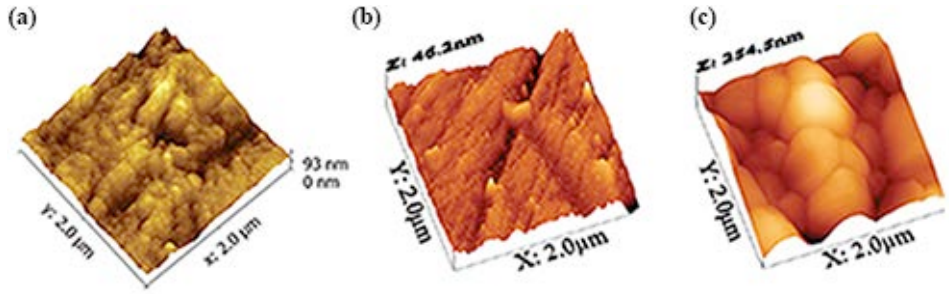

Figure 1. Micrographs taken by an AFM to describe the surfaces: **a.** BE; **b.** Ti, **c.** ZrO_2.

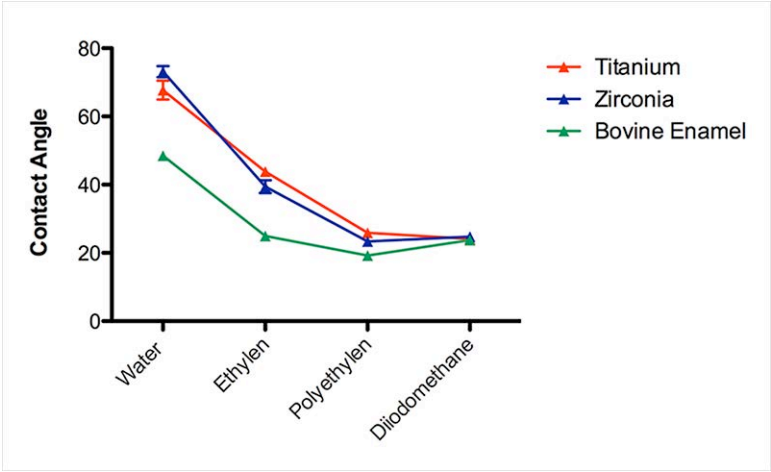

Figure 2. Effect of material surface on the wettability. In each material, four liquids were used: water, ethylene glycol, polyethylene glycol, and diiodomethane. (red) Ti, (blue) ZrO_2 and (green) BE.

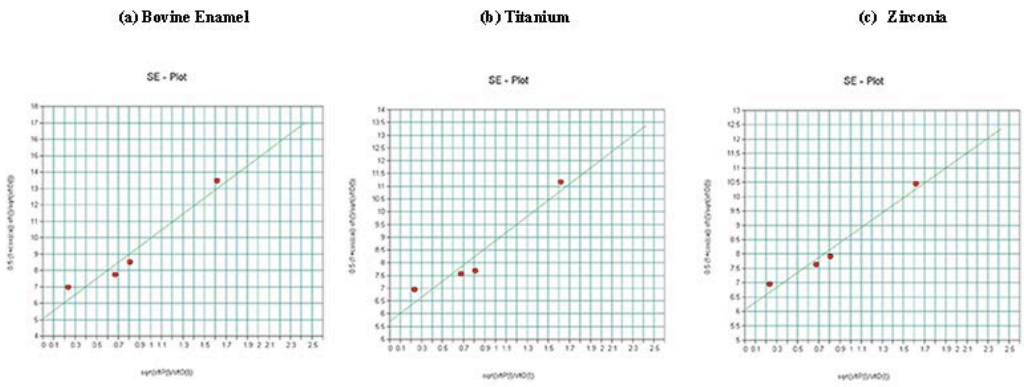

Figure 3. Energy-free surface (mN/m) analysis in surfaces: a. BE; b. Ti c. ZrO_2

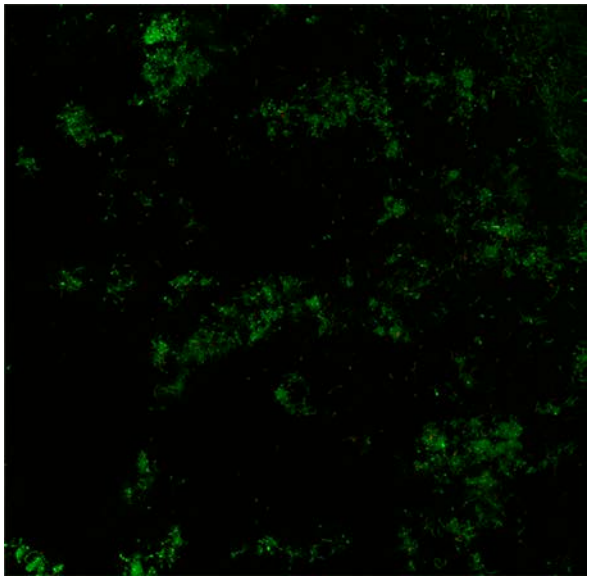

Figure 4. Biofilm of *S. mutans* UA159 were grown on different surfaces in TH medium, stained with LIVE/DEAD BacLight fluorescent dye and analyzed with CLSM. The figure shows cross-section images of biofilms after 16 hours developed on BE surfaces. Dead cells were stained red, and live cells were stained green.

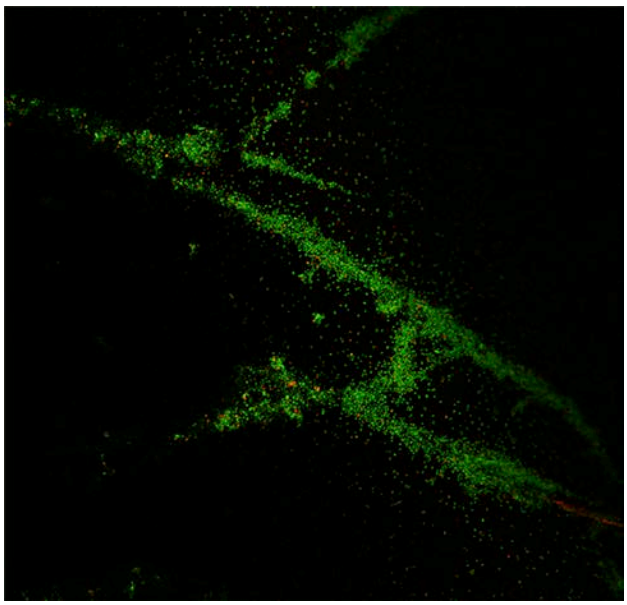

Figure 5. Biofilms of *P. gingivalis* 33277 were grown on different surfaces in BHI medium, stained with LIVE/DEAD BacLight fluorescent dye and analyzed with CLSM. The figure shows cross-section images of biofilms after 48 hours developed on BE surfaces. Dead cells were stained red, and live cells were stained green.

Table Legend

Table 1. Average value, surface roughness (Ra), peak to valley height (Maximum), median and average peak spacing (Rms) measured for the different surfaces: Ti, ZrO_2, 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

5 Capítulo 4

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THE JOURNAL OF PROSTHETIC DENTISTRY

RESEARCH AND EDUCATION

Structural and quantitative analysis of a mature anaerobic biofilm on different implant abutment surfaces

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ABSTRACT

Statement of problem. The longevity of dental implants depends on the absence of inflammation in the periimplant tissue. Similar to teeth, pathogenic bacteria can adhere on implant abutment surfaces and cause periimplant disease and consequently implant loss.

Purpose. The purpose of this in vitro study was to evaluate the influence of physical and chemical properties of 2 common materials used as implant abutments, titanium (Ti) and zirconia (ZrO_2), and the use of bovine enamel (BE) as a positive control on biofilm formation.

Material and methods. Biofilm formation was analyzed by growing *Porphyromonas gingivalis* and *Fusobacterium nucleatum* as monospecies and mixed species biofilms on the surfaces. The mean roughness (R_a) and surface free energy were evaluated for each material. Mature biofilm, formed after 7 days of incubation, was analyzed quantitatively and qualitatively by colony-forming unit and confocal laser scanning microscopy.

Results. The mean roughness in all disks was $\leq$0.21 μm and did not affect the bacterial adhesion. Titanium showed a greater degree of hydrophilicity compared with BE after 90 minutes of immersion in saliva. The surface free energy did not show differences, with the highest values for BE. Monospecies biofilms formed by *P. gingivalis* on Ti, and mixed species biofilm on ZrO_2 exhibited small numbers of cells on disk surfaces. By confocal imaging, the mixed species biofilm appeared as a thin layer on ZrO_2 surfaces.

Conclusions. Material surfaces could have a significant impact on biofilm formation. ZrO_2 implant abutment surfaces showed a decrease in anaerobic biofilm compared with Ti and BE. (J Prosthet Dent 2015;■:■-■)

Bacteria grow on both natural (tooth, mucosa) and artificial (restorative dental materials,[1] dental implants) surfaces as biofilms, which are highly organized microbial communities embedded in polymeric matrices. Although a number of other factors seem to be modifying disease expression,[2] the accumulation of biofilm on materials adjacent to the gingival tissue is a primary initiating factor for periodontal diseases.[3] When individuals lose their teeth because of periodontal disease, pathogenic bacteria remain inside the oral cavity. When dental implants replace a hopeless tooth, the microorganisms on the dental surfaces may colonize the implant abutment surfaces, similar to what happens on natural teeth, resulting in periimplant disease.[4] Periimplantitis is a lesion resulting from an inflammatory reaction induced by pathogenic bacteria around the implant surfaces.[5] Periimplant tissue inflammation is currently considered a major contributor to implant loss.[6,7] Periimplantitis is estimated to occur in approximately 17% of implants after 10 to 16 years of follow-up.[8]

The development of inflammation around oral implants is associated with the accumulation of specific bacterial biofilms. Even though more than 700 bacterial

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Structural and quantitative analysis of a mature anaerobic biofilm on different implants abutment surfaces

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ABSTRACT

Statement of problem. The longevity of dental implants depends on the absence of inflammation in the periimplant tissue. Similar to teeth, pathogenic bacteria can adhere on implant abutment surfaces and cause periimplant disease and, consequently, implant loss.

Purpose. The purpose of this in vitro study was to evaluate the influence of physical and chemical properties of 2 common materials used as implant abutments, titanium (Ti), and zirconia (ZrO_2), and the use of bovine enamel (BE) as a positive control on biofilm formation.

Material and methods. Biofilm formation was analyzed by growing *Porphyromonas gingivalis* and *Fusobacterium nucleatum* as monospecies and mixed species biofilms on the surfaces. The mean roughness (Ra) and surface free energy (SFE) were evaluated for each material. Mature biofilm, formed after 7 days of incubation, was analyzed quantitatively and qualitatively by colony forming unit (CFU/mL) and confocal laser scanning microscope (CLSM).

Results. The mean roughness in all disks was ≤0.21 μm and did not affect the bacterial adhesion. Titanium showed a greater degree of hydrophilicity compared with bovine enamel after 90 minutes of immersion in saliva. The SFE did not show differences, with the highest values for bovine enamel. Monospecies biofilms formed by *P. gingivalis* on Ti, and mixed species biofilm on ZrO_2 exhibited small numbers of cells on disk surfaces. By confocal imaging, the mixed species biofilm appeared as a thin layer on ZrO_2 surfaces.

Conclusion. Material surfaces could have a significant impact on biofilm formation. ZrO_2 implant abutment surfaces showed a decrease in anaerobic biofilm compared with Ti and BE.

CLINICAL IMPLICATIONS

Periimplant disease is mediated and modulated by the host, but pathogenic bacteria are responsible for starting the inflammatory process. The physical-chemical properties of the

implant abutment materials affect the biofilm formation. Ti and BE surfaces showed an increase of anaerobic biofilm compared with that of ZrO_2.

INTRODUCTION

Bacteria grow on natural (tooth, mucosa) and on artificial (restorative dental materials,[1] dental implants) surfaces as biofilms, highly organized microbial communities embedded in polymeric matrices. Although, a number of other factors seemed to be modifying disease expression,[2] the accumulation of biofilm on materials adjacent to the gingival tissue is a primary initiating factor for periodontal diseases.[3] When individuals lose their teeth because of periodontal disease, pathogenic bacteria remain inside the oral cavity. When dental implants replace a hopeless tooth, the microorganisms on the dental surfaces may colonize the implant abutment surfaces, similar to what happens on natural teeth, resulting in periimplant disease.[4] Periimplantitis is a lesion resulting from an inflammatory reaction induced by pathogenic bacteria around the implant surfaces.[5] Periimplant tissue inflammation is currently considered a major contributor to implant loss.[6,7] Periimplantitis is estimated to occur in approximately 17% of implants after 10 to 16 years of follow-up.[8]

The development of inflammation around oral implants is associated with the accumulation of specific bacterial biofilms. Even though more than 700 bacterial species can colonize the oral cavity,[9] only a few are implicated in the pathogenesis of periodontal disease.[10] *Porphyromonas gingivalis* is associated with chronic periodontitis and can be detected in up to 85% of the disease sites.[11] In contrast, healthy sites show low numbers of this microorganism. The presence of *P. gingivalis* in a periodontal pocket may predict imminent disease progression.[12] This organism expresses several virulence factors implicated in periodontal inflammation.[13] The pathogenic role of this bacterium in periodontal disease depends on its ability to bind to the host's cells, the acquired pellicle, and other bacteria.

Fusobacterium nucleatum is also commonly associated with periodontal disease and implant failure[14] and has been shown to exhibit co-aggregation with different bacteria, including *P. gingivalis,* which plays a central role in the development of the dental biofilm. Its opportunistic characteristic has been shown in diseased periodontal and periimplant sites because *F. nucleatum* serves as a bridge between the early and late colonizers.[15]

Prosthetic components, such as implants abutments, have an important effect on microbial adhesion, and the type of material can help increase or reduce the bacterial attachment and biofilm formation.[16-19] The most relevant surface properties influencing the bacterial attachment are roughness, wetting, and surface energy.[20-24] Overall, these physicochemical characteristics have been thought to change the implant and prosthetic component surfaces in an attempt to reduce the adherence of pathogenic microorganisms.[25-28] Titanium (Ti) is still considered the gold standard material because of its physical characteristics, including biocompatibility, stability, and corrosion resistance. The high demand for esthetic restorations, however, has favored the introduction of yttria-stabilized zirconia (ZrO_2) ceramic implant abutments. In vivo and in vitro studies of the biofilm formation on titanium and zirconia surfaces have been reported[29-34;] however, knowledge is still limited about possible differences in the bacterial adhesion mechanisms to metal compared with ceramic surfaces. Therefore, this study investigated the anaerobic biofilm formation on 2 implant abutment surfaces, titanium, and zirconia. The hypothesis was that the physical chemical composition inherent in each material interferes in the ability of microorganisms to adhere to the different tested substrates.

MATERIAL AND METHODS

Disks Preparation and Surface Analysis

Pure titanium and yttria-stabilized zirconia disks (Conexao Sistema de Protese Ltd) (8 mm in diameter and 2 mm in thickness) were used as the experimental groups, and bovine enamel disks were used as a positive control. Ti and ZrO_2 were prepared by machining, and enamel disks were cut from bovine incisors. The bovine enamel specimens were stored in 0.1% thymol solution at 4°C to inhibit the microbial activity until use. Enamel disks were obtained by using a 10-mm diamond drill for glass thread (Dinser Diamond Tools Ltd), coupled to the drill bench vise (Schulz; FSB model 16; chuck taper DIN 238-B18). The disk surfaces were prepared with abrasive paper of 800, 1200, and 4000 grit to polish the surface and reduce the roughness (grain 220; T469-SF-Noton; Saint-Gobam Abrasives Ltd).

The mean roughness (Ra) of all disks was quantitatively analyzed with a portable roughness tester (Mitutoyosurftest SJ-401; Mitutoyo Corp) with an accuracy of 0.01 mm, a reading length of 2.5 mm, an active tip speed of 0.5 mm/s, and a radius of 5 μm. To verify the reliability of the results, a device was used to stabilize and standardize the analysis. The reading at 2 different times and the intra-examiner reproducibility were assessed, calculating the intraclass correlation coefficient (ICC) with a confidence interval of 95%.

The disks were immersed in 800 μL of unstimulated human saliva for 90 minutes, and then washed once with 1 mL of sterile phosphate-buffered saline (PBS), dried at room temperature, and stored in a 24-well plate before the surface free energy (SFE) measurement with the Sessile-Drop method (Optical Contact Angle Measurements SCA-20 DataPhysics Instruments GmbH). To this end, 4 wet agents were selected from more to less polar solvents: water, ethylene glycol, polyethylene glycol, and diiodomethane. To assess the reproducibility of the experiment, 5 disks of each material were tested, and the test was repeated 3 times in each liquid. The average value of the contact angle was regarded as the mean value of the contact angle for each specimen, and software (SCA-SOFTWARE / OCA-20; DataPhysics

Instruments GmbH) was used for the SFE calculation using the concept of polar and dispersion components to the Owens, Wendt, Rabel, Kaelble (OWRK) method.[35]

The surface and elemental compositions were analyzed with a field emission environmental scanning electron microscope (SEM) (FEG-MEV; JEOL 7500F model) and energy-dispersive x-ray spectroscopy (EDS) and the data were examined by a specialist (M.J.J.). For this end, three disks of each group was used. The samples were positioned inside the SEM and 5 areas from each specimen were, randomly, chosen by a blind examiner. All disk surfaces were taken from their original package delivered directly from the supplier. Each zirconia disk was attached to an aluminum stub with adhesive conductive carbon tape. Images were made with both secondary and backscattered electrons. For EDS analysis, 7 kV accelerating voltage was used to improve peak/background ratio for light elements. Three disks of each group was used

After the disks were cleaned, the samples were randomly distributed according to the group and placed inside envelopes, i.e., each element in the population (total number of samples) had a known and equal probability of selection, to be sterilized overnight using gamma-irradiation at 25 kGy from an artificial cobalt 60 source in a nuclear reactor (ISO-11137: 2006) (Energy and Nuclear Research Institute - IPEN).

Unstimulated saliva samples were collected on the morning from 1 healthy donor adult aged 30 years, according to an approved protocol (Research Ethic Committee process number: 051/2012).[36,37] The donor had not taken any medication 3 months prior to the sample collection and did not display active periodontal disease.[37,38] The saliva was harvested on ice and clarified by centrifuging at 45 N for 15 minutes at 4°C. The supernatant was sterilized by filtration, membrane pore size 0.22 μm (Millipore), and stored at -20°C until use. The *P. gingivalis* ATCC 33277 and *F. nucleatum* ATCC 25586, used in this study, were grown on blood agar supplemented with hemin (10 mg/mL) and menadione (5 mg/mL) for 10 days in

an anaerobic chamber (atmosphere of 85% N_2, 10% H_2, and 5% CO_2). One colony from each bacterium was added to BHI broth supplemented with hemin and menadione and incubated for 48 hours in an anaerobic chamber. After growth, aliquots were transferred to another tube with BHI to supplement the growth curve determination.

To allow pellicle formation, the disks were placed into the well with saliva at room temperature for 90 minutes. The disks were washed once with PBS sterile, transferred to a new 24-well plate with culture medium in monospecies and mixed species and kept under anaerobic conditions for 24 hours. After the adhesion period, the disks were washed twice with PBS sterile, placed into a new 24-well plate with BHI medium, supplemented, and kept for 7 days under anaerobic conditions. During this time, the medium was changed every 48 hours. Then, the disks were removed from the plate, washed twice with PBS, and analyzed by Colony-Forming Unit CFU/mL and confocal.

In sequence, the disks were washed twice, and then placed in a tube with 2.5 mL of PBS to detach the biofilm using ultrasound for 20 minutes (Ultrasonic; 1440 Plus). Serial dilutions were performed, and 25 μL of each dilution was plated in duplicate on blood agar supplemented to verify the bacterial growth. The plates were kept under anaerobic conditions for 15 days. The CFU/mL was determined, and its reproducibility was evaluated by photography. Images were analyzed 2 different times at a 2-week interval. The experiments were performed in quadruplicate with 3 repetitions, giving 12 samples.

Confocal Laser Scanning Microscope and Image J analysis

Before the CLSM analysis, the disks were sequentially rinsed twice with 1 mL of sterile PBS to remove the nonadherent bacteria. To visualize the biofilm and viable cells and to assess their thickness, cell populations were stained for 15 minutes using 800 μL Live/Dead BacLight Bacterial Viability Kit (Invitrogen Corporation) for each specimen. The

final concentrations were Syto-9 = 0.01 mM and propidium iodide (PI) = 0.06 mM. In each experiment, exciting laser intensity, background level, contrast, and electronic zoom size were maintained at the same level. Three specimens of each group were used for this end. The disks were placed on a glass cover slip, with the surface to be analyzed in contact with the glass and 3 areas from each disk were, randomly, chosen by a blind examiner. A series of optical cross-sectional images was acquired at 1-μm depth intervals from the surface through the vertical axis of the specimen by using a computer-controlled motor drive. The confocal images were then exported to freeware (imageJ 1.48; National Institutes of Health [NIH]; http://imagej.nih.gov/ij/download.html), and the density of the biomass was measured by the intensity of the pixels. First, the background was subtracted to remove the noise and split the color channel images. Subsequently, the number of live bacterial cells (green color) was estimated by counting fluorescence-specific pixels in digital fluorescent images.

Data Analyses

Raw data were analyzed with software (Graph Pad Prism; GraphPad Software Inc). Descriptive statistics were used to calculate the mean and its standard deviation (SD). Data among groups were analyzed using 1-way analysis of variance (ANOVA), followed by the post hoc Tukey's HSD, for multiple comparisons (α=0.05).

RESULTS

Disk roughness was assessed by dispersion graphic to display the main pattern in the distribution of the data. Data beyond the line were excluded and the standard roughness values were included. The effect of surface roughness variables on biofilm formation was as follows: for Ti disks, Ra= 0.21 μm ±0.06; for ZrO_2 disks, Ra= 0.22 μm ±0.03; and for bovine

enamel, for which the readings were taken in perpendicular directions where the disk roughness was included as a positive control, the values ranged from 0.05 to 0.1 μm.

Contact angles were measured to evaluate the wettability of the different surfaces with a saliva coat. The contact angle for the sessile water drops on all the surfaces was less than 50 degrees, and, therefore, all of them can be considered as hydrophilic materials.[39] In contrast, the Ti surface presented more lyophobic characteristics than others because of the higher contact angle formed between the surface and diiodomethane, a nonpolar liquid (Fig. 1). Based on the results of the OWRK method, all material surfaces presented similar SFE values as determined by the intersection of the line with the y-axis, with BE and ZrO_2 presenting the highest numbers 3.1 and 3.2 mJ m^{-2}.

SEM (×500) revealed a homogenous surface, roughened for Ti and smoothed for ZrO_2. Ti surfaces showed a circular configuration of alternating plane, flattened, and rough surface areas (Fig. 2A), whereas for ZrO_2, a spherical shape was observed in granules (Fig. 2B). In contrast, for bovine enamel, a higher magnification showed smooth surfaces and some cracks, a common characteristic in enamel (Fig. 2C).[40] EDS identified the chemical elements in each material surface. Oxygen and titanium were found in Ti, and oxygen with zirconium elements was present in ZrO_2,. Bovine enamel showed calcium, phosphorus, and oxygen in its composition.

After 7 days of incubation, an analysis of variance for bacteria species showed a significant interaction between the material and the method of biofilm development as well as logarithms of CFU/mL ($P \leq .001$). There was a wide increase in the CFU/mL of *F. nucleatum* monospecies bacteria biofilm on all surfaces in comparison with *P. gingivalis* (Figure 3A,B), but the materials did not affect the numbers of *F. nucleatum* ($P < .05$). The effect of material on monospecies bacterial biofilms showed high numbers of *P. gingivalis* on ZrO_2 surfaces and a significantly lower number on BE. Then, the ability of mixed species bacteria to develop

biofilm on the experimental and control groups was tested. The highest number of colonies was found on bovine enamel, and this number was always higher for *F. nucleatum* than *P. gingivalis* (Fig. 3C).

The viability of the bacteria grown on the material surfaces was similar. The differences in bacterial distribution on the tested substrates were confirmed by CLSM. Monospecies and mixed species biofilms with *P. gingivalis* on Ti and ZrO_2 showed less colony spreading for all surfaces (Fig. 4A,B), but an intense biofilm on bovine enamel was observed (Fig. 4C). However, no statistically significant difference in CFU/mL for the *F. nucleatum* biofilm was observed; for ZrO_2, the cell numbers decreased after 7 days. High-resolution observations (×40) of *F. nucleatum* revealed relatively large bacterial colonies and high intensity, or high biovolume, on Ti disks (Fig. 5A) in contrast to ZrO_2 and bovine enamel, which had small and sparse bacterial colonies (Fig. 5B,C). Similarly, depth biofilm analysis showed that *F. nucleatum* was able to form more confluent and profound biofilms on Ti surfaces than on the other tested surfaces. In relation to mixed species bacteria biofilms, ZrO_2 showed little biofilm, with a 12-µm depth on disks and small-scattered cell clusters with large voids in comparison with Ti (Fig. 6A,B); the biofilm formed on the bovine enamel surface was notably deeper, up to 28-µm depth (Fig. 6C).

To investigate the effect of each material on the density of the biofilm, the confocal images were scanned and expressed as the bacterial number in terms of integrated intensity of pixels, with a mean and standard deviation. The data were consistent with CLSM images. The density of *F. nucleatum* biofilm on Ti surfaces was 3.2- fold higher than on bovine enamel (Fig. 7A). In contrast, ZrO_2 showed the best results, with the lowest density values of monospecies and mixed species bacteria biofilm represented by the number of pixels. The density of *P. gingivalis* as monospecies biofilm on ZrO_2 material was 3.0-fold lower than on BE and 3.4-fold lower than on Ti (Figure 7B). Mixed species biofilm formed on BE showed

biomass 1.2-fold higher than on Ti and 5.5-fold higher than on ZrO_2 (Figure 7C).

DISCUSSION

The results of this research suggest accepting the research hypothesis that the different material surfaces affect the quality and quantity of a mature anaerobic biofilm. The surface roughness of the material surfaces seems to play a crucial role in bacteria adhesion and biofilm formation.[22,27] The variable roughness was eliminated to maintain the homogeneity of the groups at an Ra of about 0.2 μm, while evaluating the real effect of each material.[28] Surface contact angle is also an important factor for the adhesion and growth of bacteria on different material surfaces. And, since implant abutment surfaces are covered with an acquired pellicle, in vivo, the disks were immersed in sterilized saliva prior to biofilm development to simulate clinical conditions. The data showed that although all the material surfaces were hydrophilic as determined by the contact angles for sessile water drops lower than 50 degrees,[39] Ti presented the highest values. Furthermore, the contact angle data between the materials surfaces and diiodomethane revealed a higher lipophilic characteristic to bovine enamel. Bacteria express a wide diversity of complex molecules, such as lipopolysaccharide (LPS) in the case of gram-negative bacteria, which gives the cell surface a net anionic charge (negative). The degree to which these structures influence the hydrophilic or hydrophobic characteristics is dependent on the structural components.[18] Surfaces with polar and nonpolar properties, such as hydroxyapatite from enamel surfaces, can become electrostatically more attracted and bound to the different bacteria species.[19] This chemical concept can explain why the mixed species bacterial biofilm exhibited the highest value for biomass of biofilm formed on enamel. Regarding the SFE, no difference among the materials was observed. A possible explanation is that the saliva coating formed onto the disks altered the surface energy of all the surfaces analyzed. The SFE of a material surface is reported to

affect the initial adherence and biofilm formation of microorganisms, suggesting that those with low SFE would attract fewer bacteria than others with higher SFE.[23] The high SFE can be interpreted as a high number of active ions on surfaces, which would increase the attraction forces for liquid (or bacteria) with the same chemical composition. However, no correlation was observed between SFE and biofilm formation, indicating that other properties from material surfaces such as surface chemical composition and the wettability of materials combined with bacterial strains and number of species used could be involved in this outcome.

Both *F. nucleatum* and *P. gingivalis* displayed intraspecies variability with regard to biofilm formation. The developed biofilm on the implant abutment surfaces, both Ti and ZrO_2, were different from that formed on a typical tooth surface. In monospecies *P. gingivalis* biofilm, ZrO_2 showed higher cell numbers than on other materials, and *F. nucleatum* did not show any differences in adhered cell numbers. These results were not consistent with CLSM, which could be considered a limitation of this in vitro study. A possible explanation is that monospecies bacterial biofilm on ZrO_2 or Ti surfaces would be significantly thinner than on bovine enamel and that the cells could be detached from the disks during the ultrasound procedure. However, in the case of mixed species biofilm, their structure (by CLSM) and log CFU/mL were significantly different when comparing bovine enamel with both implant abutment material surfaces, demonstrating thicker biofilms with higher bacterial numbers after reaching the mature state (after 7 days). Some studies have also reported low but significant numbers of bacteria adhered to ZrO_2 than to Ti surfaces.[34] *Streptococcus mutans* was found in higher numbers on ceramic than other bacteria species such as *Streptococcus sanguis*, which seemed to have more affinity for Ti materials, but the reason for this is not yet known. In another recent study, different data were observed; ZrO_2 attracted more *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus salivarius*, and *Staphylococcus*

aureus biofilm than comparable variants of Ti or Ti alloys, across the hydrophilicity or hydrophobicity characteristics.[33] Likely, the chemical elements from wall bacteria cells determine the type and quality of the interaction between microorganism and material surface.[24] All those investigations were short-term evaluations (≤ 24 hours) and study only early bacterial adhesion. In the present study mature colonizers differed significantly between the monospecies and mixed species biofilms on Ti and ZrO_2 materials. The time point corresponded to the formation of a barrier epithelium beginning at 1 to 2 weeks and completed at 6 to 8 weeks of healing.[4] This is the most important period for bacterial adherence and the development of biofilm on material surfaces because of the absence of a soft tissue protector.

CONCLUSION

The results indicated that in the case of mixed species bacterial biofilm, the number of cells and the density of the biofilm on ZrO_2 were lower than on Ti materials. Physical chemical characteristics from different materials affect the pathogenic bacteria adhesion and their growth. Further studies using an oral microbial community may be able to show exactly what happens when a complex and natural microbiota combines with different implant abutment materials.

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LEGENDS

Figure 1. Effect of material surface on contact angle for different liquids: water, ethylene glycol, polyethylene glycol, and diiodomethane (red) Ti, (blue) ZrO_2, and (black) BE.

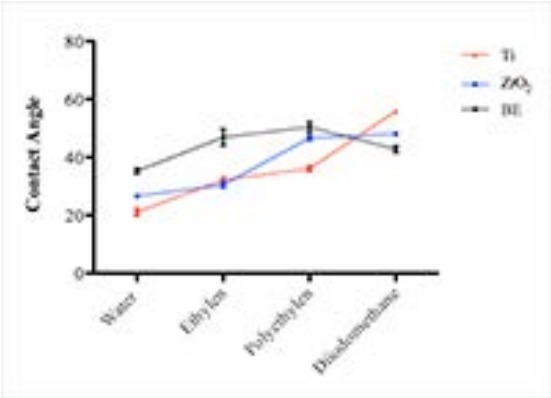
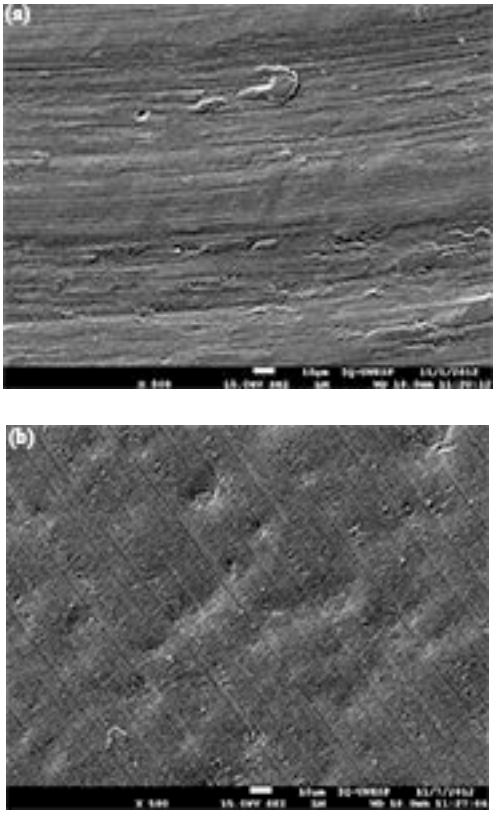


Figure 2. Scanning electron micrographs (×500) of each material surface. A, Ti. B, ZrO_2. C, Bovine enamel. The areas of interest were selected by setting each specimen at horizontal and vertical planes.



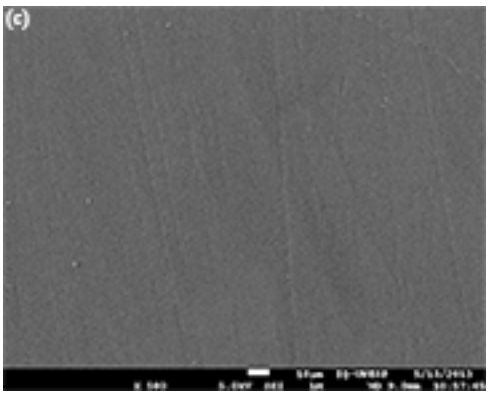
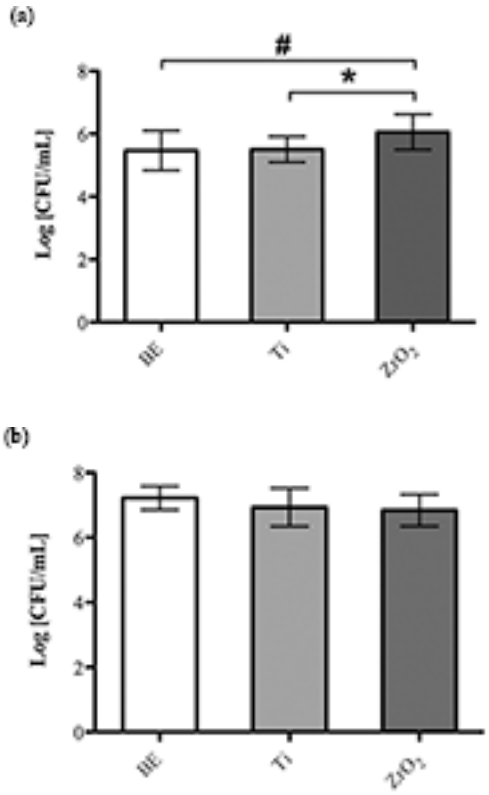


Figure 3. A, Mono *P. gingivalis*. B, Mono *F. nucleatum*. C, Mixed species bacterial biofilm formed on Ti and ZrO_2 implant abutment surfaces and on bovine enamel after 7 days incubation (log CFU/mL). Data are shown as mean ±SD (n=12), *# indicating statistically significant differences among materials, *P<.05.*



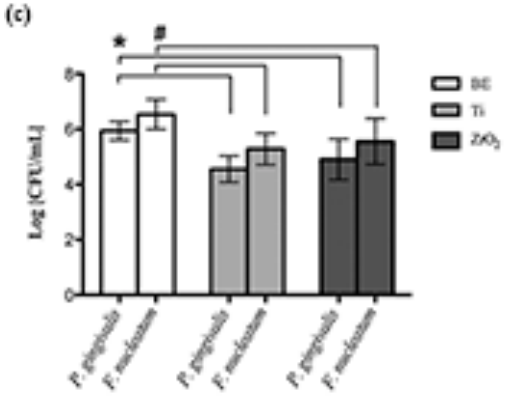


Figure 4. Cross-sectional images of *P. gingivalis* biofilms that developed after 7 days of incubation. A, Ti. B, ZrO_2. C, Bovine enamel. Dead cells stained red, live cells stained green.

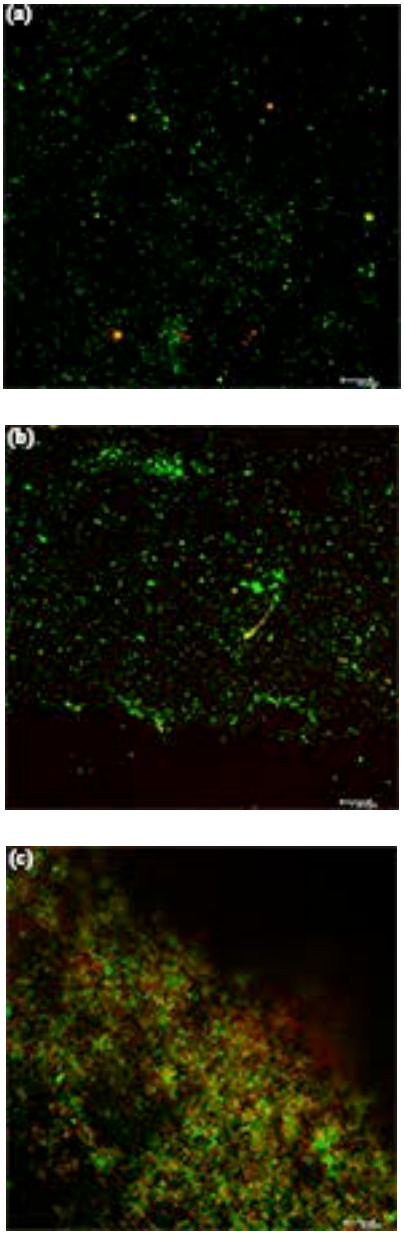


Figure 5. Cross-sectional images of *F. nucleatum* biofilms after 7 days of incubation. A, Ti. B, ZrO_2. C, Bovine enamel. Dead cells stained red, live cells stained green.

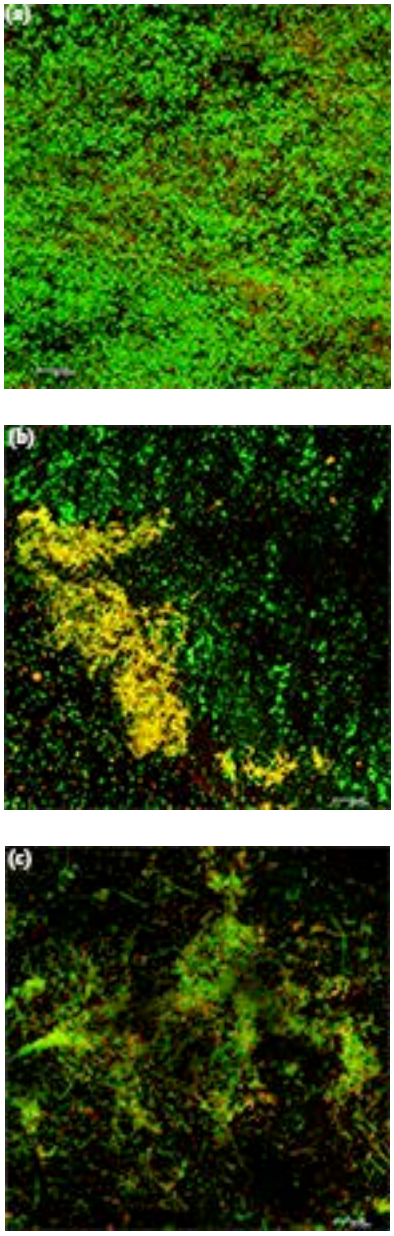


Figure 6. Cross-sectional images of mixed species *P. gingivalis* and *F. nucleatum* biofilms after 7 days of incubation. A, Ti. B, ZrO_2. C, Bovine enamel. Dead cells stained red, live cells stained green.

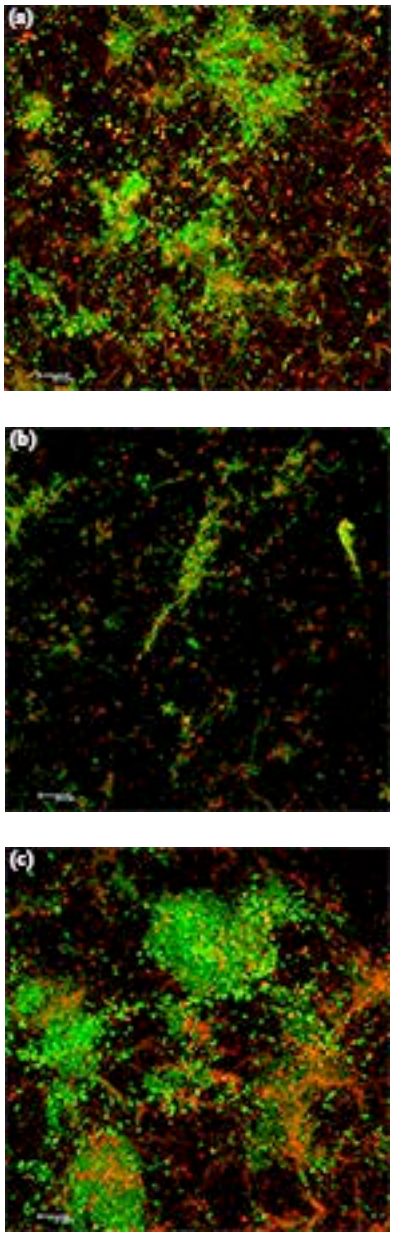
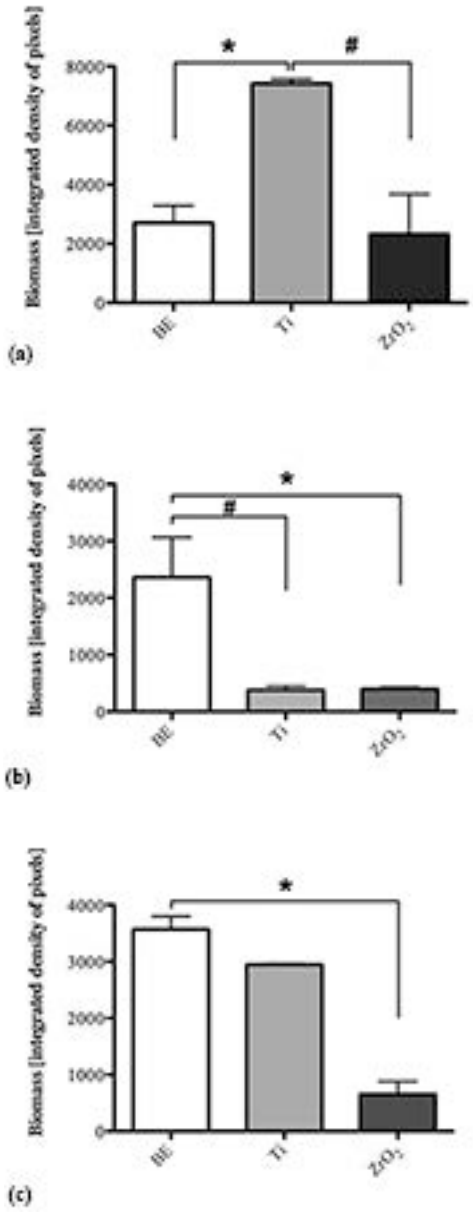


Figure 7. Quantitative analysis of biomass of monospecies and mixed biofilm for substrate. The integrated density of (a) *F. nucleatum* monospecies bacterial biofilm was significantly higher on Ti disks, *P=0.016*; (b) in case of *P. gingivalis* monospecies biofilms, integrated density was significantly higher on BE disks, *P=0.02*; (c) for mixed species biofilms, a

significantly reduced integrated density was observed on ZrO_2 disks, *p=0.0012*. Data are shown as mean ±SD (n=3), *P<0.05*, indicating a statistically significant difference among disk materials.



6 Capítulo 5

Artigo em revisão
"*Materials Science and Engineering: C*"

Analysis of a complex polymicrobial community on implants abutment surfaces

Biofilm and dental implant surfaces

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Abstract

An important factor contributing to the development of inflammation is the adherence of microorganisms on the implant abutment surfaces. Recommendation of prosthetic abutment components has been a critical part of implants treatment. This study addresses the effect of two different commercially materials on the adhesion phase and biofilm formation using a human polymicrobial oral communities. The physical chemical surface properties were investigated by different approaches. Initial adherent bacteria and biofilm formation were evaluated after 16 and 48 hours, respectively, by incubating of the discs in rich medium containing a representative saliva-derived oral microbial community. Scanning electron microscopy (SEM) and rugosimeter revealed quantitative and qualitative similarities between titanium (Ti) and zirconia (ZrO_2), with smooth surfaces. Ti presented lower hydrophobicity and surface free energy values than the ZrO_2. The amount of bacteria adhered on Ti disclosed a significant difference with 6.1-fold less bacteria. After 48 hours, detailed quantitative analysis showed that biofilm biomass and the biofilm density were lower on the Ti discs than to ZrO_2. Denaturing Gradient Gel Electrophoresis analyses demonstrated that, the quantity of phylotypes on the Ti and ZrO_2 surfaces was relatively similar during the attachment and early biofilm formation period. Although, no difference on bacteria profile was observed between both materials, independent on the time point, the highest level of colonization was on ZrO_2. Since aesthetic implications in oral rehabilitation are considered to be an indicator of success, the information contained in this work could offer a starting point to clinical studies with periodontally compromised patients.

Keywords. Peri-implantitis; Abutments; Titanium; Biofilm; Dental implants.

1. Introduction

In the last decades, dental implant rehabilitation has become an efficient method of treatment for patients with missing teeth.[1, 2] With the advance of the implant therapy, the number of complications that arise over time is on the increase at clinics. Included in the later problems, are the biologic complications of peri-implantitis, an inflammatory condition affecting the soft and hard tissues that lead to dental implant loss. Some studies have shown the prevalence of implant failure due to infection on peri-implant tissue[3-5] and the data are alarming. It is estimated that peri-implantitis occurs in about 17% of implants installed after 10 to 16 years of follow-up.[6] In a recent review, Mombelli et al. revealed that the prevalence of peri-implantitis seems to be in the order of 20% patients during 5–10 years after implantation.[7]

Although peri-implant disease is mediated and modulated by the host, bacteria are responsible to initiate the inflammatory process. It has been assumed that the same pattern of colonization that occurs in periodontal disease may occur around the subgingival surface of dental implants.[8-11] The reason for this assumption is a similar pathological bacterial flora, which forms around diseased teeth and diseased implants, though with some differences in partially and completely edentulous patients.[12] This is important information and has direct clinical implications, especially in choosing of implant surface materials. Surface attachment is the first step to biofilm development and this is an important precursor to adaptive behavior of bacteria species, becoming the base for colonization and invasion pathogens. The hydrophobic attractive forces and electrostatic charge interactions between cells and materials surfaces are considered as the key-properties of dental implant surfaces for biofilm formation.[13, 14] Furthermore, the contact between cells and surfaces promote morphological changes that facilitate cooperative behavior, rapid community growth, and migration of communities.[15, 16] So far, there is not enough evidence to make definitive conclusions on the clinical implications, but it has been known that the chemical composition

and surface characteristics of the different substrates used for abutment components may directly affect microorganism adhesion and oral biofilm maturity and, consequently, facilitate or hinder the colonization and growth of microbial species found in the oral cavity.[14] The abutment components seems to be of decisive importance for the biofilm formation due to its supra and sub gingival localization. Thus, it is desirable that materials employed for the fabrication of implant abutments inhibit bacteria colonization on their surface. An important question may rise: are there quantitatively and qualitatively differences of biofilm formed on materials applied for the fabrication of implant abutments? Titanium is the most routinely used material for implant abutments, due to its excellent biological and mechanical properties.[17] In recent years, zirconia (ZrO_2) has become a favorite material for implant abutments in the anterior maxillary region and as copings for crowns and bridges, mainly because of its presumed favorable light dynamics[18, 19] and aesthetics.[20, 21] The popularity of this material somewhat worrying considering the fact that data documenting the performance of ZrO_2 abutments in relation to biofilm formation are insufficient. While several studies have demonstrated that differences in biofilm formation occur on titanium and ceramic materials,[22-26] others observed no effect of both materials surfaces on biofilm formation.[27-29]

In order to fill in the knowledge gaps, we proposed evaluate the effect of two different commercially materials on the adhesion phase and biofilm formation using a human polymicrobial oral communities. Here, we tested the hypothesis that the different material surfaces interfere on the quality and quantity of bacteria adhered and biofilm formed onto them as well as the bacteria profile.

2. Material and Methods

2.1 Materials, sample preparation and surface analysis

Machined pure titanium (Grade 2) and yttrium stabilized zirconia discs (8 mm in diameter and 2 mm in thickness) (Conexão Sistemas de Próteses Ltda., SP, Brasil) were used in this study. In order to investigate the effect of each material surface on bacterial attachment and biofilm formation, we selected only the samples with average roughness (Ra) lower than 0.30 μm. Surface roughness was determined on the surface of all of discs using a portable roughness analyzer (Mitutoyosurftest SJ-401, Mitutoyo Corporation, Japão) with an accuracy reading of 0.01 mm, reading length of 2.5 mm, an active tip speed of 0.5 mm/s and a radius of 5 μm. For each material, two measurements of each side were carried out and mean values were calculated.

To confirm the uniformity of the samples referring to the same group, the morphology of the implant abutments surfaces was examined using a scanning electron microscopy (SEM) (FEG-MEV; JEOL 7500F model). The specimens were sputter-coated with carbon, directly mounted on aluminum stubs and SEM images were obtained in high resolution with the microscopy working between 2-15 KV. Here, three discs of each material and five randomized areas were analyzed.

The contact angles for liquid drops (aqueous phase with varying pH, water, ethylene glycol, polyethylene glycol and diiodomethane) were measured with a goniometer using the sessile-drop technique. Each wet agent was dropped on the center area of each disc and the contact angle between the surface and the liquid, was calculated by Laplace-Young equation. We used five discs of each material and repeated the procedure three times to assess the reproducibility of the experiment. Then, the average referring to each surface and each wet agent was inserted in the specialized drop-shape analysis software and the surface free energy (SFE) was calculated using the concept of polar and dispersion components to the method of Owen.[30]

Prior to use, each disc was cleaned with acetone to remove the organic material,

followed by 15 minutes rinse with ultrapure water in ultrasonic baths. Then, the discs were sterilized by gamma irradiation at 25 kGy from an artificial cobalt 60 source in a nuclear reactor (ISO-11137: 2006)

2.2 Oral microbial community and culture conditions

To simulate the clinical conditions and increase the relevance of this *in vitro* study, we worked with a cultivable microbial saliva community representative of the complex oral microbiome as model system for biofilm formation.[31, 32] Additionally, a rich new culture medium, named SHI medium, was developed to support a diversified bacterial from oral microbiota.[32]

Biofilm were grown on the microbial community anaerobically (85% N_2, 10% H_2, and 5% CO_2) 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[32], 25% filtered saliva (filters pore size 0.22 µm), 0.5% mannose, 0.5% sucrose). Adhesion phase and biofilm formation, after 16 and 48 hours incubation, respectively, were evaluated at 37°C under anaerobic conditions. For both time points, the optical density (OD) was adjusted to 0.1 in fresh medium to decrease the bacterial concentration. Eight hundred µL of the oral microbial community were placed onto discs in a sterilized 24-well polystyrene culture plate (Fisher Scientific). The oral microbial suspension used in this study was inoculated directly into the polystyrene plate to serve as positive controls for adhesion phase and biofilm formation. Additionally, sterile medium with and without Ti and ZrO_2 discs were incubated with to serve as background controls.

2.3 Colony-Forming Units/mL (CFU/mL)

After 16 and 48 hours incubation, the discs were transferred to a new 12-well polystyrene culture plate and washed three times with 1 mL phosphate-buffered saline (PBS) sterilized. Then, we transferred the discs to a new 24-well plate with 1 mL of SHI medium and scraped the surface with a pipette tip to detach the biofilm. One mL of culture was transferred to a 1.5 sterilized eppendorf tube (tube 1). Subsequently, the 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 procedure was repeated until tube 8, following the serial dilution. Twenty-five µL of each tube was spread out on a fresh SHI medium agar and the plates kept at 37°C under anaerobic conditions. The colonies were counted after 3 days. Plates containing only culture medium were also incubated and checked for sterility.

All experiments were performed in triplicate for each time point and repeated in three independent experiments to ensure reproducibility.

2.4 Crystal violet assay

To investigate the biomass of bacteria adhered and biofilm formed, after 16 and 48 hours incubation, the discs were transferred to a new 12-well plate and washed three times with 1 mL of PBS sterilized to remove the unattached bacteria. In sequence, we transferred the discs to a new 24-well plate and added 800 µl of 0.5% crystal violet solution to determine biomass accumulation onto the Ti and ZrO_2 disc surfaces. Similar procedure was conducted for control wells (no discs). The 24-well plate was incubated at room temperature for 20 minutes and then, the discs were transferred to a 12-well plate and washed four times with 5 mL PBS to remove excess crystal violet. The plates were gently shaken for 5 minutes during the last 2 PBS washes to ensure complete removal of residual dye. After the final PBS wash, the discs were transferred to a new 24-well plate and incubated at room temperature on a rotatory shaker at 250 r.p.m for 15 minutes with 800 µL of 95% ethanol. Eighty hundred µL of ethanol

solution containing the crystal violet stain retained by the biofilms was transferred into 1.5 mL cuvettes (USA Scientific**)** and the optical density at 595 nm was determined for total biomass evaluation.

All experiments were performed in triplicate for each time point and repeated in three independent experiments to ensure reproducibility.

2.5 Sample preparation, confocal laser scanning microscopy and imageJ analysis

In order to evaluate the viability, the density and thickness of the complex oral biofilm formed on Ti and ZrO_2 disc surfaces, after 16 and 48 hours incubation, the discs were rinsed three times with PBS to remove unattached bacteria and labeled with the LIVE/DEAD BacLightTM Bacterial Viability staining kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. For this end, the discs were placed onto a glass cover slip (Fisher Scientific) into a 10 μL drop of PBS with the biofilm side down. Imaging was performed using confocal laser scanning micros- copy LSM 510 (Version 4.2, Carl Zeiss MicroImaging Co., Ltd., Jena, Germany) with a laser wavelength of 488 nm and filter 505-530, and samples were imaged through a 10x dry (Plan NeoFluar NA 0.3 air) and 40x oil-immersion (Plan NeoFluar NA 1.3 oil) objectives. The scanning module of the system was mounted on an inverted microscope (Axiovert 200 M) and the biofilm images of each sample were acquired from three random positions. Biofilms were carefully screened to determine if biofilms had similar thickness throughout the whole disc area. Z-series were generated by vertical optical sectioning with the slice thickness at 1 μm. Confocal images, from the each disc were exported to imageJ 1.48 for macintosh (Version 10.2) [National Institutes of Health (NIH), Bethesda, MD, USA; freeware from http://imagej.nih.gov/ij/download.html] program and converted into a RGB color. The background was subtracted to ensure maximum removal of background noise and then, the image was assembled into color channels. The area occupied

and the density of biomass, by live bacteria within each section, was calculated by integrated density of pixels.

All experiments were performed in duplicate for each time point with two repetitions.

2.6 DNA extraction, PCR and DGGE

To detach the biofilm, each surface was scraped with a sterile pipette tip in a well containing 150 μL of PBS and the same volume placed into 1.5 mL Eppendorf Tube. The total genomic DNA was isolated by MasterPure^{TM} DNA purification kit (EPICENTRE) and the concentration of bacterial DNA determined with a Nanodrop 2000 (Thermo Scientific). For amplification, the following universal bacterial primers were used to amplify approximately 300-base-pair in length (bp): Bac1 with a GC clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAC TAC GTG CCA GCA GCC-3') and Bac2 (5'-GGA CTA CCA GGG TAT CTA ATC C-3') [33]. The PCR mixture (50 μL) contains 100 ng of DNA, 40 ρmol of each primer, 200 μM of dNTP, 4.0 mM $MgCl_2$, 5 μl 10x PCR buffer and 2.5 U *Taq* DNA polymerase (Invitrogen). The resulting PCR products were confirmed by electrophoresis in a 1.0% agarose gel. DGGE of PCR products was performed using the Bio-Rad DCode System (Bio-Rad Laboratories, Inc., Hercules, CA) at 60V and 58°C for 17 hours. Polyacrylamide gels at an 8% concentration prepared were prepared with a denaturing urea/formamide gradient between 40% and 60%. The gel was submerged in 1X TAE buffer (40 mM Tris base, 40 mM glacial acetic acid, 1 mM ethylenediaminetetraacetic acid) and, approximately 45 μL of the PCR product were loaded into each well. After electrophoresis, gels were stained with ethidium bromide to visualize the bands on the gel. Gel images were taken with the Molecular Imager Gel Documentation system (Bio-Rad Laboratories).

All experiments was performed in duplicate and repeated three times for each time point. Overnight oral saliva community culture (S) was useful as a positive control.

2.7 DNA sequencing of excised DGGE bands

Some bands of interest were excised from the DGGE gel with a sterile razor blade, put into 1.5-mL tubes containing 15 μL of sterile Milli-Q water, and macerated with a sterilized pipet. The tubes were incubated overnight at 4°C. A volume of five μL of the DNA sample was used as a template and re-amplified. Re-amplification was performed with universal primers (Bac1 and Bac2 described above) and the product was sending for sequencing using the universal primer. The 16S DNA sequences were aligned with the GenBank sequences using BLAST program in Human Oral Microbiome Database (HOMD) to identify the phenotypic from oral bacterial.

2.8 Statistical data analysis

All data are presented as mean ± standard deviation (SD), except for qualitative analysis by confocal microscopy. Statistical comparisons were performed using the unpaired *t*-test, two tailed, using GraphPad Prism version 5.0c. $*p<0.05$ indicating a statistically significant difference between Ti and ZrO_2 discs.

3. Results

3.1 Surface topography and morphology of Ti and ZrO_2 discs

For Ti discs used in this study, the roughness average (Ra) was 0.21 μm (±0.06) and for ZrO_2, 0.22 μm (±0.03). To complement the roughness analysis, we examined the morphology of Ti and ZrO_2 materials at high magnifications, 1.000 X. SEM revealed a rough homogeneous surface for Ti and a smooth surface for ZrO_2. Ti surfaces showed a circular configuration of alternating plane, flattened, and rough surface areas (Figure 1a), whereas for ZrO_2, a spherical shape characteristic was observed in granules (Figure 1b).

3.2 Ti and ZrO_2 - hydrophobic materials surfaces

In order to analyze the physicochemical characteristics of Ti and ZrO_2 surfaces, we determined the SFE by contact angle formed between different wet agents and the materials. According to the Table 1, although both materials were hydrophobic with contact angles higher than 50 degree, there was significant difference between them, with ZrO_2 presenting less wetting with water. On the other hand, there was no significant difference between the contact angle between diiodomethane and materials, revealing a similar non-polar characteristic of Ti and ZrO_2. The variability of the data collected for contact angle of each liquid to each material was evaluated by a descriptive analysis. We assumed the low standard deviation (SD) values and the finals average were inserted in 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).

3.3 Ti accumulate fewer bacteria than ZrO_2 material surfaces in attachment phase

Next, we examined whether the surface property described above had any influence on the ability of a complex oral community to adhere and develop biofilms on two abutment surfaces. The quantity and quality of bacteria adhered onto Ti and ZrO_2 materials were evaluated after 16 hours incubation using a variety of different approaches. CFU/mL disclosed a statistically significant difference in overall bacteria adhered with 6.1-fold more colonies evident on the ZrO_2 than Ti surfaces (Figure 2). The qualitatively assessment of the attached biomass via confocal microscopy confirmed that the Ti discs accumulated considerably less bacteria compared to their counterparts (Figure 3a,b). However, at a 40x magnification no evident difference in density of coverage could be observed between the two surfaces (Figure 3c,d). Further, the biofilm viability of both materials was similar, as revealed by fluorescent LIVE/DEAD stain. Detailed quantitative analysis of the confocal images

determined that the density of adherent bacteria was 1.6 higher on the ZrO_2 than on Ti discs (Figure 3e). Consistent to confocal analysis, crystal violet showed substantially higher biomass on ZrO_2 samples (2.7-fold more) than on Ti (Figure 4).

3.4 Ti accumulate fewer bacteria than ZrO_2 material surfaces in biofilm phase

Since the attached phase showed less salivary bacteria adhered to Ti discs, we evaluated if this outcome was persistent during the subsequent biofilm formation and maturation process. CFU/mL also disclosed a statistically difference in overall biofilm formed after 48 hours with 1.6-fold more bacteria adhered on ZrO_2 than on Ti surfaces (Figure 5). The difference in biofilm formation between Ti and ZrO_2 discs was confirmed by confocal microscopy analysis (Figure 6a,b). The observation under the confocal laser microscopy showed that the biofilm thickness on ZrO_2 was deeper than that of Ti group (Figure 6c,d). From the qualitative analyses from the confocal images, we found that the average biofilm density on ZrO_2 surfaces was 1.6-folds higher than that of Ti (Figure 6e). Crystal violet was coherent with previous analysis, and after 48 hours incubation the biomass of biofilm on ZrO_2 was 2.2-fold more than Ti discs (Figure 7).

3.5 Similarity of biofilm bacterial community profile on Ti and ZrO_2 surfaces

Our next goal was evaluate 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 attachment and early biofilm formation period (Figure 8). However, we observed difference on loosely adherent biofilm bacteria and strongly adherent biofilm bacteria for both time points. The intensity of bands 2 and 3 were highest during adhesion phase (16 hours incubation). The similarity on bacteria composition persisted after 48 hours, but the time affected the dominance of some bacteria

species. We found that the bands 1, 5 and 6 identified as *Fusobacterium periodonticum, Neisseria subflava* and *Alloprevotella sp.*, respectively, became deeper on Ti and ZrO_2, after 48 hours, indicating that the biofilm formation on those materials are time-dependent.

4. Discussion

Studies have shown that patients treated for periodontitis may experience more implant loss and complications around implants; thus, there has been great interest in better understanding the interface between prosthetic superstructure and implants beyond aesthetic considerations. To the best of our knowledge, this is the first study that analyzes a cultivable polymicrobial community, representative of the oral cavity,[32] on two commercial types of implant abutments. Here, our data accepted one of the hypotheses, revealing more biofilm accumulated on ZrO_2 than on Ti materials surfaces. However, rejected the other, since the type of material did not affect on the bacterial profile.

The scientific literature has shown that the surface properties of materials have marked influence particularly on the early phases of biofilm formation, suggesting that smooth surfaces and those with low SFE feature less microbial adherence than materials with higher surface roughness or SFE. [34-36] For this study, we choose a standard roughness for the experimental Ti and ZrO_2 abutments that approached the optimal roughness as previously described in the literature [37, 38] for permucosal implant abutments. Our purpose was to eliminate the roughness variable, keep homogeneity of the groups and focus on the effect of abutment materials on bacteria attachment and biofilm formation. SEM also showed a homogenous roughened microstructure alternating flat and slightly rougher surface areas for both material surfaces. Potential differences in bacterial colonization and biofilm formation were presumably the result of differences in the chemical composition and, consequently, differences in SFE (electrical conductivity). In our study, based on analysis of the total

number of bacteria adhered, in the initial culture stage and in a mature biofilm, to different surfaces, more bacteria accumulation was observed on the ZrO_2 surfaces, the most hydrophobic material with the highest value of surface tension. This correlation between SFE and bacterial adhesion has been studied previously [39, 40] and has been reported to have an influence in bacterial adhesion, [41] because high SFE materials attracted microorganisms with relatively high SFE. [42] In 2010, Burgers et al. investigated the adhesion of *Candida albicans* on Ti and ZrO_2 substrates and observed that Ti held a lower count of cells than ZrO_2. According to these authors, the SFE has an important impact adhesion and biofilm formation. [43] This could explain the results obtained in our investigation.

We demonstrated by different approaches that Ti reduced the amount of oral community-derived bacteria adhered to the surface. The amount of bacteria adhered on Ti disclosed a significant difference with 6.1-fold less bacteria. This reduction was also reflected by reduced biomass and biofilm density. Notably, the reduction in biofilm formation was sustained after 48 hours. Detailed quantitative analysis showed that biofilm biomass and the biofilm density were 2.2 and 1.6-fold, respectively, lower on the Ti discs in comparison to ZrO_2. Our data are in contrast to the most studies found in the literature, which shows no difference between biofilm formation on both materials or fewer bacteria adhered on ZrO_2 in comparison to Ti substrate.[27, 28, 44, 45] However, it is important to consider that the chemical and physical properties of the surfaces (surface wettability of materials) combined with one or a few number of bacteria species do not reflect the real effect of the material in the oral cavity.[27, 28, 45, 46]

Previous investigation[47] evaluated the biofilm formation on material surfaces applied for the implant abutments fabrication and no differences were reported on Ti and ZrO_2 substrates. However, the authors kept the samples immersed in saliva for 2 hours before the biofilm development, which may have affected the SFE, making it similar between the

materials.[47] Consequently, the interactions between the hydrophobic/hydrophilic region of the outer cell wall[48] and the material surface may also have been similar. An intriguing observation in this study was the determined number of microorganisms in biofilm multispecies exposing the specimens to a suspension of *Streptococcus gordonii, Streptococcus mutans, Actinomyces naeslundii,* and *C. albicans*.[47] Since bacteria express a wide variety of complex molecules that can contribute to the overall tendencies for microorganisms to interact with other cells, the selected microorganisms species can create a different final energy and could change the interaction to their environment.[49, 50] Since communication among microorganisms is essential for initial colonization and subsequent biofilm formation on dental surfaces, the limitation of *in vitro* biofilm models described in the literature, so far, may explain the inconsistent results.

Human saliva has been used in many *in vitro* studies as a nutrient source for sustaining oral microbial flora.[47, 51, 52] However, the use of natural saliva has some disadvantages, such as the difficulty of enhancing the complex oral microbial communities growth with a specific culture medium and the reproducibility of the experiment. And here, to simulate clinical conditions and keep the experimental environments reproducible, we used a cultivable polymicrobial community representative of the oral cavity as a model system, sustained by a bacterial growth medium (SHI-FSMS) containing saliva and blood components.[32]

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.[53] Hence, the physicochemical characteristic inherent to each material may interfere on the degree and nature of initial bacterial adhesion and, consequently, define the late profile of the biofilm.[49, 50] Since Ti accumulated fewer bacteria than ZrO2 into the surface, we examined if the material properties would also affect the profile of bacteria colonization. In our study, no differences were

observed in bacterial composition in the attachment phase. The effect of Ti and ZrO_2 beyond the early stage continued over time. After 48 hours incubation, the bacterial profile, measured by the intensity of the bands, did not show differences between materials, but the intensity was different in comparison to the first time point. Ti and ZrO_2 are considered highly hydrophobic materials due to their chemical composition; the low polarity of their surfaces will attract molecules with the same chemical composition. These concepts can explain the similarity of the bacterial profile during the both time points. One limitation of this study may be related to the methodology employed. DGGE analysis is an important tool has been extensively employed to examine the microbial populations found in subgingival plaque, endodontic infections, and dental plaque microbiome.[54, 55] The sophistication of DGGE is that it allows the varied PCR-amplified gene products of similar lengths. However, this technique analysis of the high-quality diversity patterns is restricted to a visual comparison and interpretation.[33]

5. Conclusion

Collectively, our findings indicated that initial attachment phase and biofilm formation are affected by the substrate type. ZrO_2 accumulated significant more bacteria and biofilm into the material surfaces in comparison to Ti. However, the results of this *in vitro* study should be interpreted with cautions considering the limitations of an *in vitro* investigation, and more studies are needed to examine the mechanisms by which properties of ZrO_2 affect the bacterial colonization. Additionally, since aesthetic implications in oral rehabilitation are considered to be an indicator of success, the information contained in this study could offer a starting point in future design of implants abutments, such as the creation of anti-microbial surfaces, to improve the implant longevity.

Acknowledgments

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Conflict of interest

The authors declare that there is no conflict of interest in this study.

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FIGURES LEGENDS

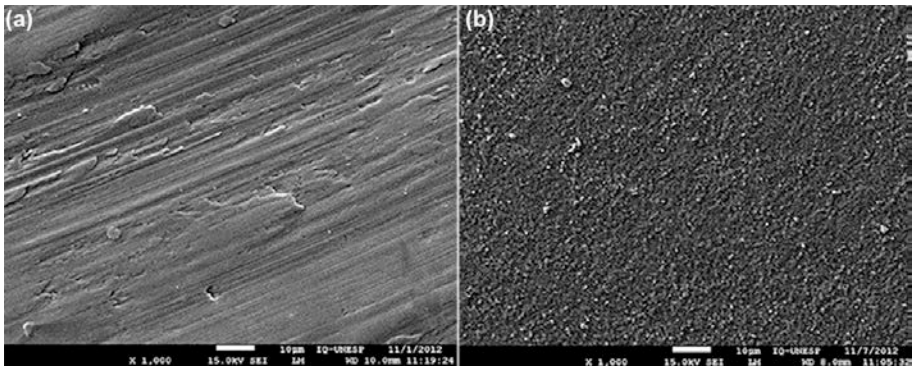

Figure 1. Scanning electron micrographs (SEM) of Ti (a) and ZrO_2 (b) discs (Bar=10μm) with high magnification (1000x) showing the surface topography.

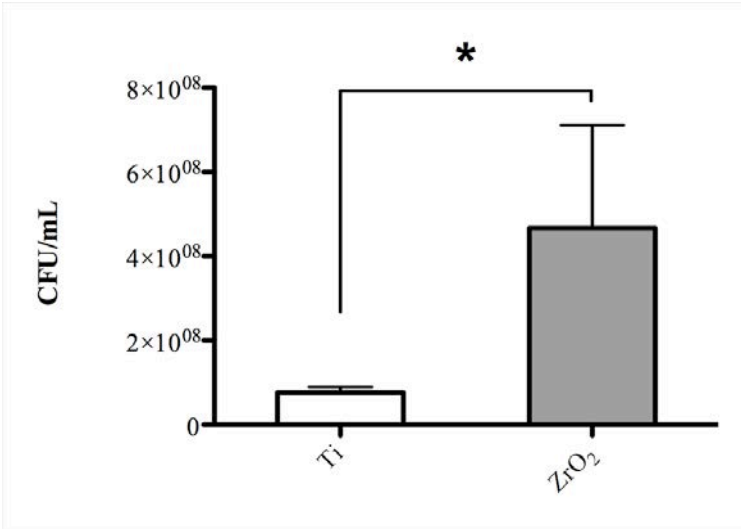

Figure 2. The effect of Ti and ZrO_2 materials surfaces on bacterial attachment was evaluated after 16 hours incubation via quantitative culture counts (in colony-forming units). Unpaired t test, two tailed, indicated that the CFU/ml was significantly reduced on Ti discs, p=0.019. Data are shown as the mean ± SD (n=9), *$p<0.05$ indicating a statistically significant difference between Ti and ZrO_2 discs.

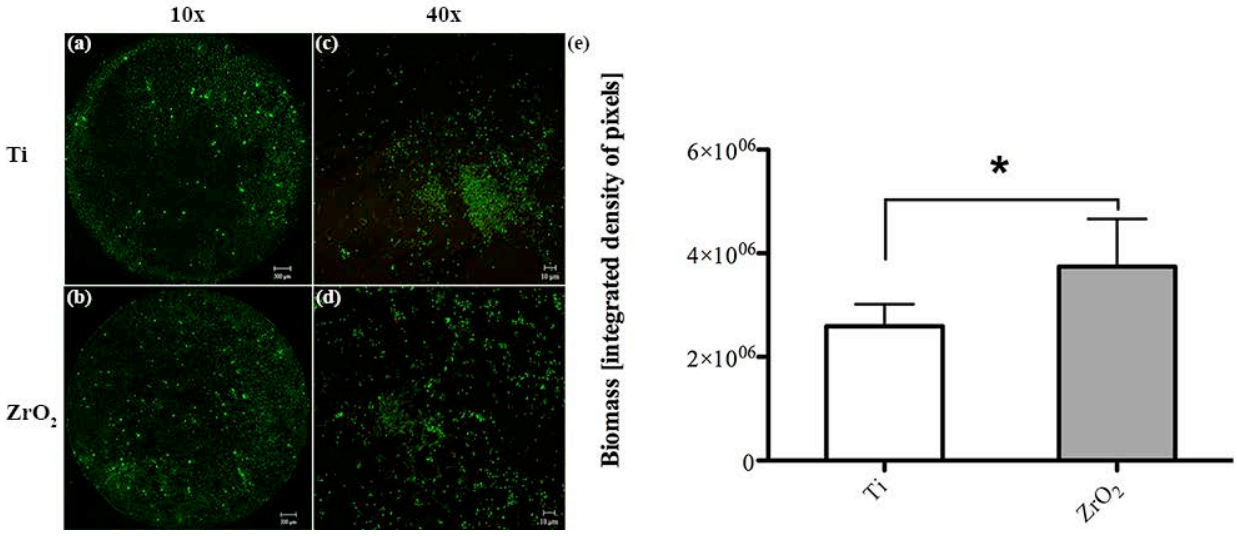

Figure 3. Representative microscopic images of oral microbial communities after 16 hours incubation were evaluated by confocal. Images in 10x resolution of **(a)** Ti and **(b)** ZrO_2 discs surfaces. CLMS in high resolution (40x) of **(c)** Ti and **(d)** ZrO_2 discs surfaces, revealed similar bacteria spreading on both materials substrate. **e.** Unpaired t test, two tailed, indicates that integrated density integrated density was significantly reduced on Ti discs (white bar, Ti), p=0.030. Data are shown as the mean ± SD (n=9), *$p<0.05$ indicating a statistically significant difference between Ti and ZrO_2 discs.

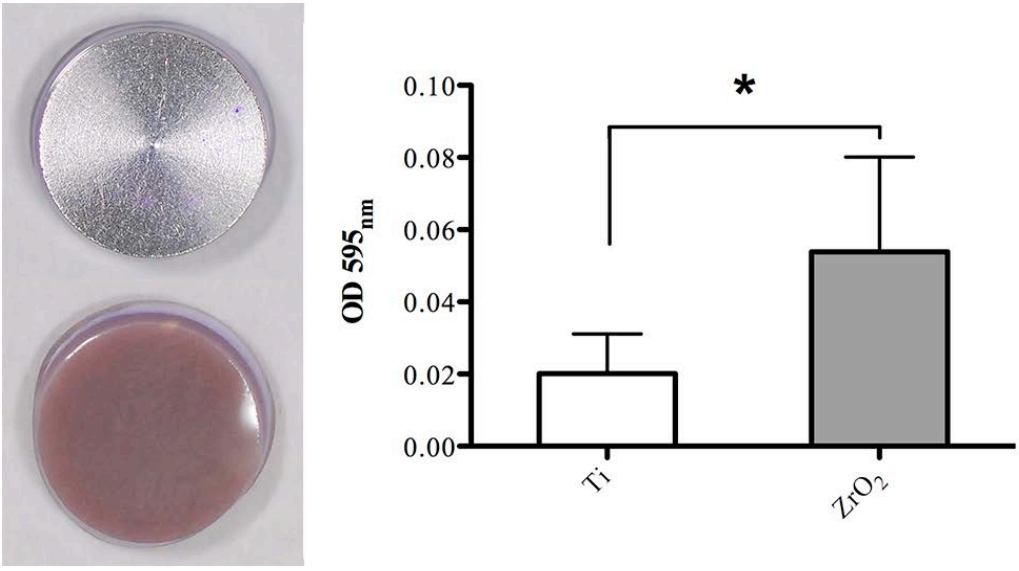

Figure 4. The effect of Ti and ZrO_2 materials surfaces on biomass of bacterial attachment was evaluated after 16 hours incubation via crystal violet staining. The unpaired test, two tailed, indicated statistically higher biomass of bacterial attachment on ZrO_2 (gray bar, ZrO_2) than Ti discs (white bar, Ti), p=0.029. Data are shown as the mean ± SD (n=9), *$p<0.05$ indicating a statistically significant difference between Ti and ZrO_2 discs.

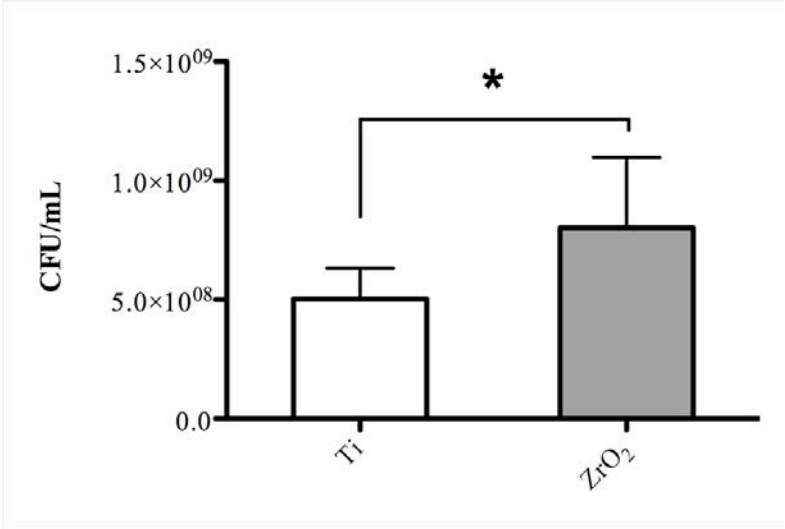

Figure 5. The effect of Ti and ZrO_2 materials surfaces on biofilm formation was evaluated after 16 hours incubation via quantitative culture counts (in colony-forming units). Unpaired t test, two tailed, indicated that the CFU/ml was significantly reduced on Ti discs (white bar, Ti), p=0.046. Data are shown as the mean ± SD (n=9), *$p<0.05$ indicating a statistically significant difference between Ti and ZrO_2 discs.

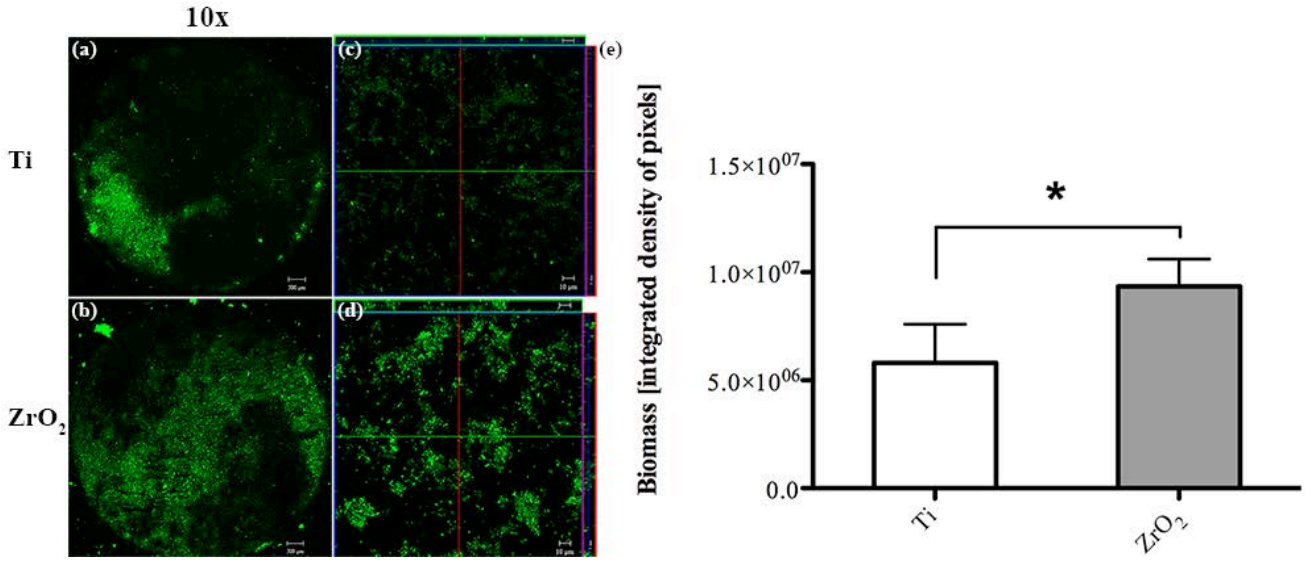

Figure 6. Representative microscopic images of oral microbial communities biofilm developed after 16 hours incubation. Images in 10x resolution of **(a)** Ti and **(b)** ZrO_2 discs surfaces. Images in z-stacks revealed biofilm thickness formed on **(e)** Ti and **(f)** ZrO_2 discs surfaces (bar=10μm). **e.** Unpaired t test, two tailed, indicates that integrated density was significantly reduced on Ti discs (white bar, Ti), p=0.041. Data are shown as the mean ± SD (n=9), *$p<0.05$ indicating a statistically significant difference between Ti and ZrO_2 discs.

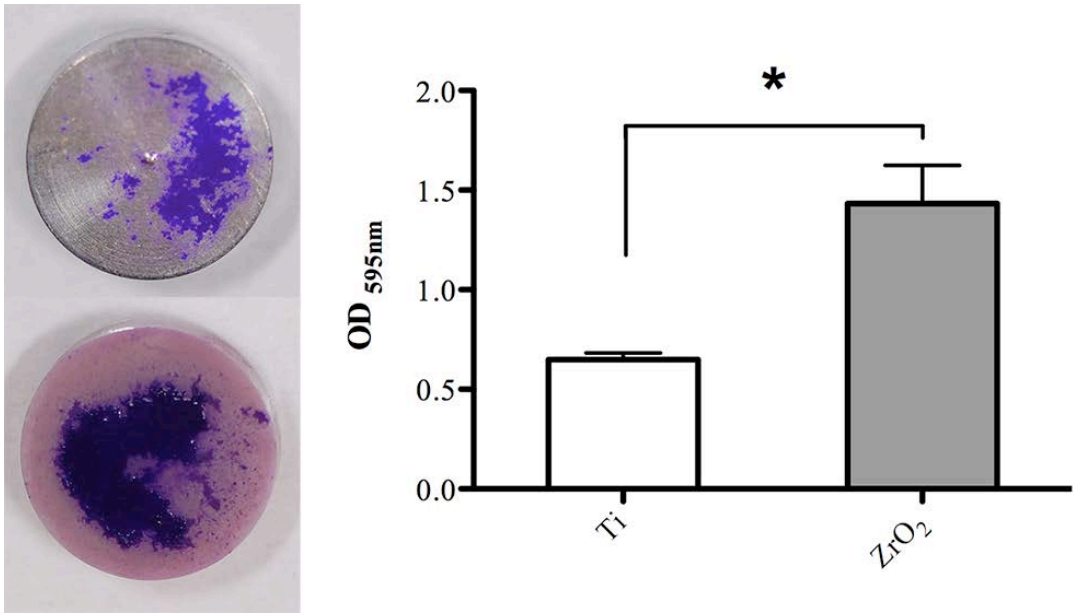

Figure 7. Biomass of biofilm formed on Ti and ZrO_2 discs surfaces were evaluated by crystal violet after 48 hours incubation. Unpaired t test, two tailed indicated that the biomass was significant high on ZrO_2 discs (gray bar, ZrO_2), p=0.002. Data are shown as the mean ± SD (n=9), *$p<0.05$ indicating a statistically significant difference between Ti and ZrO_2 discs.

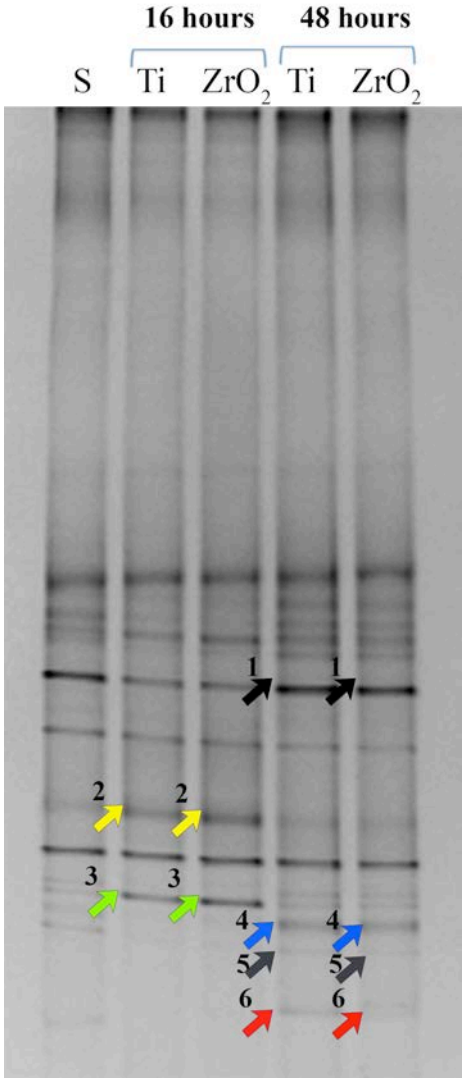

Figure 8. DGGE analysis of oral microbial communities formed on Ti and ZrO_2 discs surfaces after 16 hours and 48 hours. Bands indicated by arrows were excised and the DNA sequenced. Microbial identities are as follows: (1) *Fusobacterium periodonticum*, (2) *Streptococcus sp*, (3) *Gemella sanguinis*, (4) *Veillonella parvula*, (5) *Neisseria subflava* and (6) *Alloprevotella sp.*

TABLES LEGENDS

Table 1. Mean and standard deviation (SD) of the contact angle (θ) of liquid for each sample.

Wetting Agents	Samples	
	T	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)
Diiod	24,09 (1,82)	24,77 (1,56)

Table 2. Strains of bacterial communities identified from the excised bands of DGGE profiles.

DGGE Band	Descriptions	% Identity	Accession number
1	*Fusobacterium periodonticum*	100%	201BS011
2	*Streptococcus sp.*	95.2%	074-T4E3
3	*Gemella sanguinis*	100%	757C24KA
4	*Veillonella parvula*	98.9%	161BU083
5	*Neisseria subflava*	91.3%	476_9291
6	*Alloprevotella sp.*	83.2%	279CW034

7 Capítulo 6

Artigo Aceito
"*Journal of the International Academy of Periodontology*"

Aesthetic Implant Rehabilitation in Periodontally Compromised Patient: A Surgical and Prosthetic Rationale

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Abstract

Two cases of aesthetic implant abutment rehabilitation in the maxillary anterior area in periodontally compromised patients following conventional periodontal therapy and tooth extractions are presented. For the two cases of anterior tooth loss due to advanced periodontal disease progression, atraumatic flapless extractions were performed followed by the placement of immediate implants and provisional restorations. For the first case, lithium disilicate cemented over the abutment was used to achieve excellent aesthetic results. In the second case, custom zirconia abutments were used as prosthetic components. The results after three-year follow-up showed absence of inflammation and/or infection on the peri-implantar tissue with satisfactory aesthetic and excellent biological / clinical results achieved with reduced treatment time and morbidity for both patients. Total absence of infection and frequent plaque control after implant placement are mandatory before selection of the abutment material. The planning of the final treatment as specified by the concept of comprehensive dental care is outlined, and the final outcome is discussed according to the literature.

Keywords. Aesthetics; dental implants; periodontitis; bone loss; bone graft.

Introduction

Periodontitis is a chronic inflammatory condition caused by the host inflammatory response to plaque biofilm accumulation, which leads to tooth-supporting soft and hard tissue destruction (de Molon *et al.*, 2014; de Molon *et al.*, 2015c). When the individuals lose their teeth by periodontal disease, dental implants appear as an alternative to replace missing teeth. Such implants, mainly in the anterior maxillary area, have become one of the most important needs of patients attending clinics to restore aesthetics and/or function (de Molon *et al.*, 2015a). However, some studies have assumed that periodontally compromised patients present a potentially higher risk for implant failure than healthy individuals (Aguirre-Zorzano *et al.*, 2015; Mombelli and Decaillet, 2011). This assumption is due to the observation that similar pathological bacterial flora are present around both diseased teeth and diseased implants (Apse *et al.*, 1989). However, several mutual confounding factors for periodontitis and peri-implantitis have been identified, including smoking, uncontrolled diabetes (de Molon *et al.*, 2013c), genetic predisposition and oral hygiene (Levin *et al.*, 2011). In addition, a small number of periodontal maintenance patients seem to be refractory to treatment and continue to experience significant tooth loss. A history of periodontal disease is an important information and different clinical implications regarding the proposed treatment for these types of patients must be respected. Because bacteria are responsible for initiating the inflammatory process, and surface attachment is the first step in biofilm development (Patrick and Kearns, 2012), the choice of the abutment design and material in prosthetic implant restorations is essential.

Abutment surfaces are typically prone to subgingival biofilm formation due to an increased contact area with peri-implant gingival tissues. Routinely, titanium abutments are the first choice of material. However, in recent years, ceramic surfaces have been introduced in prosthetic implant dentistry. Due to less discoloration at the gingival margin compared to metal abutments (Bidra and Rungruanganunt, 2013; Nakamura *et al.*, 2010) ceramic abutments were particularly favourable for anterior esthetic restorations. Among the ceramic

materials, zirconia and glass ceramic systems have received growing interest because of their biological, mechanical and aesthetic properties. The initial adhesion of microorganisms to the substrate surface has been shown to have a relevant impact on the etiopathogenesis of infections related to biomaterials (Quirynen and Bollen, 1995) and, consequently, on the longevity of implant rehabilitation. Thus, an important question in relation to implant therapy in periodontally susceptible patients with tooth loss is whether these patients can be rehabilitated with aesthetic prosthetic components. Recently, investigations focusing on the aesthetic material substrate have reported encouraging data. According to previous study (Grossner-Schreiber *et al.*, 2009a), higher total rates of bacterial colonization were detected on titanium surfaces compared to zirconia. Concurrently, no significant differences were observed in the diversity of the identified bacterial species among all of the surfaces examined (Grossner-Schreiber *et al.*, 2009b). Similar to these results, in a recent in vivo study, lower bacterial counts were detected on zirconia materials than on titanium components (Nascimento *et al.*, 2014). When treating patients' at a high risk for peri-implant disease, it is mandatory to identify implant and prosthetic component characteristics as well as patients' needs for aesthetic demands to ensure periodontal health and patients' satisfaction.

In the following context, we present two complex cases of implant rehabilitation in the anterior maxillary in periodontally compromised patients following conventional periodontal therapy and tooth extractions will be presented aiming at an optimal aesthetic rehabilitation, a single appointment combined approach was chosen. The treatment of a periodontally hopeless tooth involved the following: (i) atraumatic tooth extraction, (ii) immediate implant placement, (iii) particulate bone graft, and (iv) immediate restoration (de Molon *et al.*, 2015a; de Molon *et al.*, 2015b). Furthermore, the relation between type of implant abutment material and the success of the treatment defined by the longevity as well as aesthetic results and satisfaction of the patient are discussed in the presented cases.

Case Descriptions

Case Description 1

A 66-year-old woman was referred for dental treatment due to mobility of the maxillary central incisor and presence of periodontal abscess. She had no relevant medical history and never smoked. Clinical and radiographic examinations revealed a central incisor with signs of Class III Miller mobility, 5 mm of probing depth, abscess, and the absence of buccal bone wall, creating a functional defect requiring bone augmentation (*Figure 1 A-E*). The initial treatment was a local curettage to allow abscess drainage. Then, standard disinfection was accomplished, and the Widman flap surgical technique was performed to provide improved visual access to the periodontally involved tissues. The vertical periodontal defects were filled with Osteogen® (resorbable hydroxyapatite, granulated to 300 to 400 microns), a bone regeneration material followed by interrupted silk sutures (*Figure 2 A-D*). To minimize postoperative tooth sensitivity and improve bone repair, the patient was submitted to 4 sessions of laser therapy at a low frequency accordingly to previous published study (Gomes *et al.*, 2015) and the application of Duraphat® on the tooth. The patient was recalled every four months for plaque control (*Figure 3A*). However, after 5 years, the mobility of the maxillary incisors persisted and increased (*Figure 3 B-D*), and based on clinical and radiographic examinations, immediate implant placement followed by regenerative procedures and immediate provisionalization of the crown were proposed and accepted by the patient. Written informed consent was obtained prior to the initial treatment.

In this specific case, the patient received prophylactic antibiotic (Klinge *et al.*, 2015) with oral administration of Amoxicillin 500 mg/Clavulanate 125 mg, every 8 hours (three times a day), 7 days before the surgery, and after one week post-surgery due to the deep periodontal pocket (Klinge *et al.*, 2015). The maxillary incisors were atraumatically extracted

under local anesthesia (2% mepivacaine and 1:100.00 epinephrine - Mepiadre®, DFL, Rio de Janeiro, RJ, Brazil) using a flapless technique to preserve the buccal bone architecture and osseous structures around the fresh socket (*Figure 4A*). After extraction of the teeth, the alveoli were curetted, and two narrow dental implants 3.5 x 13.0 mm Cone Morse (Flash Porous NP by Conexão Sistema de Prótese) were immediately inserted (*Figure 4B*) respecting the minimum distances necessary to establish optimal aesthetic results. The initial stability of the implant was 60 and 45 Ncm to the left and right maxillary incisor, respectively, allowing immediate provisionalization of the crown. In sequence, a temporary resin crown was placed over the provisional titanium abutments using RelyX™ Ultimate Adhesive Resin Cement (RelyX Temp NE - 3M ESPE, St. Paul, MN, USA; (*Figure 5 A-D*). Because the buccal bone plate was lost due to the inflammatory process, the mesial and distal gap between the bone and implant was filled with Bio-Oss® (Geistlich, 7 Wolhusen, Switzerland) to allow bone remodeling, according to previous study (De Santis *et al.*, 2011); *Figure 6A*). The patient was seen one week after surgery for suture removal and provisional resin crown adjustments (*Figure 6B*). Postoperative visits included oral hygiene instructions and plaque control every month for four months after surgery (*Figure 6C*). After four months postoperatively, periapical radiography was taken (*Figure 6D*), the provisional restoration was removed, and the prosthetic procedures were initiated by transferring tissue architecture using a pick-up technique with modified squared impression copings (squared impression copings with 2-mm prolongations) created with autopolymerizing acrylic resin (de Avila *et al.*, 2013). A UCLA custom abutment overcast in cobalt-chromium (Conexao Sistema de Protese, Sao Paulo, SP, Brazil) was selected to increase the contact between this component and the implant. Here, it was used a common method to mitigate the poor appearance of the peri-implant tissue caused by the cobalt-chromium metal, altering the metallic color by applying porcelain on the component. To achieve this goal, Ivoclar press ceramics were applied on the titanium

abutment materials, followed by copings fabricated with IPS e.max Press (Conexao Sistema de Protese, Sao Paulo, SP, Brazil). Next, the feldspathic porcelain crown IPS Empress II - lithium-disilicate glass-ceramic restoration (Ivoclar, Vivadent) was fabricated and cemented with RelyX™ Ultimate Adhesive Resin Cement (3M ESPE, St. Paul, MN, USA) over the abutment to achieve excellent aesthetic results (*Figure 7, A-D*).

Clinical evaluation of the soft tissue and radiographic evaluation have been done to assess bone level at the implant site. The patient was seen monthly during the first year to evaluate the periodontal status of the patient (gingival index, plaque scores, and bleeding on probing), followed by professional prophylaxis and oral hygiene instructions (OHI). Maintenance visits, every 6 months, consisted of reinforcement of OHI and professional prophylaxis. The three-year follow-up results demonstrated an improved clinical situation, allowing an optimal aesthetic outcome without probing depths or gingival recession (*Figure 8 A-B*). Additionally, there was no bleeding on probing. The periapical radiographs showed the correct position of the implant in relation to the adjacent teeth and an increase in vertical bone formation completely filling the osseous vertical defect without marginal bone loss (*Figure 8 C*). The functional and aesthetic expectations of the patient were achieved relative to the pretreatment situation. It is important to mention that the patient was observed frequently for oral hygiene instructions and plaque control.

Case Description 2

A 71-year-old woman was referred for dental treatment due to periodontal disease. She was also disappointed with her teeth and complained about the aesthetics of the maxillary central incisor (*Figure 9 A-B*). With regard to the occlusal relationship and because it was impossible to obtain an adequate crown-root ratio after tooth treatment, it was decided to extract the roots orthodontically and replace them with a dental implant (de Molon *et al.*, 2013b). It is

important to note that this case report was planned with a multidisciplinary team, *i.e.*, an orthodontist, a periodontist, and a prosthodontist. The initial phase of treatment consisted of the reduction and control of plaque accumulation, oral hygiene instructions and reinforcement of the patients' hygienic efforts, followed by supra and subgingival scaling and root planning. The periodontal treatment and oral hygiene instructions were performed over 4 months according to previous protocol (Gkantidis *et al.*, 2010). The orthodontic treatment was initiated using brackets (Abzil-3M, São José do Rio Preto-SP, Brazil). The central incisor brackets were positioned cervically and 0.018″ stainless steel wire was used to allow tooth extrusion (*Figure 10, A-C*). Periapical radiographs were obtained to monitor the bone profile progress (Korayem *et al.*, 2008); *Figure 10D*). The orthodontic extrusion was completed after 8 months and the implant procedure was planned considering the bone gain obtained from orthodontic treatment (de Avila *et al.*, 2012).

The extraction was initiated by incision of the buccal and lingual soft tissues around the teeth, as well as on the contralateral aspect of each adjacent tooth (Figure 11A). The implant placement treatment was successful using Flash Porous NP® 3.5 x 13.0 (Conexão Sistema de Prótese; *Figure 11B*). Due to an initial implant stability of 60 Ncm, immediate provisionalization of the crowns was possible. The gap between the vestibular bone and implant was filled with Bio-Oss® (Geistlich, Wolhusen, Switzerland), and in sequence, the provisional crowns were cemented, using RelyX™ Ultimate Adhesive Resin Cement (RelyX Temp NE - 3M ESPE, St. Paul, MN, USA, taking great care during the removal of excess cement, which may lead to peri-implant inflammation in this region (*Figure 12 A-D*). The reason for cemented crowns' choice can be explain since this type of restoration provided superior accessibility and had better porcelain fracture rates than screwed crowns according to previous studies (Shadid *et al.*, 2011; Torrado *et al.*, 2004). Additionally, cemented crowns have shown lower rates of peri-implant diseases in comparison to screwed crowns (Nissan *et*

al., 2011). The definitive prosthesis was confectioned at three months after the surgical procedure. To achieve this goal, an impression technique with squared, splinted copings using metal drill burs and Pattern resin was chosen to copy the peri-implanter anatomy (de Avila *et al.*, 2014). Two custom zirconia abutments were confectioned using computer-aided design and computer-aided manufacturing (CAD/CAM) and cemented on the Cone Morse System (*Figure 13*).

This case has been followed up for 3 years, and the clinical and radiographic examinations showed no signs of inflammation. Optimal aesthetic results (Figure 14, A-B) and patient satisfactions were achieved. Through a multidisciplinary approach, this case was successfully rehabilitated and showed stable results after three years of follow-up. In addition, it is important to emphasize that plaque control was performed every four months during these three years.

Discussion

Oral rehabilitation of a periodontally compromised partially edentulous patient requires special attention, considering biologic, aesthetic and technical aspects. The reduced periodontal support around the prospective abutment teeth and potential transmission of periodontal pathogens from the teeth to the implants, may affect the long-term survival and success of the prosthodontics treatment. In addition, the decision to use either titanium or ceramic abutments must be based on the available scientific evidence, skill/experience of the operator, oral and systemic conditions, and patient preference. Using two case reports, the present study attempted to demonstrate evidence-based conclusions for oral rehabilitation of periodontally compromised partially edentulous patients with aesthetic abutment implants. Healthy and stable clinical conditions were seen after 3 years of follow-up.

The scientific literature shows a high long-term survival rate of implants in implant-

supported restorations in periodontitis patients, given that adequate infection control and an individualized hygienic program are provided. However, minimal information is available for the long-term survival rates with regard to abutments material in periodontally compromised patients (Dhingra, 2012). Previous studies investigating biofilm formation on abutment materials have demonstrated differences among these materials, with alloys featuring thick biofilms with low variability, and ceramic materials featuring thin biofilms with high viability (Busscher *et al.,* 2010). However, other investigations have demonstrated no differences or less biofilm adhesion on titanium material surfaces (Nascimento *et al.,* 2014). Further, soft tissue responses to ceramic materials using well-controlled *in vivo* studies in humans are lacking (Klinge *et al.,* 2006). The inconsistencies of the results provided by the scientific literature are critical for professionals who must make clinical decisions. The clinician is often confronted with difficulties related to selecting the appropriate treatment to ensure long-term successful outcomes in terms of function and aesthetics.

In the first case, lithium disilicate glass ceramic covering metal abutments were used due to the low cost in relation to zirconia materials. The advantage of this material is its superior optical properties. In terms of mechanical characteristics, it is known that, in general, ceramics are inherently brittle materials and prone to breakage under inadvertent bending forces (Charlton *et al.,* 2008). In contrast, the structure of monolithic lithium disilicate can resist masticatory stress, by dissipating it throughout the restoration (Kang *et al.,* 2013). The molecular structure of lithium disilicate has demonstrated good biological results that encourage its use in anterior areas. However, its application requires further study since, to the best of our knowledge, only one report has discussed biofilm formation using this type of material (Bremer *et al.,* 2011). In this study, the authors have demonstrated that the biofilm formation on various types of dental ceramics differed significantly, *in vivo*, with zirconia accumulating lower plaque than that identified with the lithium disilicate glass-ceramic. The

limited number of participants selected for this work and a short in vivo growth period analyzed restricts the information on the marked differences found in the present study (Bremer *et al.*, 2011). In the second case, it was selected a zirconia abutment as a prosthetic component. Zirconia implant abutments have been used for anterior aesthetic restorations for more than 10 years, and in relation to mechanical considerations, this material can be considered as a viable treatment in the maxillary anterior area for single unit crowns (Passos *et al.*, 2014). The presented cases showed excellent aesthetic results after 3 years of follow-up and are supported by previous clinical investigations that evaluated zirconia implant abutments from 1 to 5 years in anterior and posterior regions. In those studies, the authors identified good technical and biological performances of this material over a short-term period (Passos *et al.*, 2014; Zembic *et al.*, 2013).

In both cases, atraumatic flapless extraction of the hopeless teeth was performed to preserve the reminiscent bone and to improve the functional and aesthetic outcomes, as described previously (de Molon *et al.*, 2015a). The advantages of flapless surgery allow maintenance of the soft tissue architecture with conservative tissue manipulation, leaving an intact periosteum to preserve the blood supply and increase the predictability and success of the implant. Moreover, flapless implants are feasible, and the scientific literature has described excellent results (de Molon *et al.*, 2015b). A recent systematic review showed that implants placed immediately in fresh extraction sockets yielded a low annual failure rate of 0.82%, translating into a 2-year survival rate of 98.4% (Lang *et al.*, 2012). However, a number of factors may affect the outcomes of procedures other than the timing of implant placement alone, including the type of bone, the dimensions of the edentulous area and the history of oral and systemic diseases. Here, an implant with a narrow diameter implant was chosen because of the limited bucco-lingual width of the alveolar bone. In addition, provisionalization of the crown was performed to provide immediate implant loading, which

is possibly accompanied by an increased bone remodeling as demonstrated in an animal experiment (Romanos *et al.*, 2002).

Extensive loss of tooth supporting structures, e.g. bone dehiscence and fenestrations, as a consequence of periodontal diseases may impede prosthetic rehabilitation (de Molon *et al.*, 2013a; de Molon *et al.*, 2014). As an attempt at restoring the original anatomy and hence improving the aesthetic results of implant therapy, such defects should be corrected. Therefore, in the second case, orthodontic extrusion was planned to increase the amount of available vertical bone and/or gingival tissue and to avoid autograph bone graft procedures. During the orthodontic extrusion, mechanical stresses exerted onto the alveolar bone led to activation of angiogenic growth factors, which would contribute to the formation of new support tissue (Shiu *et al.*, 2005). Because tooth movement occurs in the coronal direction, the gingiva and bone attached by the periodontal ligaments migrate in the same direction of the movement, resulting in a coronal shift of the bone at the base of the defect (Sterr and Becker, 1980; de Barros *et al.*, 2013). The criteria required for the satisfactory application of this procedure are as follows: the apical third of the root must maintain an intact fiber apparatus, and the patient should not present systemic problems (de Molon *et al.*, 2013c; Salama and Salama, 1993). Overall, the literature recommends that in patients with periodontal disease, the orthodontic treatment should be initiated 2-6 months after periodontal therapy to allow for periodontal healing and stabilization (Gkantidis *et al.*, 2010). Based on these observations, orthodontic extrusion was initiated at 4 months after conclusion of initial periodontal treatment and when absence of inflammatory processes was confirmed by clinical and radiographic examinations.

Research has provided evidence those patients with a history of periodontitis present with an increased risk for peri-implantitis (Aguirre-Zorzano *et al.*, 2015; Lindhe *et al.*, 2008). Early studies have shown that the microbiota around failing implants and periodontally

involved teeth yield similar compositions (Aguirre-Zorzano *et al.*, 2015). Therefore, periodontal patients who wish to replace lost teeth with implants should be informed that the scientific literature supports the impact of this risk factor on the onset of biological complications related to implants over time. Another example of risk to develop this infection is due to submucosal cement persistence (Wilson, 2009). It has been reported that residual excess cement after placement of fixed dental prostheses has been associated with clinical and radiographic signs of peri-implant disease. In regard to control the risks of peri-implantitis, the frequency of supportive periodontal care appointments has been proposed as a part of an ongoing periodontal maintenance program. Additionally, the clinicians should establish an early diagnosis and intervention, which will contribute to more effective management of peri-implant diseases (Rosen *et al.*, 2013).

The success of implant restoration in anterior aesthetic areas is resulted of a harmonious relationship between the implant and peri-implant tissue and the remaining natural teeth. Thus, to achieve aesthetic results is necessary complete absence of biological, technical and aesthetic complications. Therefore, the choice of the prosthetic component material should be considered together with the total absence of any infection followed by frequent plaque control after implant placement.

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Figure Legends

Figure 1 – A. Initial clinical aspect of the maxillary central incisors; B. Periodontal probing showing 5 mm loss of palatal bone. C-D. Presence of fistulae between the right and left central incisors. E. Periapical radiography showing generalized bone loss.

Figure 2 – A. Clinical view after basic periodontal therapy. B. Widman surgical flap to allow adequate scaling and root planning followed by (C) xenogenous bone graft to fill the vertical bone defect. D. Uninterrupted silk suture to maintain the flap in an apical position.

Figure 3 – A-C. Clinical view of the maxillary teeth showing the progression of the bone loss over time and migration of the gingival tissue to an apical position after the Widman surgical access. (D) 5-year clinical and radiographic outcomes after surgery.

Figure 4 – A. Atraumatic tooth extraction preserving the reminiscent buccal bone followed by immediate implant placement (B) following the rule of restorative-driven 3-dimensional placement.

Figure 5 – A. Occlusal clinical view of the implants placed in an ideal position. B. Provisional abutment installation followed by (C) provisional copping for immediate crown provisionalization. D. Provisional crown immediate confectioned after implant placement.

Figure 6 – A. To fill the gap between the implant and the bone defect, particulate xenogenous bone was placed in position. B. Immediate crown installation after bone graft. C-D. After four months postoperatively, clinical and radiography images showing optimal aesthetic results and absence of infection and bone loss.

Figure 7 – A-C. Installation of the definitive abutment and crowns. D. Clinical view with the final prosthesis installed.

Figure 8 – A-B. Three-year follow-up revealed optimal aesthetic outcome without probing depths or gingival recession. C. The periapical radiograph showed the correct position of the

implant in relation to the adjacent teeth and an increase in vertical bone formation completely filling the osseous vertical defect without marginal bone loss.

Figure 9 – A-B. Frontal and occlusal clinical views of the maxillary central incisors and the occlusal relationship.

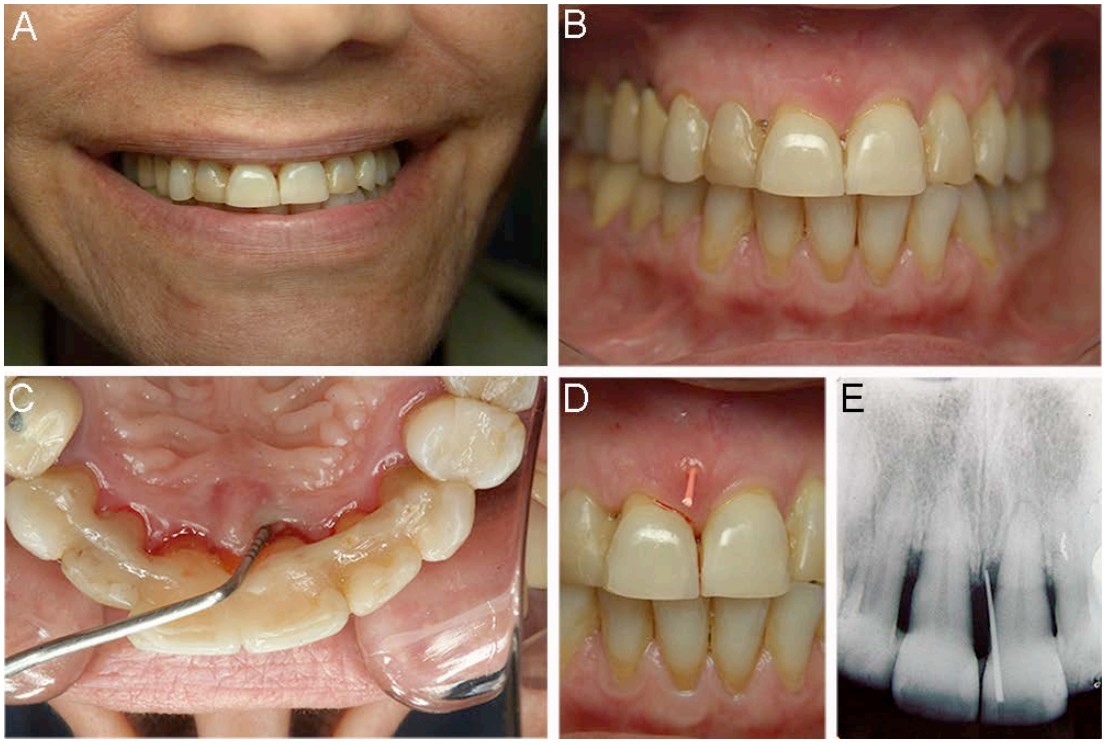
Figure 10 – A-C. Orthodontic treatment for teeth extrusion allowing vertical bone gain prior to the teeth extractions. D. Periapical radiography showing the initial situation (left) and after 8 months of active orthodontic treatment (right).

Figure 11 – A. Atraumatic teeth extraction followed by immediate implant placement (B).

Figure 12 – A-B. Provisional abutment and copping installation. C. Bone graft was placed over the implant to fill the gap between the defect and the implant. D. Installation of the provisional crowns.

Figure 13 – A-B. Clinical view after 3 months postoperatively. C. Two custom zirconia abutments were confectioned using CAD/CAM system.

Figure 14 – A. Final clinical aesthetic outcomes and B. Periapical radiography after 3 years of follow-up.



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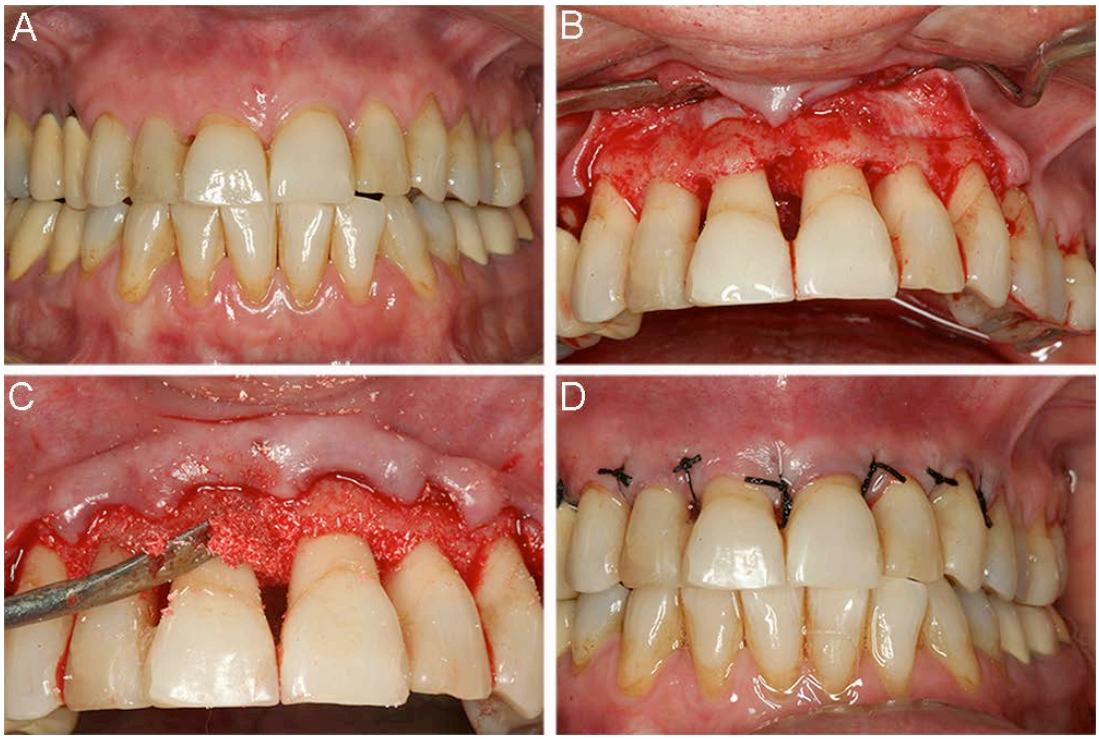

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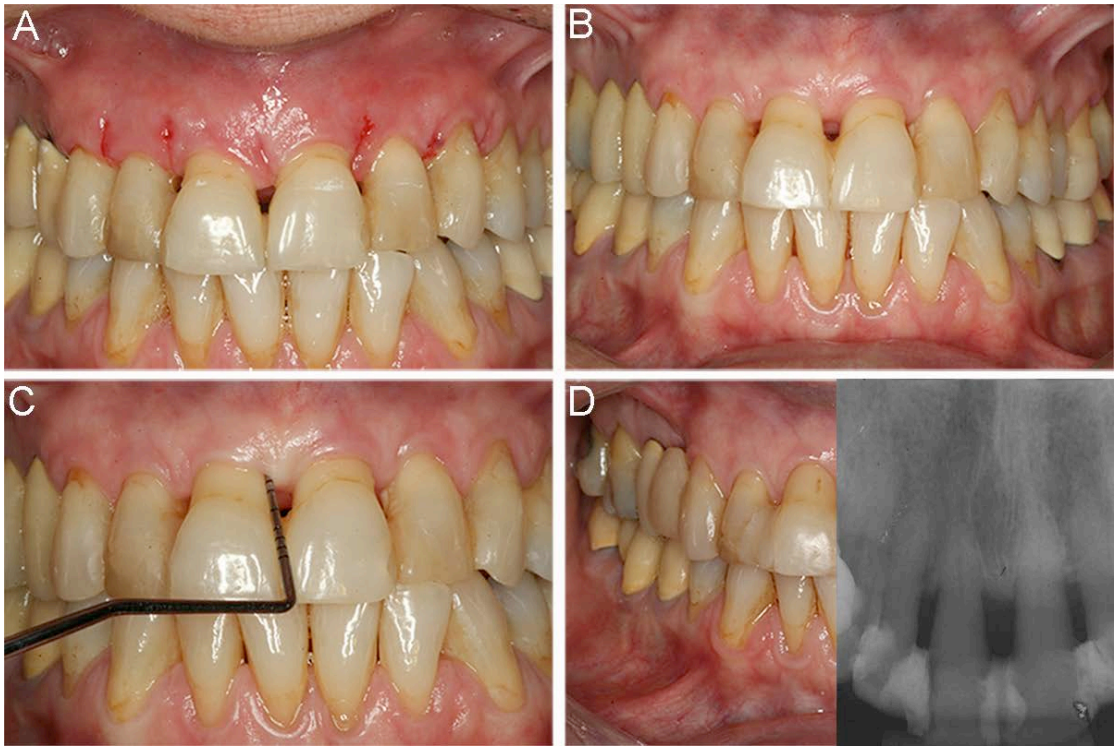

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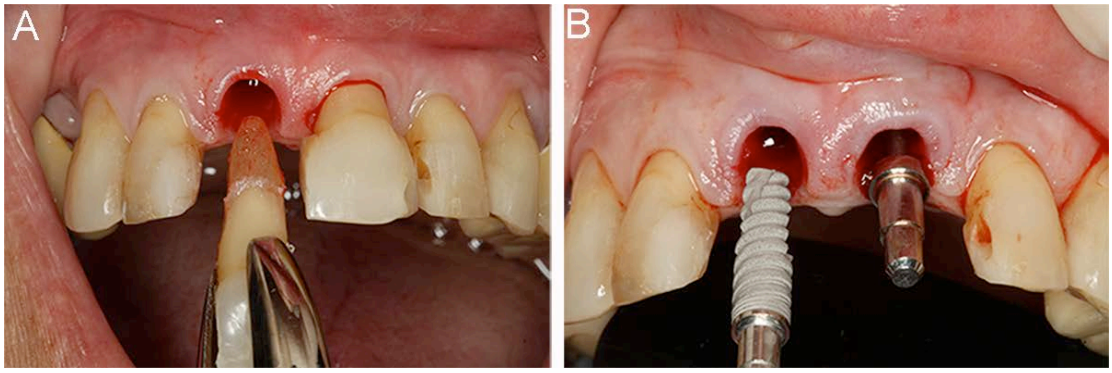

Figure 4 – A. Atraumatic tooth extraction preserving the reminiscent buccal bone followed by immediate implant placement (B) following the rule of restorative-driven 3-dimensional placement.

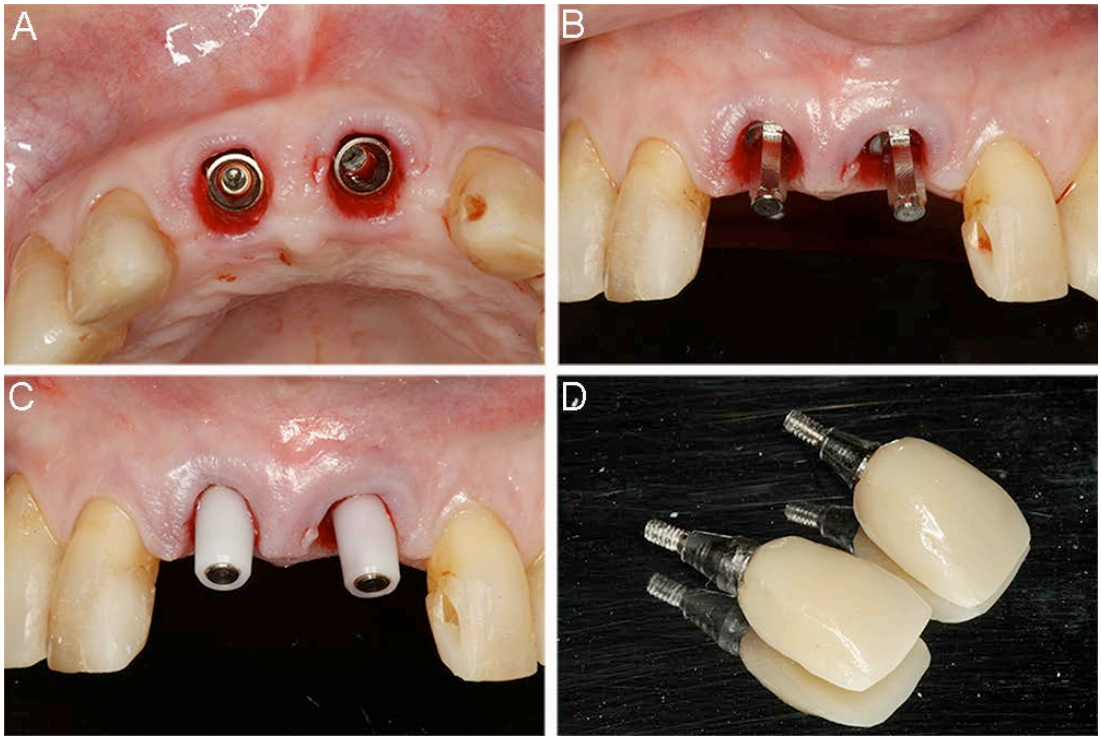

Figure 5 – A. Occlusal clinical view of the implants placed in an ideal position. B. Provisional abutment installation followed by (C) provisional copping for immediate crown provisionalization. D. Provisional crown immediate confectioned after implant placement.

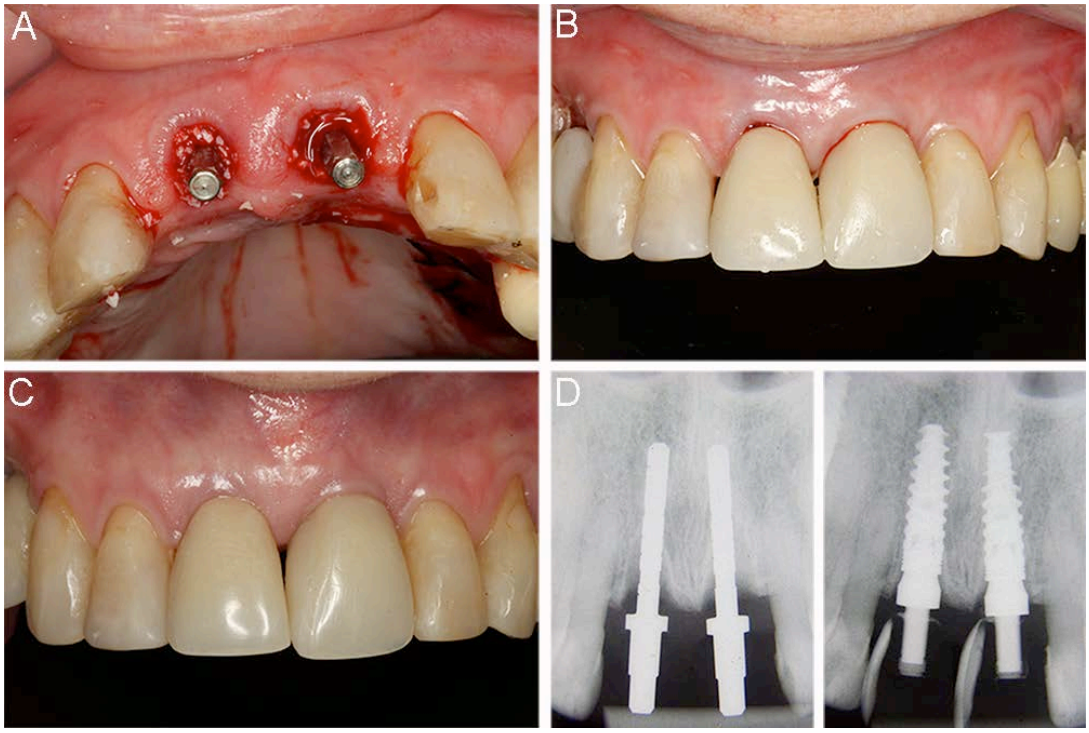

Figure 6 – A. To fill the gap between the implant and the bone defect, particulate xenogenous bone was placed in position. B. Immediate crown installation after bone graft. C-D. After four months postoperatively, clinical and radiography images showing optimal aesthetic results and absence of infection and bone loss.

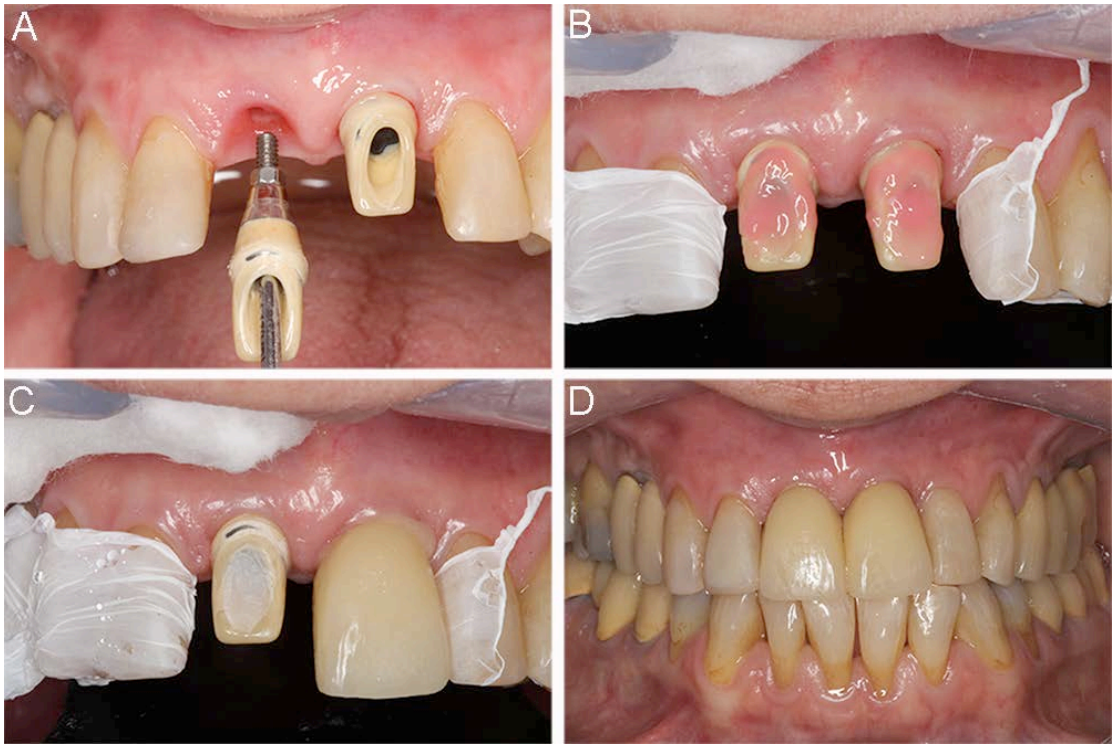

Figure 7 – A-C. Installation of the definitive abutment and crowns. D. Clinical view with the final prosthesis installed.

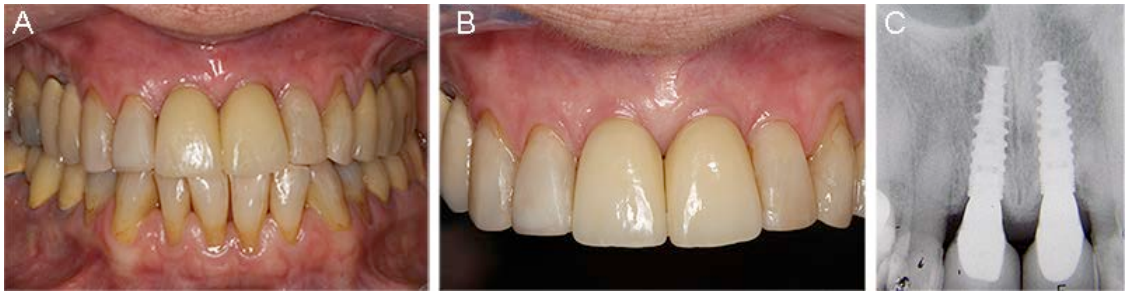

Figure 8 – A-B. Three-year follow-up revealed optimal aesthetic outcome without probing depths or gingival recession. C. The periapical radiograph showed the correct position of the implant in relation to the adjacent teeth and an increase in vertical bone formation completely filling the osseous vertical defect without marginal bone loss.

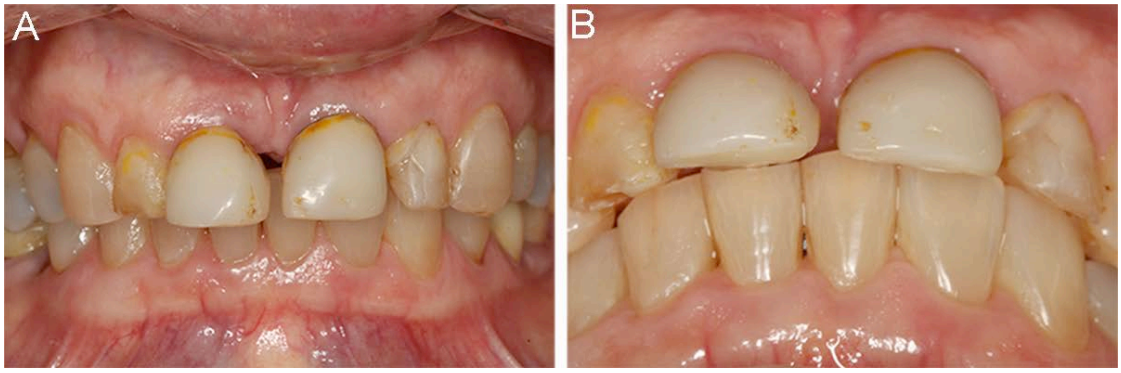

Figure 9 – A-B. Frontal and occlusal clinical views of the maxillary central incisors and the occlusal relationship.

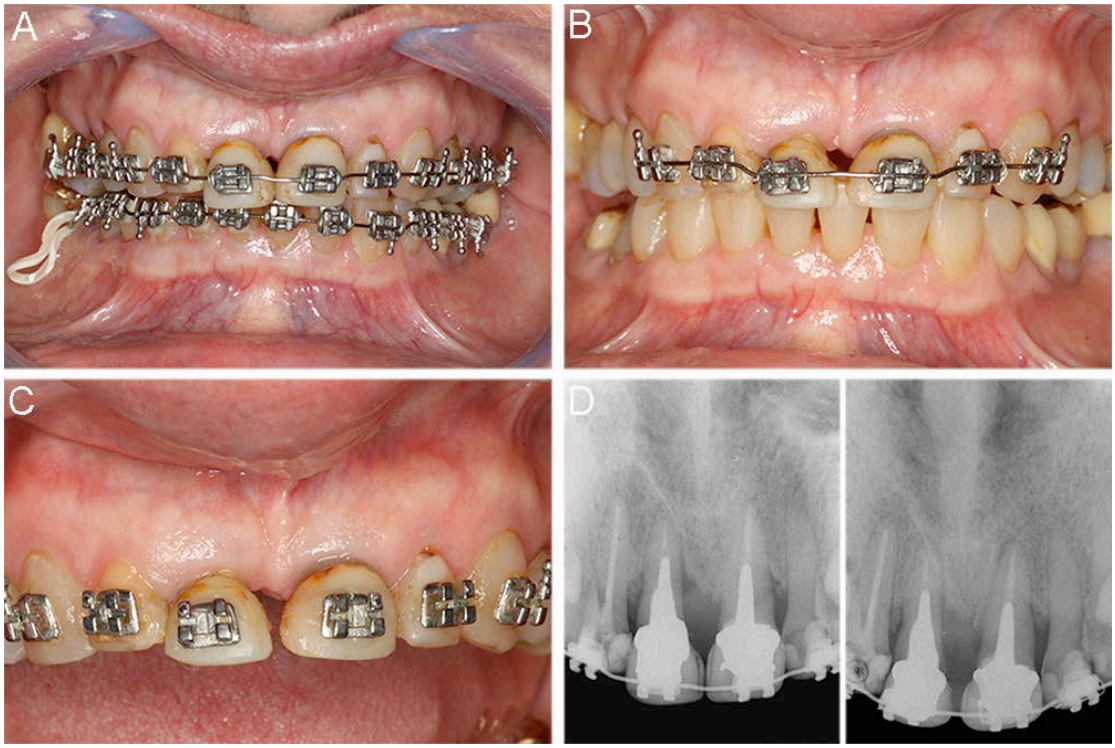

Figure 10 – A-C. Orthodontic treatment for teeth extrusion allowing vertical bone gain prior to the teeth extractions. D. Periapical radiography showing the initial situation (left) and after 8 months of active orthodontic treatment (right).

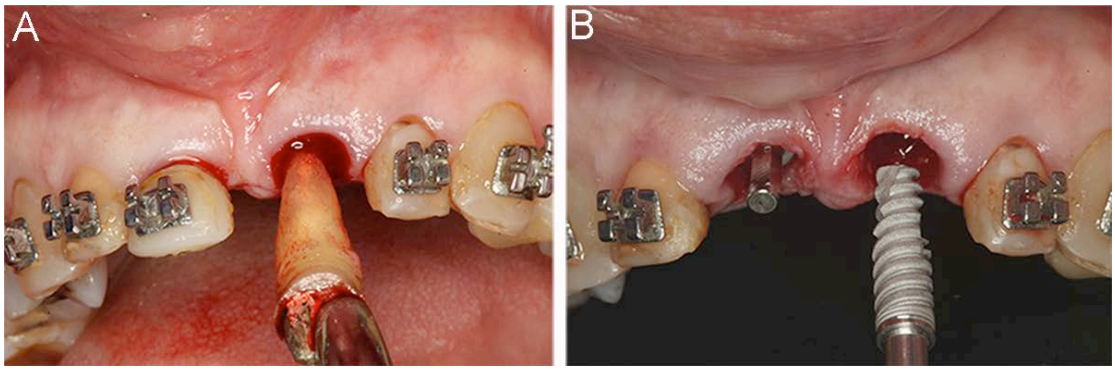

Figure 11 – A. Atraumatic teeth extraction followed by immediate implant placement (B).

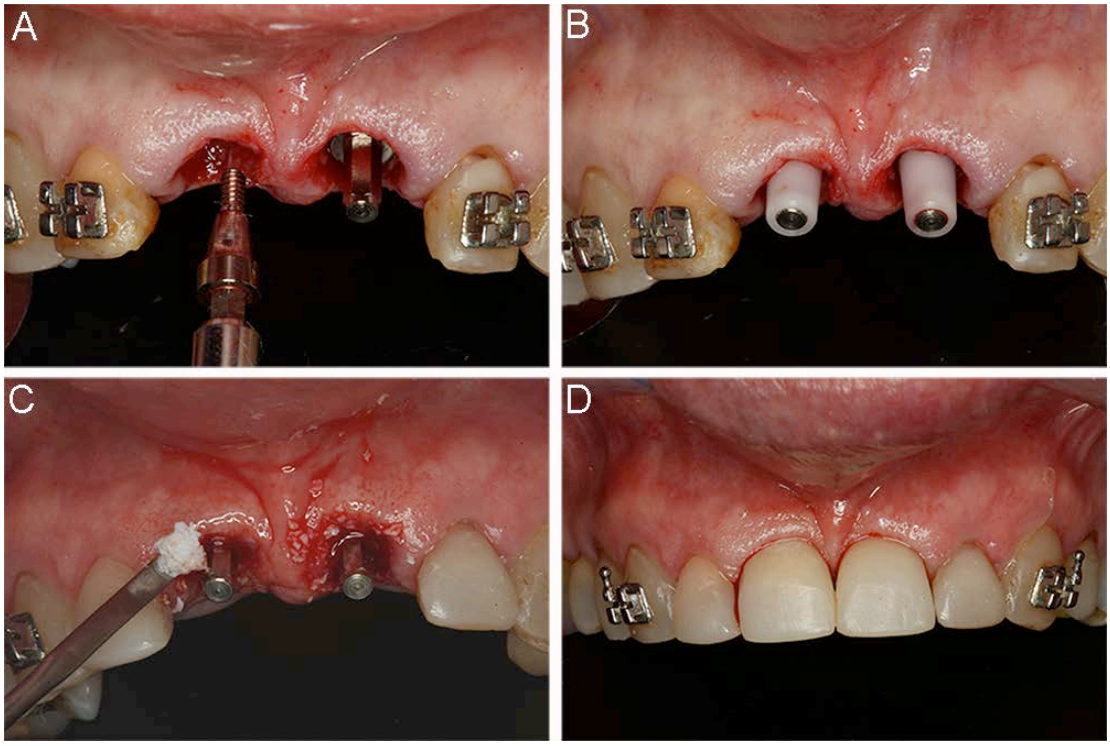

Figure 12 – A-B. Provisional abutment and copping installation. C. Bone graft was placed over the implant to fill the gap between the defect and the implant. D. Installation of the provisional crowns.

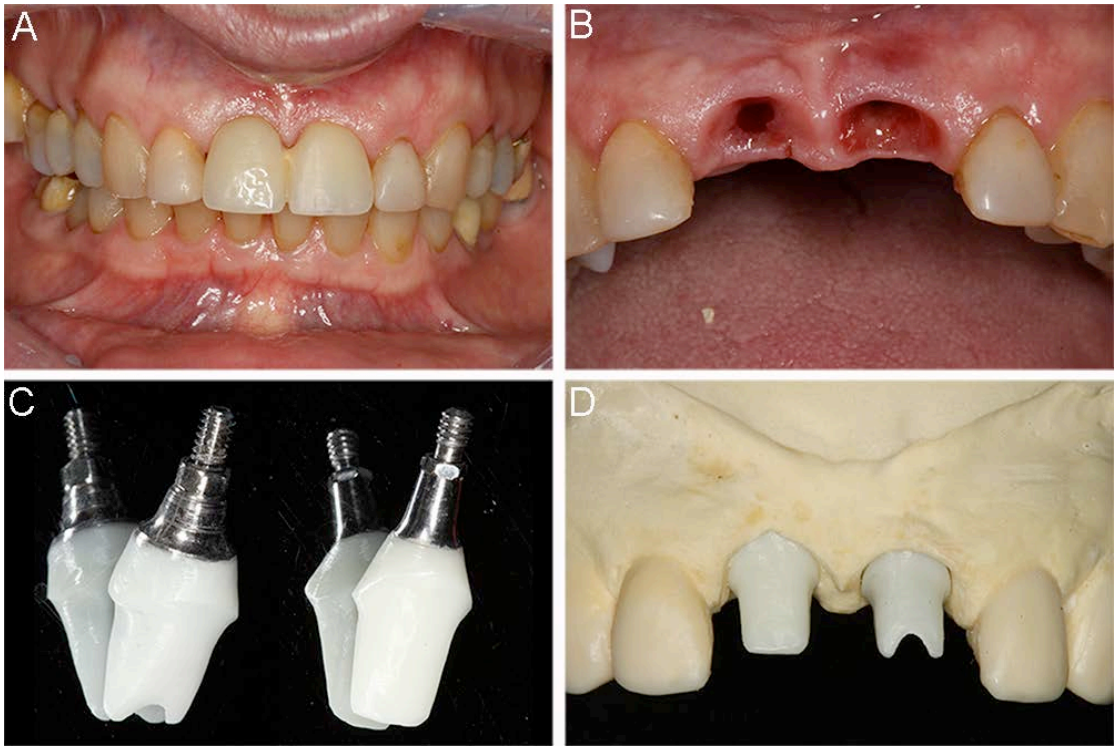

Figure 13 – A-B. Clinical view after 3 months postoperatively. C. Two custom zirconia abutments were confectioned using CAD/CAM system.

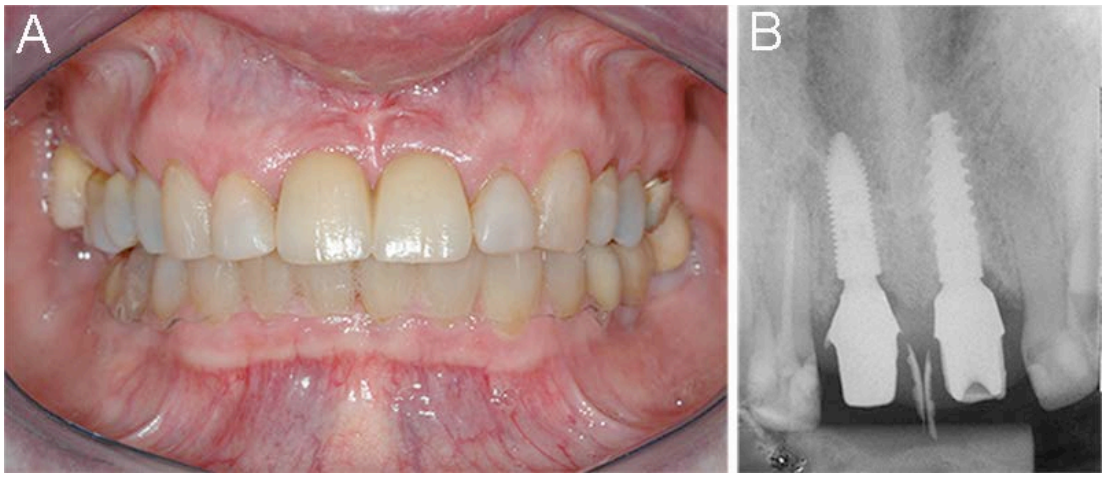

Figure 14 – A. Final clinical aesthetic outcomes and B. Periapical radiography after 3 years of follow-up.

8 Capítulo 7

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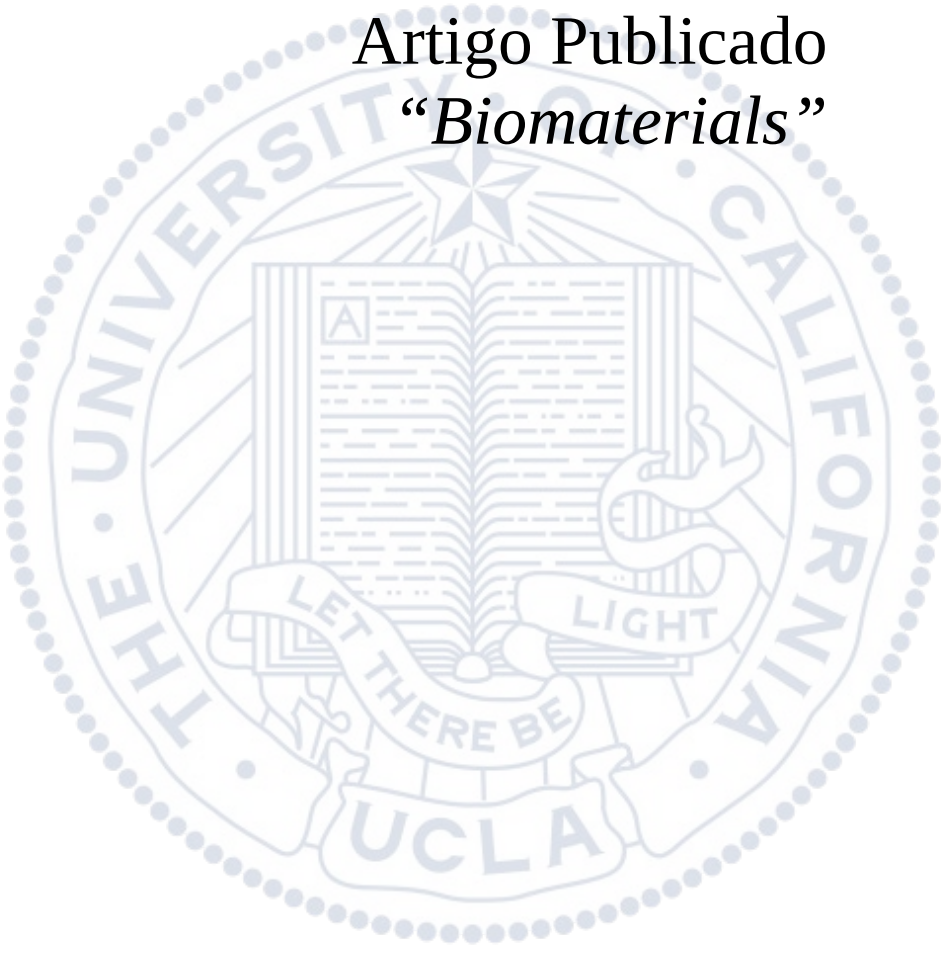

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Effect of UV-photofunctionalization on oral bacterial attachment and biofilm formation to titanium implant material

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ABSTRACT

Bacterial biofilm infections remain prevalent reasons for implant failure. Dental implant placement occurs in the oral environment, which harbors a plethora of biofilm-forming bacteria. Due to its transmucosal placement, part of the implant structure is exposed to oral cavity and there is no effective measure to prevent bacterial attachment to implant materials. Here, we demonstrated that UV treatment of titanium immediately prior to use (photofunctionalization) affects the ability of human polymicrobial oral biofilm communities to colonize in the presence of salivary and blood components. UV-treatment of machined titanium transformed the surface from hydrophobic to superhydrophilic. UV-treated surfaces exhibited a significant reduction in bacterial attachment as well as subsequent biofilm formation compared to untreated ones, even though overall bacterial viability was not affected. The function of reducing bacterial colonization was maintained on UV-treated titanium that had been stored in a liquid environment before use. Denaturing gradient gel-electrophoresis (DGGE) and DNA sequencing analyses revealed that while bacterial community profiles appeared different between UV-treated and untreated titanium in the initial attachment phase, this difference vanished as biofilm formation progressed. Our findings confirm that UV-photofunctionalization of titanium has a strong potential to improve outcome of implant placement by creating and maintaining antimicrobial surfaces.

1. Introduction

Dental implants have become a popular restorative choice with an initial success rate of up to 98% [1]. Depending on implant type, this success rate declines over time and ranges between 90.1 and 95.4% after 5 years, with a further reduction to about 89% and 83% after 10 and 16 years, respectively – the longest observation period reported so far [2]. Older patients, those with systemic conditions, smoking status [3,4] or prior periodontal disease [5–7] are affected by an overall higher failure rate. Most complications can be attributed to lack of sufficient osseointegration and infection. Hence, complete and infection-free establishment of bone-implant integration has become a persistent challenge in oral rehabilitation. The major causes for implant-related infections and inflammatory responses are microbial biofilms, which can form on all currently employed implant materials [8–10]. Biofilm formation is a multi-step process that starts with the bacterial attachment to natural or artificial surfaces. This initial interaction between bacteria and surface can occur directly via charged groups (e.g. phosphoryl-, carboxyl-, and amino-groups) present on their complex cell surface layer [11]. Since the bacterial cell surface is in direct contact with the environment, their charged cell surface layer groups are able to interact with ions or charged molecules present on the implant material surfaces [12]. In addition to this direct interaction, microorganisms can exploit other molecules including host proteins that adhere to the implant material to achieve surface colonization [13]. In the oral environment relevant for dental implant dentistry, molecules derived from saliva such as the proteins involved in

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0142-9612/

Effect of UV-photofunctionalization on Oral Bacterial Attachment and Biofilm Formation to Titanium Implant Material

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Abstract

Bacterial biofilm infections remain prevalent reasons for implant failure. Dental implant placement occurs in the oral environment, which harbors a plethora of biofilm-forming bacteria. Due to its transmucosal placement, part of the implant structure is exposed to oral cavity and there is no effective measure to prevent bacterial attachment to implant materials. Here, we demonstrated that UV treatment of titanium immediately prior to use (photofunctionalization) affects the ability of human polymicrobial oral biofilm communities to colonize in the presence of salivary and blood components. UV-treatment of machined titanium transformed the surface from hydrophobic to superhydrophilic. UV-treated surfaces exhibited a significant reduction in bacterial attachment as well as subsequent biofilm formation compared to untreated ones, even though overall bacterial viability was not affected. The function of reducing bacterial colonization was maintained on UV-treated titanium that had been stored in a liquid environment before use. Denaturing gradient gel-electrophoresis (DGGE) and DNA sequencing analyses revealed that while bacterial community profiles appeared different between UV-treated and untreated titanium in the initial attachment phase, this difference vanished as biofilm formation progressed. Our findings confirm that UV-photofunctionalization of titanium has a strong potential to improve outcome of implant placement by creating and maintaining antimicrobial surfaces.

1. Introduction

Dental implants have become a popular restorative choice with an initial success rate of up to 98% [1]. Depending on implant type, this success rate declines over time and ranges between 90.1 and 95.4% after 5 years, with a further reduction to about 89% and 83% after 10 and 16 years, respectively – the longest observation period reported so far [2]. Older patients, those with systemic conditions, smoking status [3, 4] or prior periodontal disease [5-7] are affected by an overall higher failure rate. Most complications can be attributed to lack of sufficient osseointegration and infection. Hence, complete and infection-free establishment of bone-implant integration has become a persistent challenge in oral rehabilitation. The major causes for implant-related infections and inflammatory responses are microbial biofilms, which can form on all currently employed implant materials [8-10]. Biofilm formation is a multi-step process that starts with the bacterial attachment to natural or artificial surfaces. This initial interaction between bacteria and surface can occur directly via charged groups (e.g. phosphoryl-, carboxyl-, and amino-groups) present on their complex cell surface layer [11]. Since the bacterial cell surface is in direct contact with the environment, their charged cell surface layer groups are able to interact with ions or charged molecules present on the implant material surfaces [12]. In addition to this direct interaction, microorganisms can exploit other molecules including host proteins that adhere to the implant material to achieve surface colonization [13]. In the oral environment relevant for dental implant dentistry, molecules derived from saliva such as the proteins involved in pellicle formation that provide additional bacterial adhesion sites as well as blood components can attach to the implant material and change certain surface characteristics [14-16] Therefore, implant surface characteristics and the molecules from relevant bodily fluids that can attach to the implant material are important determinants in the amount and composition of bacterial biofilm adhered.

Recent approaches to address the challenge of implant failure include ultraviolet (UV)-mediated photofunctionalization of titanium (Ti) [17-19], a popular implant material due to its excellent biocompatibility, corrosion/wear resistance and its ability to promote osseointegration [20-23]. UV irradiation leads to the modification of titanium implant surfaces from a hydrophobic to a superhydrophilic state and removes hydrocarbon contamination [17, 24, 25]. These extreme changes in surface properties have been studied extensively for their effect on enhancing osteoblast attachment and proliferation, which leads to greatly improved osseointegration of titanium implants [26-29]. Despite this very encouraging extensive research regarding bone-implant integration, very little is known to date regarding the effect of photofuntionalization on bacterial attachment and biofilm on titanium surfaces despite its importance for lasting implant success [30]. A recent report demonstrated that UV treatment of Ti surfaces can reduce attachment and monospecies biofilm formation of *Staphylococcus aureus* and *Streptococcus pyogenes* [30], the major pathogens for orthopedic implant infections. While this is a very promising observation, dental implants are exposed to a more challenging environment: the microbiota of the oral cavity. Extensive 16S rRNA gene sequencing and microbiome studies revealed that over 600 different oral microbial taxa colonize the various surfaces present in the mouth [31]. The bacterial species implicated in dental implant-associated diseases such as peri-mucositis and peri-implantitis are generally very similar to those associated with periodontal diseases [32, 33]. Many of these bacteria are able to readily attach to surfaces and employ saliva and/or blood-derived proteins for enhanced attachment [16, 34]. This ability to exploit host fluids for attachment presents an additional challenge in biofilm prevention, especially during implant placement, when the sterile surface becomes exposed to the oral environment and the surgical wound site.

In this study, we investigated if a short 15 min UV-treatment of titanium surfaces, which in contrast to the standard 48 hours irradiation period could provide a convenient chair side application, has an effect on the attachment and biofilm formation of complex oral microbial communities. UV-irradiation-induced titanium surface properties were evaluated and bacterial biomass accumulation at different time points reflecting initial attachment and early biofilm formation events were determined in the presence of salivary and blood components. Community profiles of the attached microorganisms were compared between UV-treated and untreated titanium surfaces.

2. Materials and methods

2.1 Titanium disc preparation, surface analysis and UV treatment

Titanium (Ti) discs (20 mm in diameter and 1.5 mm in thickness) were prepared by machining commercially pure titanium (Grade 2). Titanium discs were treated with UV light for 12 minutes with a photo device (TheraBeam SuperOsseo, Ushio), while control discs were left untreated. The surface morphology and chemistry of the discs were examined using scanning electron microscopy (XL30, Philips, Eindhoven, Netherlands) and energy dispersive X-ray analysis (EDX) (JSM-5900LV, Joel Ltd, Tokyo, Japan), respectively [26]. The hydrophilic and hydrophobic properties of the titanium discs were evaluated by measuring the contact angle of 10 µl ddH_2O.

2.2 Oral microbial community and culture conditions

We used a previously described cultivable microbial community representative of the complex oral microbiome as model system for bacterial attachment and biofilm formation [35, 36]. The microbial community was grown anaerobically (80% N_2, 10% H_2, and 10% CO_2) at 37°C in a modified rich medium (SHI-FSMS) developed to support a high number of

oral taxa from human saliva samples (50% SHI medium [36], 25% filtered saliva, 0.5% mannose, 0.5% sucrose). Initial attachment of cells and biofilm formation were evaluated after 3 and 16 hours incubation, respectively. For evaluation of attachment at the 3 hours time point, the optical density (OD) at 660 nm of the bacterial suspensions was adjusted to 1. For measurement of biofilm formation after 16 hours, the OD_{600nm} of overnight oral microbial community cultures was adjusted to 0.1 in fresh SHI-FSMS medium. For both types of experiments, one ml of the oral microbial community at the respective concentration was placed onto titanium discs immediately after UV-photofunctionalization or directly onto untreated discs in sterilized 12-well polystyrene culture plates (Fisher Scientific). Oral microbial community cultures at the two relevant concentrations used in this study were also inoculated directly into the polystyrene plate to serve as positive controls for bacterial attachment and biofilm formation. Additionally, UV-irradiated and untreated titanium discs as well as wells without discs were incubated with sterile medium to serve as background controls. Samples were statically incubated at 37°C under anaerobic conditions for 3 or 16 hours corresponding to the respective experiments. To evaluate the continuous effect of UV-treatment, UV-treated and untreated titanium discs were immersed in fresh SHI-FSMS medium for 24 hours at 37°C**,** under anaerobic conditions prior to inoculation of bacteria. After this pre-incubation period, the medium was removed and overnight oral community culture diluted to an OD_{600nm} of 0.1 was added to disc for analysis of biofilm formation after 16 hours incubation. Concurrently, the diluted community culture was placed on UV-treated and untreated titanium discs surfaces that had not undergone pre-immersion in medium for comparison. For all experiments, medium was removed at the end of the incubation period, discs were gently transferred into 6-well polystyrene culture plates and washed three times with 5 ml sterile phosphate-buffered saline (PBS) prior to further processing.

2.3 Crystal violet assay

A 0.5% crystal violet solution was used to determine biomass accumulation onto the titanium discs surface and control wells. The PBS-washed titanium discs were placed into a 12-well plate, submerged in one ml crystal violet solution and incubated at room temperature for 20 min. The discs were then carefully transferred to a 6-well plate and washed four times with 5 ml PBS to remove excess crystal violet. The plates were gently shaken for 5 minutes during the last two PBS washes to ensure complete removal of residual dye. After the final PBS wash, the discs were transferred to a new 12-well plate. One ml of 95% ethanol was added and the plate was incubated at room temperature on a rotatory shaker (VWR rocking double platform shaker model 200) at 250 rpm for 15 minutes. The ethanol solution containing the crystal violet stain retained by the biofilms was transferred into 1.5 ml cuvettes (USA Scientific**)** and the optical density at 595 nm was determined for total biomass evaluation. All experiments were performed in triplicate for each time point and repeated three times to ensure technical and biological reproducibility with the exception of the experiments, in which untreated and UV-photofunctionalized titanium discs were pre-incubated in SHI-FSMS medium for 24 hours prior to bacterial inoculation. These were only repeated two times as technical triplicates.

2.4 Sample Preparation, Confocal Laser Scanning Microscopy and ImageJ analysis

Samples for assessment of bacterial attachment and biofilm formation after 3 and 16 hours incubation as described above on untreated and UV-treated titanium disc surfaces were rinsed three times with PBS to remove unattached bacteria prior to fluorescent labeling with the LIVE/DEAD BacLightTM Bacterial Viability staining kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The titanium discs were placed onto a glass cover slip, 35x50 mm (Fisher Scientific) into a 10 µl drop of PBS with the biofilm side down.

Samples were visualized with a PASCAL LSM5 confocal laser-scanning microscope (Zeiss, Jena, Germany). The scanning module of the system was mounted on an inverted microscope (Axiovert 200 M) and samples were imaged through 10x dry (Plan NeoFluar NA 0.3 air) and 40x oil-immersion (Plan NeoFluar NA 1.3 oil) objectives. Excitation wavelengths of 488 nm (Ar laser) and 543 nm (HeNe laser) in combination with 505 to 530 nm bandpass and 560 nm longpass filters, respectively, were employed to reveal live dead distribution of bacterial cells as well as the accumulated biomass.

Image analysis was performed with ImageJ 1.48 [National Institutes of Health (NIH), Bethesda, MD, USA; freeware from http://imagej.nih.gov/ij/download.html]. To measure the area occupied by bacteria, representative confocal images were exported and converted into 16bit. A median filter with a radius of 2 pixels was applied to reduce noise and an intensity threshold was applied to separate the fluorescent bacteria from the background. Next, the thresholded image was converted into a binary image and using the 'analyze particles' function, all the groups of cells bacteria with a minimal surface area of 2 μm^2 were calculated. Analysis of biofilm density was measured by conversion of confocal images into RGB color. First, background subtraction was applied using a sequentially decreasing rolling-ball radius to ensure maximum removal of background noise. Then, the image was assembled into color channels and the integrated density of pixels was calculated. All experiments were performed in technical duplicates for each time point with two biological repetitions.

2.6 DNA Extraction, PCR and DGGE

Microbial cells were harvested from the titanium discs by scraping with a sterile pipette tip and placed into 1.5 ml Eppendorf Tube containing 150 µl PBS. Total genomic DNA was isolated using the MasterPure^{TM} DNA purification kit (EPICENTRE). The concentration of bacterial DNA was determined with a Nanodrop 2000 (Thermo Scientific). Primers Bac1 with

a GC clamp (5’-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAC TAC GTG CCA GCA GCC-3’) and Bac2 (5’-GGA CTA CCA GGG TAT CTA ATC C-3’) were used to amplify a region approximately 300-base-pair in length (bp) of the 16S ribosomal RNA gene [37]. PCR amplification was confirmed by electrophoresis in a 1.0% agarose gel. Polyacrylamide gels at an 8% concentration were prepared with a denaturing urea/formamide gradient ranging from 40% to 60%. Approximately 45 µl of the PCR product were loaded into each well. The gel was submerged in 1X TAE buffer (40 mM Tris base, 40 mM glacial acetic acid, 1 mM ethylenediaminetetraacetic acid) and the PCR products were separated by electrophoresis for 17 hours at 58°C using a fixed voltage of 60 V in a Bio-Rad DCode System (Bio-Rad Laboratories, Inc., Hercules, CA). Gels were stained with ethidium bromide to visualize the bands on the gel. Gel images were taken with the Molecular Imager Gel Documentation system (Bio-Rad Laboratories). All experiments were performed in triplicate for each time point.

2.7 DNA Sequencing of excised DGGE bands

Bands of interest were excised from the DGGE gel with a sterile razor blade, placed into 1.5-mL tubes containing 15 µl sterile Milli-Q water. The tubes were incubated overnight at 4°C to allow the DNA to diffuse into the water. Five µl of the DNA sample were used as template for re-amplification with the universal primers, Bac1 and Bac2, and the product was sent to Laragen Sequencing & Genotyping (Culver City, CA, USA) for sequencing. For identification, the 16S rDNA sequences were compared with the Human Oral Microbiome Database (HOMD) using BLAST.

2.8 Statistical data analysis

All data are presented as a mean ± standard deviation. Statistical comparisons were performed using the unpaired *t*-test, one tailed, except for evaluation of the effect of UV treatment on titanium disc surfaces immersed for 24 hours in SHI-FSMS medium prior to bacterial inoculation compared to discs that did not undergo the pre-immersion step. In this case, one way analysis of variance (ANOVA) was employed with a Tukey's posthoc test using GraphPad Prism version 5.0c; $p < 0.05$ was considered statistically significant.

3. Results

3.1 UV-photofunctionalization increased the hydrophilicity of titanium surfaces without affecting its topography

SEM analysis confirmed that after a 12 minute UV-photofunctionalization treatment, the titanium discs used in this study maintained their close packed structure and meso-scale parallel traces typical for machined titanium surfaces (Fig. 1a). Hydrophilicity testing demonstrated that this short UV-treatment resulted in a drastic change in titanium wettability to ddH_2O. Untreated control titanium discs were highly hydrophobic with the 10 µl droplet of ddH_2O remaining in a semispherical form without spreading and a contact angle higher than 80° (Fig. 1b, d). In contrast, after UV-treatment the titanium discs became very hydrophilic, as evident by the immediate spreading of the ddH_2O droplet and a contact angle of less than 5° (Fig. 1c, d). The corresponding EDS spectra revealed that untreated titanium disks contained a clear elemental peak of carbon indicative of high carbon concentrations on the titanium surface (data not shown). This peak was significantly reduced in UV-treated disks with the percentage of surface carbon being approximately about half of the amount present on untreated titanium disks ($p<0.0001$). The effect of UV-treatment on surface characteristics was maintained for more than 24 hours (Fig. 2). While the untreated control discs remained

hydrophobic regardless if they were stored in air or in a liquid environment (Fig. 2a, c), UV-treated discs retained their hydrophilic characteristics especially when the discs were kept in a liquid environment (Fig. 2b, c). Even though UV-treated titanium surfaces were still relatively hydrophilic after 24 hours of storage in air compared to their untreated counterparts, surface wettability reversed significantly towards more hydrophobic during the experimental time period (Fig. 2b, c). This difference in contact angle between UV-treated discs subjected to different storage conditions was statistically significant ($p<0.0001$).

3.2 UV-treatment reduced bacterial attachment on titanium surfaces

Next, we examined whether the surface property changes of titanium surfaces induced by the 12 min UV-treatment described above had any influence on bacterial attachment and early biofilm formation. The biomasses attached to titanium discs that were subjected to UV-treatment and those without were evaluated after three hours of incubation with a cultured mixture of salivary bacteria using a variety of different approaches. Crystal violet staining disclosed a statistically significant difference in overall bacterial attachment with 2.6-fold less biomass evident on the UV-treated titanium surfaces compared to untreated ones (Fig. 3). Qualitative assessment of the attached biomass via confocal microscopy confirmed that the UV-treated titanium discs amassed considerably less bacteria compared to their untreated counterparts (Figs. 4a, b). Consistent with the observed difference in bacterial attachment, bacterial colonies on untreated surfaces were generally larger and more abundant (Fig. 4c) compared to the sparse colonization on UV-treated titanium (Fig. 4d). Fluorescent live/dead staining revealed that viability of the attached bacteria was very similar between both types of titanium discs (Fig. 4a-d). Detailed quantitative analysis of the confocal images determined that density and area coverage of the attached biomass was significantly reduced (3.2- and

2.8-fold, respectively) on titanium discs that underwent UV-treatment (Figs. 4e, f), even though the height was not affected (data not shown).

3.3 UV- photofunctionalization treatment reduced bacterial biofilm formation on titanium surfaces

Since our UV-treatment drastically reduced the attachment of salivary bacteria to titanium discs, we evaluated if this effect is sustained during the subsequent biofilm formation and maturation process. Crystal violet staining revealed that after 16 hours of biofilm formation the UV-treated titanium discs had accumulated significantly less (3-fold) biomass compared to untreated discs (Fig. 5). The difference in biofilm formation between UV-treated and untreated titanium discs was confirmed by confocal and scanning electron microscopy analysis. An obvious difference in the overall density of coverage as well as the frequency and size of microcolonies was apparent between the two different surfaces (Figs. 6a, b), while biofilm viability as revealed by a fluorescent live/dead stain was similar. Higher magnification imaging with confocal and scanning electron microscopy revealed that colonization of UV-treated surfaces is clearly more sparse with fewer, smaller and more scattered cell clusters as compared to untreated titanium discs, which were covered with larger, taller and more widespread microcolonies (Figs. 6c-f). Consistent with these qualitative microscopic impressions and above overall biomass determination via crystal violet staining, we found that the average biofilm density and the area coverage of biofilm cells on untreated surfaces were 2.7-fold and 4.2-fold, respectively, higher than on UV-treated titanium discs (Fig. 6g-h).

In order to investigate if the effect of UV-photofunctionalization on bacterial biofilm formation is maintained in an aqueous environment, UV-treated and untreated titanium discs were immersed in SHI-FSMS medium for 24 hours prior to biofilm development for 16 hours

as described in Material and Methods. Concomitantly, we developed biofilms for 16 hours on UV-treated and untreated titanium surfaces without pre-immersion in medium and evaluated the corresponding biomass accumulation as a control. While there was no meaningful difference between pre-immersed and directly used untreated or UV-treated titanium discs, the previously observed significant effect of UV treatment on biofilm formation was sustained even after pre-incubation of the discs in SHI-FSMS medium for 24 hours (Fig. 7). The differences between the respective untreated and UV-treated titanium surfaces were about 4-fold for both the pre-immersed and the directly used discs (Fig. 7).

3.5 UV- photofunctionalization treatment does not affect the bacterial biofilm community profile on titanium surfaces

Our next goal was evaluate whether the UV-treatment in addition to reducing biofilm accumulation had an influence on the type of bacteria that adhered to and formed biofilms on titanium surfaces. Community profiling via DGGE disclosed that during the 3 hours attachment and early biofilm formation period, the bacterial compositions for untreated and UV-treated surfaces was very similar with a possible difference in the dominance of certain taxa (Fig. 8). The overall profile was maintained in the samples collected after 16 hours of biofilm formation with minor differences in banding pattern compared to the 3 hours samples. Sequencing of selected bands that exhibited different intensities and comparison to the Human oral Microbial Database (HOMD) identified the corresponding microorganisms as *Fusobacterium periodonticum* (band 1), two *Streptococci* of the Mitis group (bands 2 and 3), *Porphyromonas sp* [HOT_279] (band 4), *Gemella sp* (band 5), *Peptostreptococcus stomatis* (band 6) and an additional *Streptococcus sp* (band 7).

4. Discussion

Photofunctionalization of titanium has previously been demonstrated to provide many benefits to this popular implant material including greatly enhanced osteoblast attachment and proliferation, improved osseointegration as well as reduced attachment of dangerous wound pathogens [17, 26, 30]. The surface property changes induced by UV-treatment such as super-hydrophilicity and removal of hydrocarbon contamination have significant impact on implant failure, which can often be linked to infection and/or incomplete osseointegration. Typical UV-treatment times, however, last for 48 hours [17, 19, 26, 38], which is prohibitive for convenient chair side application. In this study, we demonstrated that a brief 15 min UV treatment of titanium in a specialized patented photo device is sufficient to alter titanium surface properties (Fig 1) similar to those reported previously for longer treatment times [19, 26]. Even upon this short UV-exposure time titanium surfaces displayed a drastic change from hydrophobic to very hydrophilic accompanied by a significant reduction in hydrocarbon contamination present on the surface. Importantly, we demonstrated that these properties were maintained in an aqueous environment, while they slowly reversed upon exposure to air. Maintenance of surface hydrophilicity after implant placement is a critical factor during the wound healing and bone formation processes to achieve the most favorable outcome by enhancing interaction between the implant material and host cells. The short photofunctionalization treatment employed here was recently shown to result in accelerated and enhanced osseointegration in orthopedic animal models [29], similar to the longer UV-treatment times employed in earlier studies [39].

In addition to maximizing osseointegration, preventing the colonization of implant surfaces with bacteria is another essential consideration in implant surgery. Bacterial contamination during surgery is especially a concern for the placement of dental implants, which occurs in the challenging non-sterile environment of the oral cavity that harbors

billions of bacteria comprised of over 600 different species [40, 41]. To date polymicrobial infections such as peri-implantitis remain an important cause for implant failure, especially in patients with a previous history of periodontal diseases [5, 42]. To address the potential of our 15 min UV-treatment to improve this critical aspect of implant failure, we investigated if the changes in titanium surface properties had an effect on bacterial attachment to and biofilm formation on titanium surfaces. Overall, most pathogenic bacteria have been described as rather being hydrophobic, which plays a critical role in attachment and biofilm formation [43-45]. Ultimately, however, the diversity of oral microbial population and their interspecies interactions as well as the environmental factors modulate the contact with the surface [46, 47]. As a model system, we used a cultivable polymicrobial community representative of the oral cavity [36] in combination with a bacterial growth medium (SHI-FSMS) containing saliva and blood components that are typically present during dental implant surgery. Especially salivary proteins are known to play important roles in the surface attachment of oral bacteria and many species have evolved specialized adhesins that specifically recognize distinct saliva components [48, 49]. We demonstrated via several independent approaches that the convenient 12 min photofunctionalization of machined titanium reduced the amount of oral community-derived bacteria that attach to the surface by about 3 to 4-fold during the critical period after the initial implant placemen, when the bone-generating osteoblasts compete with microorganisms for space on the newly available surface. This reduction in biomass accumulation was also reflected by reduced biofilm density and surface area coverage, even though bacterial viability was not affected. These results are very encouraging, considering the importance of surface availability in the competition between bacterial and eukaryotic cell attachment [50]. These findings are consistent with a previous study by Yamada and co-workers, who examined the early stages of biomass accumulation by two important wound pathogens, *Staphylococcus aureus* and *Streptococcus pyogenes*, on different

titanium surfaces subjected to UVA and UVC irradiation for 48 hours in a monospecies culture model [30].

Notably, this reduction in bacterial attachment and biofilm formation on the UV-treated titanium surface is sustained for at least 16 hours and thus well beyond the six hours post-implantation period during which implants are considered to be particularly susceptible to bacterial colonization [51, 52]..We also observed that the biomass attached to UV-treated titanium remained sparse, predominantly consisting of individual attached cells and small cell clusters compared to untreated surfaces that were mostly covered in maturing biofilms comprised of large colonies that appeared to be encased in matrix material. In addition to preventing host cell attachment to the implant by competing for space, biofilms have also been shown to elicit much stronger inflammatory response – an important reason for bone loss – compared to planktonic bacteria [53, 54]. Furthermore, bacteria contaminating titanium implants have been demonstrated to migrate into and infect surrounding tissues, while apparently not being cleared by macrophages [55]. A weaker immune response against biofilm cells compared to their planktonic counterparts has been described [56, 57] which makes prevention of biofilm formation on implant materials a key factor in averting infections. Another promising result of our study for clinical applications is the finding that even after pre-immersion of the titanium surfaces for 24 hours in the saliva and blood components containing SHI-FSMS medium, the reducing effect of photofunctionalization on biomass accumulation is maintained. This prolonged protective effect against bacterial colonization in combination with the enhancement of osteoblast attachment and proliferation [17, 19, 26, 39] has a strong potential to not only promote continued implant osseointegration but also reduce the risk of implant loss due to bacterial biofilm infections. The time period tested in this study is sufficient to allow for initial wound healing and formation of a protective clot that minimizes bacterial access from the oral cavity to the wound site.

Furthermore, if bacteria were able to enter the implant placement site they would not be able to efficiently attach to the implant surface and form a biofilm but rather stay planktonic and thus be easier to clear by the host immune system.

Since photofunctionalization induces a drastic change in titanium surface properties from hydrophobic to superhydrophilic and hydrophobic/philic interaction play an important role in initial bacterial attachment, we examined if the altered surface properties would also affect the profile of bacteria colonizing the untreated versus the UV-treated titanium discs. DGGE analysis revealed a notable difference between UV-treated and untreated surfaces in the bacterial community profile and prevalence of the corresponding microorganisms attached to the respective titanium surfaces during the 3 hours initial attachment period. Surprisingly, streptococci of the Mitis group as well as *Porphyromonas sp*, which have been described as generally hydrophobic [58] are more prevalent in the communities isolated from the superhydrophilic UV-treated titanium discs. Other studies, however, demonstrated that hydrophobicity of titanium surfaces is not a factor in *P. gingivalis* and *S. sanguinis* attachment [59] and that cell surface hydrophobicity of *P. gingivalis* greatly vary in a strain-dependent manner [60, 61] *Fusobacterium periodonticum* also appeared to preferentially attach to the UV-treated surfaces, which is consistent with a previous report showing that fusobacteria colonize hydrophilic surfaces at a higher rate [59]. Overall microorganisms have been found to display a wide range of hydrophobicities depending on environmental conditions. Currently, there is not a clear consent on the role of cell surface characteristic in the ability of bacteria to attach to hydrophilic or hydrophobic surfaces, even though hydrophobic interactions generally seem to favor biofilm formation [62]. Furthermore, the salivary and blood components used in our model system that are relevant in the clinical context of implant placement are likely an important factor for the profile of initial bacterial attachment. Especially salivary components are known to provide important binding sites for the surface

colonization with oral bacteria [63, 64]. The distinct difference in bacterial profiles between UV-treated and untreated titanium was limited to the initial surface attachment. After 16 hours of biofilm formation, the attached bacterial communities were identical, regardless if the discs were pre-immersed in medium for 24 hours or not. This could be due to the fact that after the initial attachment, bacteria start conditioning the surface with matrix material and thus would mask the actual titanium surface properties. Interestingly, the profiles after 16 hours largely resemble the community attached to untreated titanium surfaces after 3 hours with only the appearance of an additional streptococcus species as a noticeable difference.

In conclusion, we demonstrated in our study that the changes in titanium surface properties induced by a relatively short photofunctionalization treatment leads to a significant reduction in the attachment and biofilm formation by oral bacteria. Importantly, this effect is maintained in the presence of salivary and blood components, which are typically present in the oral environment of a dental implant placement site during time periods that exceed those considered critical for bacterial contamination and initial wound healing. Therefore, this chairside-friendly application for modification of titanium properties that was already shown to significantly enhance osseointegration in previous studies can provide the additional benefit of reducing bacterial implant contamination during the surgical process. These qualities have a strong potential to significantly reduce dental implant-relaled diseases and failure.

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Figures Legends

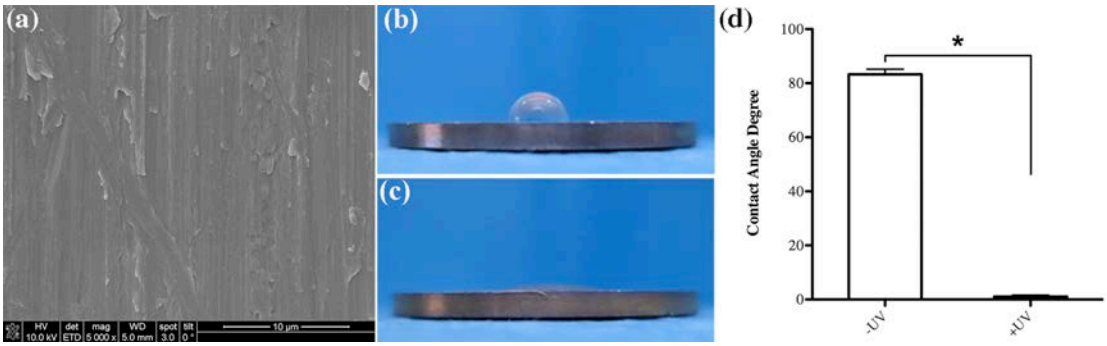

Fig. 1 Surface characterization of machined titanium. (**a)** scanning electron microscopy of UV-treated discs showing surface topography at a 5000x magnification, evaluation of hydrophilicity by contact angle measurement of 10µl ddH_2O **(b)** before and **(c)** after UV-treatment on the Ti discs and (**d)** statistical significance testing of contact angle values for -UV and +UV titanium discs. Statistically significant differences are indicated as: * $p<0.0001$. Data represent the mean ± SD of one independent experiment.

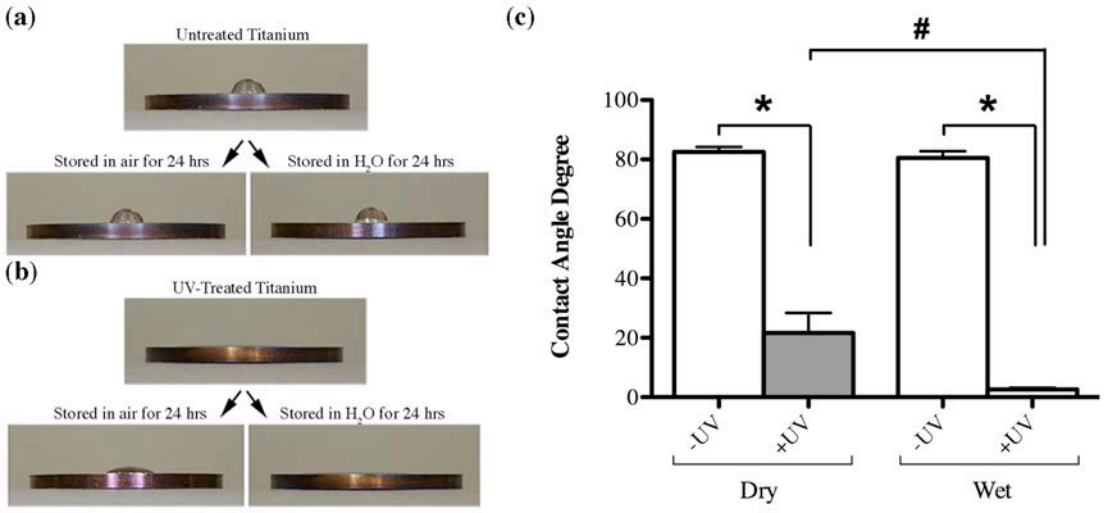

Fig. 2 Measurement of the contact angle of 10µl ddH_2O to evaluate changes in hydrophilicity of titanium discs surfaces **(a)** without and **(b)** after UV-photofunctionalization treatment as well as subsequent storage in air or a liquid environment for 24 hours. Comparison of contact angles values (**c)** between untreated (white bars, - UV) and UV-treated (gray bars, +UV) after storage in air (Dry) or a liquid environment (Wet). Statistically significant differences are indicated as: * $p<0.0001$, # $p=0.0042$. Data represent the mean ± SD of one independent experiment.

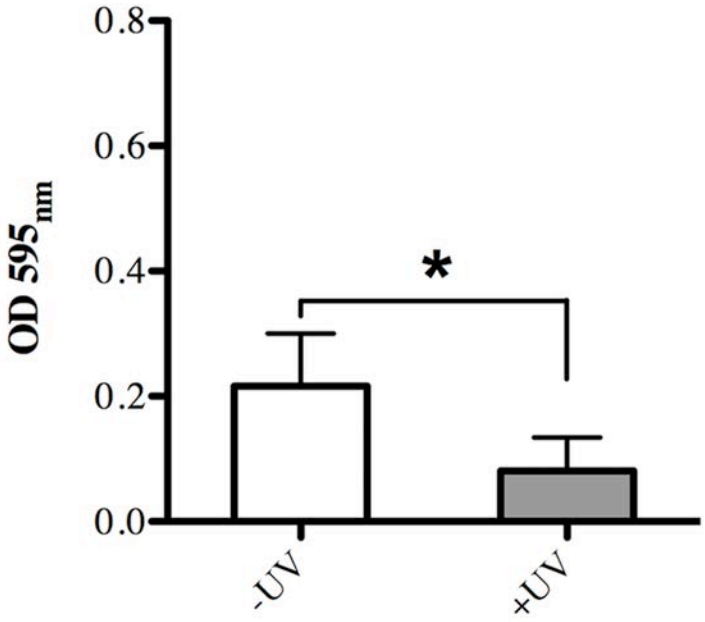

Fig. 3 Effect of UV-treatment of titanium surfaces on bacterial attachment after 3 hours incubation evaluated via quantitative measurement of crystal violet staining as indicator of biomass accumulation on untreated (white bar, –UV) in comparison to UV-treated (gray bar, +UV) titanium discs. Each value represents the mean ± SD of nine samples comprised of three technical replicates of three independent biological experiments. * indicates a statistically significant difference ($p<0.05$).

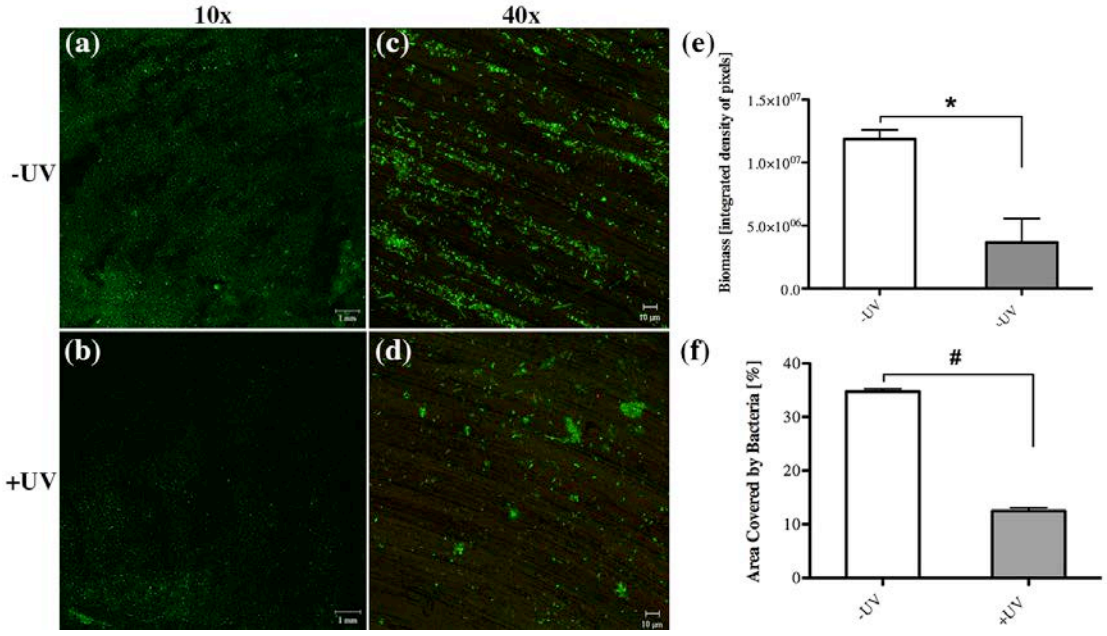

Fig. 4 Effect of UV-treatment of titanium surfaces on bacterial attachment after 3 hours incubation evaluated via confocal microscopy imaging through **(a,b)** 10x and **(c,d)** 40x objectives with representative images illustrating the live/dead distribution of bacterial cells (green for live cells, red for compromised cells) accumulated on **(a,c)** untreated and **(b,d)** UV-treated titanium discs. Quantitative comparison of **(e)** accumulated biomass and **(f)** the area covered by bacteria between untreated (white bar, –UV) in comparison to UV-treated (gray bar, +UV) titanium discs. Each value represents the mean ± SD of four samples comprised of two technical replicates of two independent biological experiments. Statistically significant differences are indicated as: * p=0.0145, # p=0.0003.

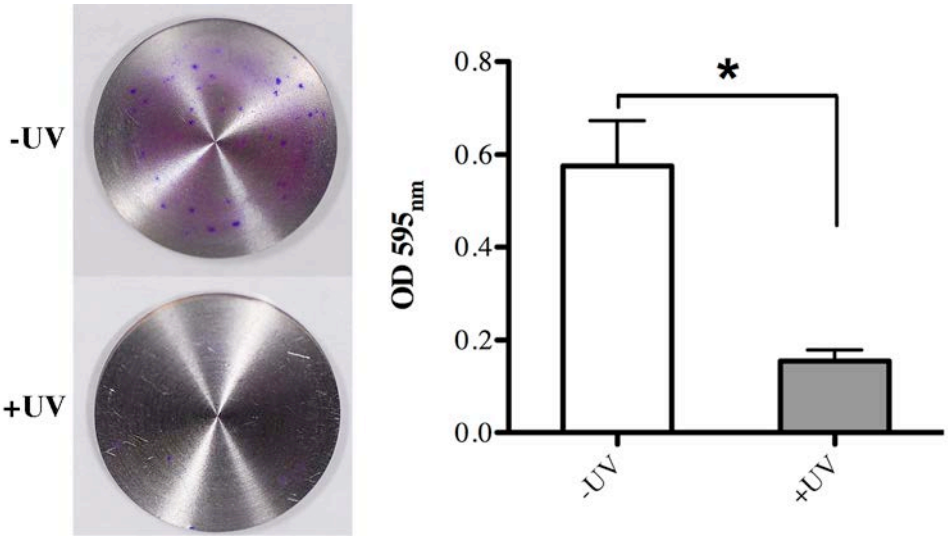

Fig. 5 Effect of UV-treatment of titanium surfaces on bacterial biofilm formation after 16 hours incubation evaluated via quantitative measurement of crystal violet staining as indicator of biomass accumulation on untreated (white bar, –UV) in comparison to UV-treated (gray bar, +UV) titanium discs. Each value represents the mean ± SD of nine samples comprised of three technical replicates of three independent biological experiments. * indicates a statistically significant difference ($p<0.001$).

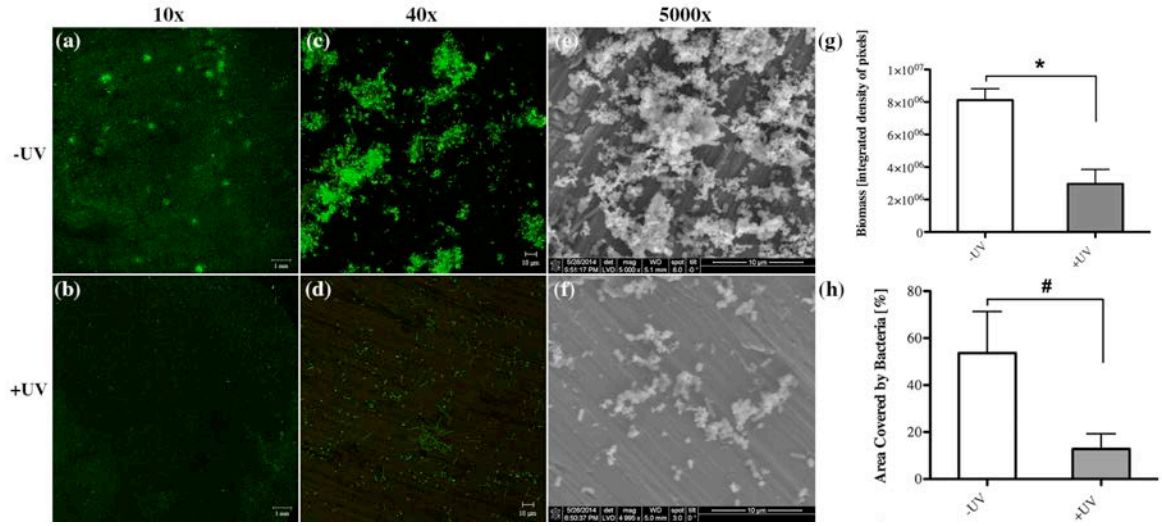

Fig. 6 Effect of UV-treatment of titanium surfaces on bacterial biofilm formation after 16 hours incubation evaluated via confocal microscopy imaging through **(a,b)** 10x and **(c,d)** 40x objectives with representative images illustrating the live/dead distribution of bacterial cells (green for live cells, red for compromised cells) accumulated on **(a,c)** untreated (-UV) and **(b,d)** UV-treated (+UV) titanium discs. Scanning electron microscopy revealing the biofilm structure on **(e)** -UV and **(f)** +UV titanium discs. Quantitative comparison of **(e)** accumulated biomass and **(f)** the area covered by bacteria between untreated (white bar, –UV) in comparison to UV-treated (gray bar, +UV) titanium discs. Each value represents the mean ± SD of four samples comprised of two technical replicates of two independent biological experiments. Statistically significant differences are indicated as: * $p<0.0116$), # $p<0.0461$.

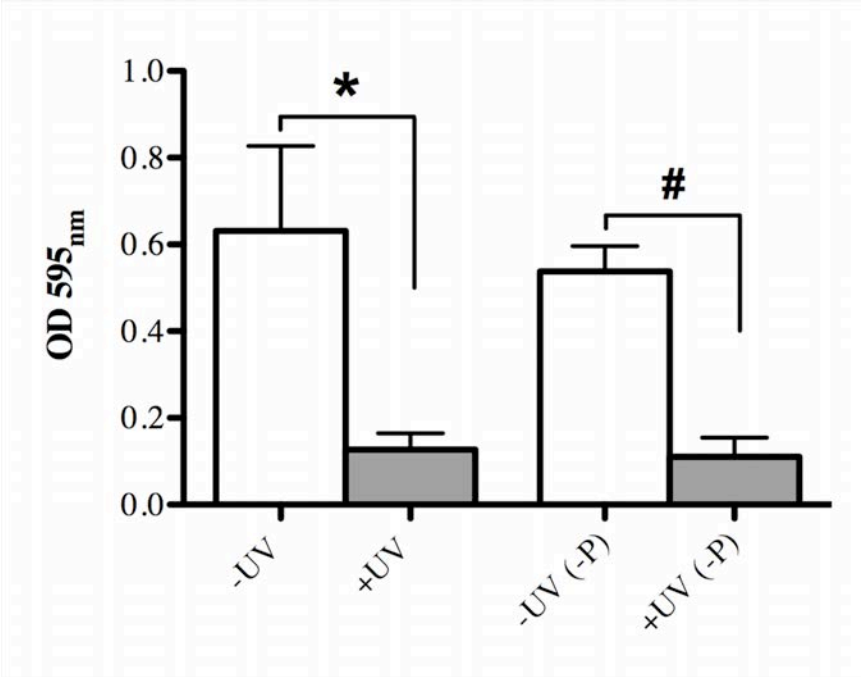

Fig. 7 Sustainability of UV-treatment of titanium surfaces and the effect on 16 hours bacterial biofilm formation after immersion of titanium discs in liquid SHI-FSMS medium for 24 hours prior to incubation with bacteria. Quantitative measurement of crystal violet staining showing biomass accumulation on untreated (white bar, –UV) in comparison to UV-treated (gray bar, +UV) titanium discs for directly used samples and those pre-immersed in liquid SHI-FSMS (-P). Each value represents the mean ± SD of four samples comprised of two technical replicates of two independent biological experiments. Statistically significant differences are indicated as: * $p=0.0012$, # $p<0.0001$.

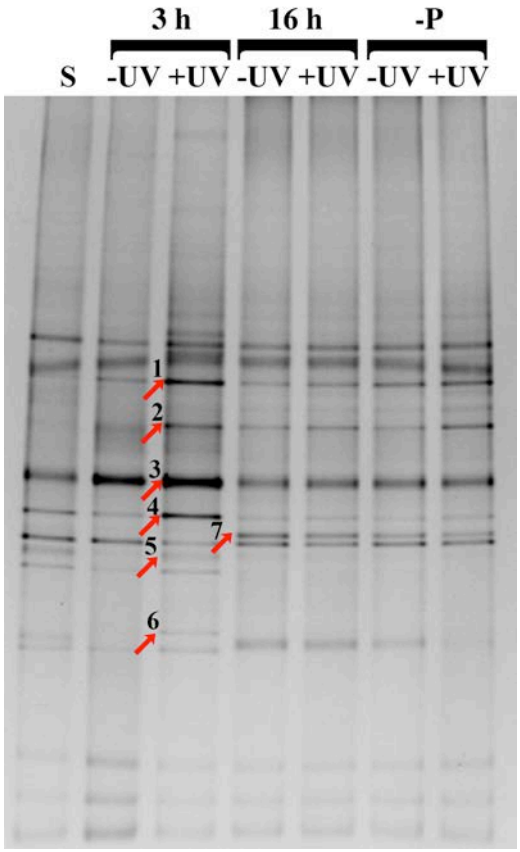

Fig. 8 - DGGE analysis of oral microbial communities formed on untreated (-UV) and UV-treated (+UV) titanium discs surfaces after 3 hours and with and without 24 hour pre-immersion in liquid SHI-FSMS (-P) medium after 16 hours. Bands that were excised for DNA sequencing are indicated by an arrow. Microbial identities are as follows: (1) *Fusobacterium periodonticum*, (2) *Streptococcus* (Mitis group), (3) *Streptococcus* (Mitis group), (4) *Porphyromonas sp* [HOT_279], (5) *Gemella sp*, (6) *Peptostreptococcus stomatis*, and (7) *Streptococcus sp.*

9 Conclusão

9 CONCLUSÃO

Com base nas condições experimentais, e considerando as limitações do presente estudo, foi possível concluir que:

1. A alteração da propriedade físico-química das superfícies de Ti por meio da luz UV-photofunctionalization reduziu significantemente a adesão de bactérias e a formação de biofilme por microrganismos presentes na cavidade oral.
2. Quando comparamos o efeito dos materiais utilizados para confecção de abutments de implantes, Ti e ZrO_2, com o EB, utilizando bactérias de diferentes classes (Gram-positiva e Gram-negativa) nós não encontramos diferenças na fase de adesão entre os materiais testados. Contudo, uma maior densidade de células foram observadas nos discos de EB.
3. Discos de ZrO_2 mostraram menor acúmulo de bactérias quando desenvolvemos biofilme anaeróbico em multiespécie, em comparação com o Ti e o EB.
4. Não obstante, quando utilizamos uma complexa comunidade de microrganismos advindos da saliva humana, a ZrO_2 favoreceu a adesão e formação de biofilme; e o acúmulo de bactérias sobre este material foi significantemente maior em comparação com os discos de Ti.

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ANEXO

Comitê de Ética em Pesquisa

UNIVERSIDADE ESTADUAL PAULISTA " JÚLIO DE MESQUITA FILHO"

FACULDADE DE ODONTOLOGIA DE ARARAQUARA

Comitê de Ética em Pesquisa

Certificado

Certificamos que o projeto de pesquisa intitulado *"ESTUDO SOBRE A ARQUITETURA E O COMPORTAMENTO DOS BIOFILMES SIMPLES E MISTO–FUSOBACTERIUM NUCLEATUM E PORPHYROMONAS GINGIVALIS – FRENTE A DUAS DIFERENTES SUPERFÍCIES DE ABUTMENTS DE IMPLANTES"* sob o protocolo nº **91/11**, de responsabilidade do Pesquisador (a) ***FRANCISCO DE ASSIS MOLLO JUNIOR*** está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa–FOAr, com validade de 02 (dois) anos, quando será avaliado o relatório final da pesquisa.

Certify that the research project titled *"STUDY ABOUT THE ARCHITECTURE AND BEHAVIOR OF SIMPLE AND MIXED BIOFILMS, FUSOBACTERIUM NUCLEATUM AND PORPHYROMONAS GINGIVALIS – FACING TWO DIFFERENT IMPLANT SURFACES ABUTMENTS"*, protocol number **91/11**, under Dr ***FRANCISCO DE ASSIS MOLLO JUNIOR*** responsability, is under the terms of Conselho Nacional de Saúde/MS resolution # 196/96, published on May 10, 1996. This research has been approved by Research Ethic Committee, FOAr-UNESP. Approval is granted for 02 (two) years when the final review of this study will occur.

Araraquara, 3 de abril de 2012.

Prof. Dr. Maurício Meirelles Nagle

Coordenador