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In vitro antimicrobial effect of titanium anodization on complex multispecies subgingival biofilm

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ABSTRACT

Anodization is a routine industrial galvanic method that produces a titanium oxide layer on the surface of titanium. Considering the possibility that this technique could influence microbial adsorption and colonization, this *in vitro* study was conducted to evaluate the impact of a process of anodization applied to a titanium surface on the microbial profile of multispecies subgingival biofilm. Titanium discs produced by using two different processes—conventional and Anodization—were divided into two groups: conventional titanium discs with machined surface (cpTi) Control Group and titanium discs with anodic oxidation treatment (anTi) Test Group. Subgingival biofilm composed of 33 species was formed on the titanium discs that were positioned vertically in 96-well plates, for 7 days. The proportions and the counts of microbial species were determined using a DNA–DNA hybridization technique, and data were evaluated using Mann-Whitney test ($p < 0.05$). Mean total bacterial counts were lower in Test Group in comparison with Control Group ($p < 0.05$). Nine bacterial species differed significantly, and were found in higher levels in Control Group in comparison with Test Group, including *T. forsythia, E. nodatum, and F. periodonticum*. In conclusion, titanium discs with anodization could alter the microbial profile of the biofilm formed around them. Further clinical studies should be conducted to confirm the clinical impact of these findings.

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KEYWORDS
Biofilms; dental implants; peri-implantitis

Introduction

Peri-implantitis, a pathological condition occurring in tissues around dental implants, is characterized by inflammation of the connective tissues and progressive loss of supporting bone (Schwarz *et al.* 2018). This disease is associated with a complex dysbiotic microbiota around the implant-supported restoration (Berglundh *et al.* 2018; Cosgarea *et al.* 2019). Thus, a most important factor for the long-term outcome of implant therapy is thought to be the reduction of bacterial colonization and biofilm formation around the titanium components.

The bacteria species of subgingival microbiota were classically divided into seven complexes (groups), named by different colors: yellow, purple, green, orange, red and the actinomyces (indicated with the blue color). The yellow, purple, green, and actinomyces complexes were associated with health conditions. The orange complex is related to the transition from healthy to disease state while the red complex is strongly associated with periodontal disease conditions (Table 1). In a similar way, it is well known that bacterial biofilm continues to be the main etiologic factor of peri-implant diseases and the microbiota associated with peri-implantitis is more complex than the type found under healthy peri-implant conditions (Shibli *et al.* 2008; Retamal-Valdes *et al.* 2019). The sequence of bacterial adhesion into the subgingival peri-implant biofilm emerges to be very analogous to periodontitis; additionally, the dysbiotic biofilm seems to be comparable between these diseases (Berglundh *et al.* 2018). Therefore, *Phorphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* (the red complex) is called to be related to the etiology of peri-implant diseases, and *P. intermedia* plays a vital position in peri-implantitis development (Perez-Chaparro *et al.* 2016; Lafaurie *et al.* 2017)

Therefore, it is of vital importance to find alternative approaches to reduce colonization and biofilm formation around dental implants. Indeed, several

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Table 1. Species organized by the complexes.

Species	ATCC
Actinomyces gerencseriae	23840
Actinomyces israelii	12102
Actinomyces naeslundii	12104
Actinomyces oris	43146
Actinomyces odontolyticus	17929
Veillonella parvula	10790
Streptococcus gordonii	10558
Streptococcus intermedius	27335
Streptococcus mitis	49456
Streptococcus oralis	35037
Streptococcus sanguinis	10556
Aggregatibacter actinomycetemcomitans	29523
Eikenella corrodens	23834
Capnocytophaga ochracea	33596
Capnocytophaga gingivalis	33624
Capnocytophaga sputigena	33612
Fusonucleatum polymorphum	10953
Fusonucleatum vincentii	49256
Fusobacterium periodonticum	33693
Prevotella intermedia	25611
Eubacterium nodatum	33099
Campylobacter gracilis	33236
Campylobacter showae	51146
Streptococcus constellatus	27823
Parvimonas micra	33270
Tannerella forsythia	43037
Porphyromonas gingivalis	33277
Selenomonas noxia	43541
Streptococcus anginosus	33397
Gemella morbillorum	27824
Probionibacterium acnes	11827
Eubacterium saburreum	33271
Streptococcus mutans	25175

approaches have been evaluated (Kaiser *et al.* 2020), and one of these strategies could be the use of titanium components with anodized surfaces.

An electrochemical method, known as anodization or anodic oxidation, is a well-established surface modification technique that will produce a protective layer on metals, with different indications in industries (Brunette *et al.* 2001). This nano-modification technique has been successfully used as a surface treatment for orthopedic and dental implants (Yao and Webster 2006). Succinctly, anodizing is an electrolytic passivation process used to increase the thickness of the natural oxide layer on a metal surface, including titanium surfaces. This enables different colors to form on titanium surfaces and can improve their abrasive resistance, corrosion resistance, and color stability (Li *et al.* 2013; Li *et al.* 2014; Wang *et al.* 2019).

It is well established that electrochemical anodization of the titanium surface, used in different clinical applications, including implant dentistry, can contribute to improving osseointegration as well as long-term implant survival (Chrcanovic *et al.* 2016, Wennerberg *et al.* 2018). In addition, recent *in vitro* studies suggested that anodized-surfaces enhanced initial growth of fibroblasts and preosteoblasts (Wheelis *et al.* 2018), adhesion of epithelial cells and fibroblasts (Mussano *et al.* 2018), thereby contributing to soft tissue healing. Wang *et al.* (2020) observed that the use of titanium abutments that had been colored by anodic oxidation could improve the esthetic appearance of peri-implant soft tissues. Another clinical influence of anodizing the titanium surface is on biofilm formation around this metal. An *in vitro* study showed a significantly lower adherence of oral *streptococci* to anodized, in comparison with commercially pure titanium disks (Dorkhan *et al.* 2014). Similar data was also reported by Yue *et al.* (2014). Since the streptococci bugs are the initial colonizers of peri-implant surfaces, we hypothesized that anodization of implant surfaces may reduce the adhesion of biofilm.

Therefore, based on the foregoing clinical benefits and applications, it is important to understand the behavior of bacterial biofilm on anodized titanium surfaces. In this context, no studies to date have made an in-depth the profile of subgingival multispecies biofilm formation around this type of modified titanium surface. So, this study aimed to compare the microbial profile formed on commercially pure titanium discs in comparison with anodized titanium discs in an *in vitro* model of multispecies subgingival biofilm.

Material and methods

Experimental groups

Ten commercially pure titanium (cpTi) and ten anodized titanium (anTi) disks (5-mm in diameter and 3 mm thick (Bionnovation biomedical, São Paulo, Brazil) were prepared, polished (sequential series of 320, #400, #600 grit abrasive paper) and cleaned in a detergent solution for 15 min, followed by rinsing with pure distilled water (Milli-Q; Millipore, Bedford, MA, USA) for 15 min. Afterwards, all discs were sterilized by Gamma radiation. The discs of both groups (cpTi and anTi) were then sterilized individually and used in two rounds (five discs each round) of biofilm formation.

Biofilm formation

The species used to form multispecies biofilm were *Actinomyces gerencseriae* ATCC 23840, *Fusonucleatum polymorphum* ATCC 10953, *Actinomyces israelii* ATCC 12102, *Fusonucleatum vincentii* ATCC 49256, *Actinomyces naeslundii* ATCC 12104, *Fusobacterium periodonticum* ATCC 33693, *Actinomyces odontolyticus* ATCC 17929, *Prevotella intermedia* ATCC 25611,

Eubacterium nodatum ATCC 33099, *Streptococcus gordonii* ATCC 10558, *Aggregatibacter actinomycetemcomitans* ATCC 29523, *Streptococcus intermedius* ATCC 27335, *Tannerella forsythia* ATCC 43037, *Streptococcus mitis* ATCC 49456, *Eikenella corrodens* ATCC 23834, *Streptococcus oralis* ATCC 35037, *Capnocytophaga ochracea* ATCC 33596, *Streptococcus sanguinis* ATCC 10556, *Selenomonas noxia* ATCC 43541, *Streptococcus anginosus* ATCC 33397, *Gemella morbillorum* ATCC 27824, *Capnocytophaga gingivalis* ATCC 33624, *Probionibacterium acnes* ATCC 11827, *Campylobacter gracilis* ATCC 33236, *Campylobacter showae* ATCC 51146, *Streptococcus constellatus* ATCC 27823, *Capnocytophaga sputigena* ATCC 33612, *Parvimonas micra* ATCC 33270, *Eubacterium saburreum* ATCC 33271 and P*orphyromonas gingivalis* ATCC 33277, *Actinomyces oris* ATCC 43146, *Streptococcus mutans* ATC 25175, *Veillonella parvula* ATC 10790 (Table 1).

All of the species used in the model were allowed to grow for 24 h, under anaerobic conditions, on plates of tryptone soy agar with 5% sheep blood for all the species (except for Eubacterium). For *P. gingivalis*, the agar was enriched with 1% hemin, 5% menadione. For *T. forsythia*, the media used contained tryptone soy agar with yeast extract enriched with 1% hemin, 5% menadione, 5% sheep blood, and 1% N-acetylmuramic acid. Eubacterium was cultured on fastidious anaerobic agar with 5% sheep blood. After 24 h of growth, all bacterial species were transferred to glass tubes with Brain Heart Infusion (BHI) culture medium (Becton Dickinson, Sparks, MD) supplemented with 1% hemin and allowed to grow for another 24 h.

After then, the optical density (OD) was adjusted to obtain inoculum with approximately 10^8 cells/ml of each species in each tube. The individual cell suspensions were diluted to obtain 10^6 cells in each tube, and 100 µL aliquots containing 10^6 cells from each species were mixed with 11700 µL of BHI broth supplemented with 1% hemin and 5% sheep blood to obtain a final biofilm inoculum of 15 ml (Miranda *et al.* 2019; Pingueiro *et al.* 2019).

Round-shaped titanium discs were placed vertically in a 96-well plate to act as substrate for biofilm formation. There were two groups of titanium disks: cpTi Group (Control Group), consisting of discs with a machined surface; and anTi Group (Test Group), which were the same titanium discs, but with an anodized surface. An aliquot of inoculum with a total volume of 150 µL, containing 10^4 cells of each species was added into each well with titanium discs, and plates were incubated at 37 °C under anaerobic conditions. After 72 h of incubation, the culture media was replaced with fresh BHI broth (supplemented with 1% hemin and 5% sheep blood) and maintained at 37 °C under anaerobic conditions for a period of seven days of biofilm formation. We used 7 days of formation because the subgingival biofilm is mature after this period through an *in vitro* method (Soares *et al.* 2015). Two independent experiments were performed (Miranda *et al.* 2019; Pingueiro *et al.* 2019).

DNA-DNA hybridization (checkboard DNA-DNA)

Ten titanium discs coated with 7-day biofilms of each of the groups were transferred to Eppendorf tubes containing 100 µL of TE buffer (10 mM Tris-HCl, one mM EDTA [pH 7, 6]), and then 100 µL of 0.5 M NaOH was added. The tubes containing the discs and the final solution were boiled for 10 min and the solution was neutralized by adding 0.8 ml of ammonium 5 M. The samples were analyzed individually for the presence and quantity of the 33 bacterial species, using DNA-DNA hybridization technique. Briefly, upon lysis of the samples, the DNA was plated onto a nylon membrane using a Minislot device (Immunetics, Cambridge, MA). After the DNA became attached to the membrane, it was placed in a Miniblotter 45 (Immunetics). Digoxigenin labeled with DNA probes of the entire genome for the subgingival species used were hybridized to individual lanes of Miniblotter 45. After hybridization, the membranes were washed, and DNA probes were detected using a specific antibody to digoxigenin conjugated to phosphatase alkaline. The signals were detected using AttoPhos substrate (Amersham Life Sciences, Arlington Heights, IL), and the results were obtained by using Typhoon Trio Plus (Molecular Dynamics, Sunnyvale, CA). Two lanes in each race contained the standards with $10^5$and 10^6 cells of each species. Signals obtained with the Typhoon Trio were converted to absolute counts, by comparison with the patterns on the same membrane. Failure to detect a signal was recorded as zero. The values obtained after experimental period for Test Group were compared with the values of the Control Group (Miranda *et al.* 2019; Pingueiro *et al.* 2019).

Statistical analysis

For microbiological analyses, mean counts (x10^5), the percentage of the total DNA probe counts, and total count of specific bacterial species within biofilms

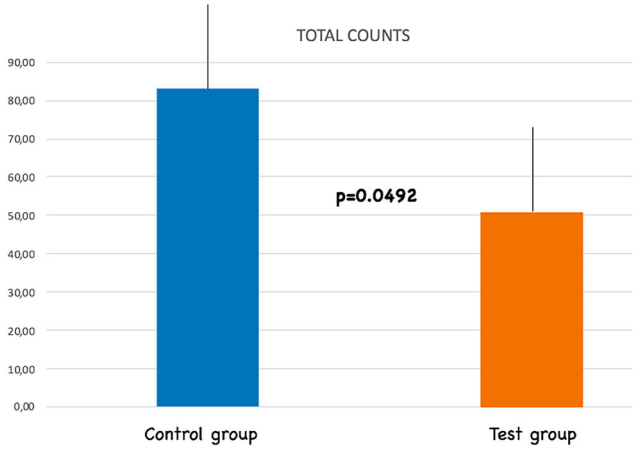

Figure 1. Total counts (x 10^5) of all bacterial species included in the biofilm formed on surface of the two groups of discs. * means statistical significance between the two groups by Mann-Whitney U test.

were determined initially in each sample. The significance of differences between groups was sought using the Mann-Whitney U test. The microbiological analyses were adjusted for multiple comparisons (Socransky *et al.* 1991).

Results

Figure 1 shows total counts of bacterial species formed on surface of the two groups of discs. Significant difference was observed between groups. Mean total bacterial counts were lower in Test Group in comparison with Control Group ($p = 0.0492$)

Figure 2 shows the mean counts (x 10^5) of 33 species evaluated in biofilm formed in the two groups. Nine species differed significantly between groups. *T. forsythia*, *E. nodatum, A. naeslundii F. periodonticum, S. intermedius, A. israelli, E.saburreum, S.noxia and S. showae* were elevated in the Control Group *($p < 0.05$).* No species in the Test Group had higher mean levels when compared with the Control Group ($p > 0.05$).

The mean percentage of DNA probe counts of the 33 individual species evaluated in the biofilm model are shown in Figure 3. The proportions of three bacterial species were significantly higher in the Control group (*T.forsythia*, *C.sputigena and E.saburreum*) while the proportions of two bacterial species were higher in the Test group (*A.gerencseriae and S.anginosus*).

Discussion

In this study, subgingival biofilm formation on modified titanium surfaces was investigated. The surfaces were prepared by anodic oxidation in the Test Group, while the control disc surfaces were machined. Our data showed that anodization or anodic oxidation may have an impact on biofilm colonization around titanium surfaces. According to these data, the electrochemical surface modification technique known as anodization promoted a lower level of total bacterial colonization on titanium surfaces. This included a lower level of colonization of species that are important for initial biofilm formation, such as *S. intermedius, A.israelli,* and species of peri-implant pathogens from the orange and red complex such as *E. nodatum, F. periodonticum, C.showae and T.forsythia*.

The bacteria *T. forsythia,* is a member of the red complex and has been associated with the establishment of periodontitis in association with *P. gingivalis* and *T. denticola*, other members of the red complex. Moreover, *T. forsythia* seems to be even more relevant in the pathogenesis of peri-implantitis than in periodontal disease. Long-term evaluations of dental implant survival have demonstrated that this microorganism might be found in higher numbers at implant sites than in the adjacent teeth, and that it was positively associated with severity of the disease (Eick *et al.* 2016; Eckert *et al.* 2018)

E. nodatum, F. periodonticum, C.showae, members of the orange complex, were expected to be responsible for the transition from a healthy situation to a diseased state by supporting the colonization of red complex members. However, their specific role in peri-implant disease still needs to be determined. A recent follow-up study demonstrated that dental implants with higher total counts and frequencies of *P. gingivalis*, *T. denticola*, and *F. nucleatum* were more likely to develop the disease (Costa *et al.* 2019). For this reason, several procedures have been studied to prevent biofilm colonization around dental implants (Kaiser *et al.* 2020), and among them, anodization is outstanding.

Anodization is a routine industrial galvanic method that produces a titanium oxide layer on the surface of titanium. By using appropriate electrolytes and electrochemical process parameters, a dielectric breakdown is induced, which results in sparks running chaotically over the titanium surface and forms a thick, rough, porous oxide layer. These characteristics have been shown to affect bacterial adherence (Dorkhan *et al.* 2014; Jung *et al.* 2015; Zhang *et al.* 2018). Zhang *et al.* (2018) demonstrated the effect of this electrochemical titanium surface modification against *Staphylococcus aureus*. In addition, Jung *et al.* (2015) found antibacterial effect against a very important peri-implant pathogen, *P. gingivalis*. More

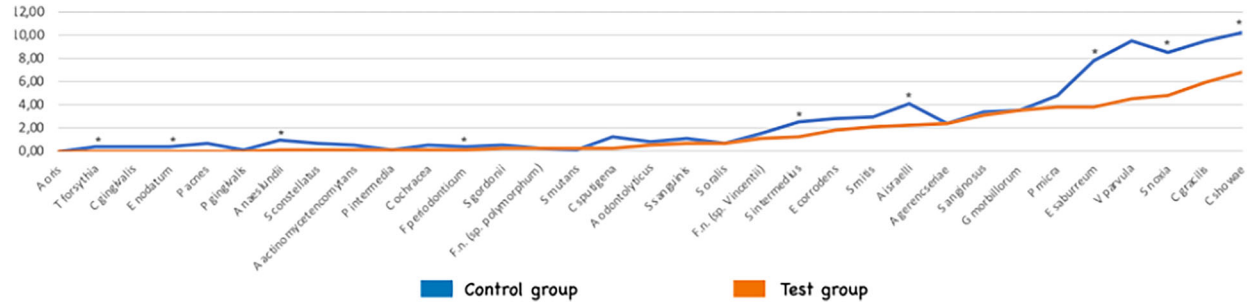

Figure 2. The mean counts (x 10^5) of all of the 31 species evaluated in biofilm formed in the two groups. * means statistical significance for the counts of the specific bacterial species between the two groups by Mann-Whitney U test.

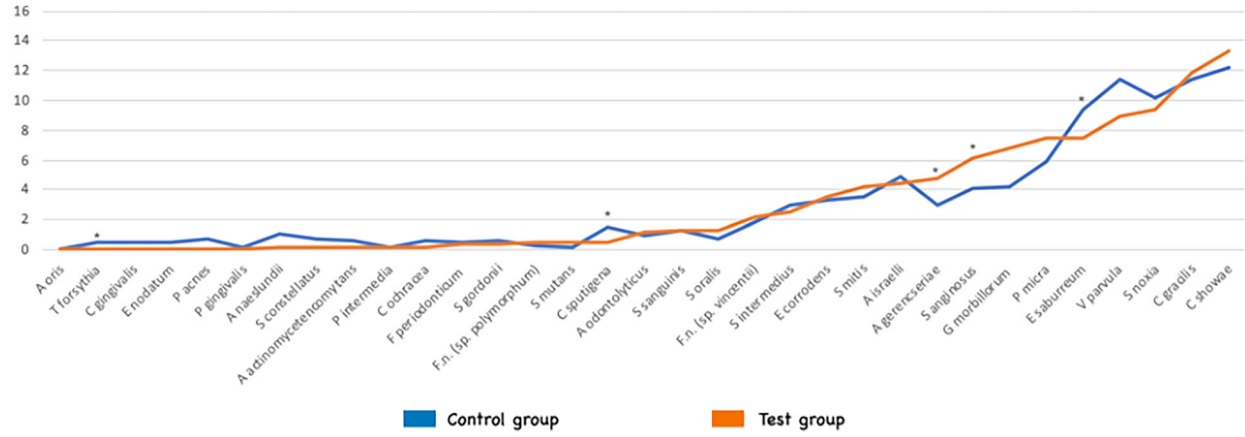

Figure 3. The mean percentage of DNA probe counts of the 31 individual species. * means statistical significance for the percentage of the specific bacterial species between the two groups by Mann-Whitney U test.

recently, Astasov-Frauenhoffer *et al.* (2019) investigated the antibacterial effect of several electrochemical treatment protocols, and found the benefit of this metal modification against the level of *P.gingivalis,* by decreasing the viable bacterial count in the environment surrounding the titanium surface. These data are in agreement with the results of the present study that found a reduction in the levels of nine bacterial species, including the lower levels and proportion of bacterial species from the orange and red complexes.

Interesting data are the lower levels and proportion of *T.forsythia* on the surface of anodized titanium in the Test Group, since the literature has clearly demonstrated that the presence and frequency of higher numbers of T. forsythia are likely to induce the development and increase the severity of both periodontal and peri-implant diseases (Salvi *et al.* 2017; Deng *et al.* 2017; Daubert and Weinstein 2019).

The mechanism of bacterial adhesion to the anodized titanium should be investigated more fully because up to now this process is still unclear. Thus, since improving soft tissue attachment and reducing bacterial colonization on titanium surfaces are key factors for the long-term maintenance of healthy soft and hard peri-implant tissues, the process of anodization on titanium surfaces could have a beneficial clinical impact on reducing the prevalence of peri-implantitis.

Lindhe *et al.* (1992), in a preclinical study in which the breakdown of peri-implant and periodontal tissue was evaluated, showed that clinical and radiographic signs of tissue destruction were more pronounced around implants than around teeth, and that the size of the soft tissue lesion was larger at implants. Whereas, Berglundh *et al.* (2007) suggested that the progression of peri-implantitis is more pronounced at implants with a moderately rough surface than at implants with a polished surface. Therefore, future *in vivo* studies should evaluate the efficacy of dental implants, titanium mesh or dental implant abutments made of anodized titanium, relative to clinical outcomes around dental implants.

The limitation of this study lies on the *in vitro* model. It is important to bear in mind that the scientific evidence supporting the clinical treatment procedures is based on systematic reviews with meta-analysis and on randomized clinical trials. Hence, the results of present article should not support changes in dental implantology, but the clinical benefits of this process should be further investigated.

In conclusion, anodization of titanium surfaces reduced and modified the *in vitro* biofilm profile, by reducing the levels of orange and red bacterial species, when compared to non-anodized surfaces.

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