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Titanium particles and ions favor dysbiosis in oral biofilms

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Abstract

Objective: To evaluate the effect of titanium (Ti) particles and ions on oral biofilm growth and composition.

Background: Particles and ions of Ti released from dental implants can trigger unfavorable biological responses in human cells. However, their effect on oral biofilms composition has not been tested.

Methods: In this blind in situ study, volunteers wore a palatal appliance containing Ti disks for 7 days to allow biofilm formation. Disks were then collected and biofilms were treated, in vitro, with Ti particles (0.75% and 1%), ions (10 and 20 ppm), or a combination of both (1% particles + 20 ppm ions). Biofilms exposed only to medium was used as control group. After 24 hours, biofilms were collected and analyzed by checkerboard DNA-DNA hybridization. Direct effects of Ti particles and ions on biofilm/cellular morphology were evaluated by transmission electron microscopy (TEM).

Results: Ti particles affected biofilm composition, increasing population of four bacterial species ($P < .05$), while Ti ions showed higher levels of putative pathogens from the orange complex with reduction in species from the yellow complex ($P < .05$), compared with control. The combination of particles + ions increased green complex and reduced yellow complex proportions ($P < .05$). TEM showed clusters of particles agglomerated in extracellular environment, while Ti ions were precipitated in both extracellular and intracellular sites.

Conclusions: Ti products, especially Ti ions, have the potential to change the microbiological composition of biofilms formed on Ti surfaces. Therefore, the presence of Ti products around dental implants may contribute to microbial dysbiosis and peri-implantitis.

KEYWORDS

biofilms, ions, particle, titanium

1 | INTRODUCTION

Titanium (Ti) is the main dental implant material due to its excellent physical-chemical properties and high biocompatibility with oral tissues.[1] Nonetheless, once exposed to the oral environment, dental implants can be affected by mechanical and chemical degradation processes, such as surface corrosion and wear,[2] which leads to the release and accumulation of Ti particles in the peri-implant surrounding tissues.[2-4] In contact with biological fluids, these particles can dissolve and generate Ti ions.[5] Although Ti particles and ions may be found in healthy and diseased peri-implant surrounding tissues,[6] higher concentrations of these products have been found in peri-implantitis sites.[2] In addition, recent studies have suggested that both Ti particles and ions can influence the pathogenesis of peri-implantitis.[7,8]

The release of Ti products on peri-implant tissues can trigger a complex inflammatory response, characterized not only by an early acute inflammatory cell infiltration,[7,9] but also by a later presence of chronic inflammatory cells.[10] Local osteoblasts have also been described as being able to phagocyte Ti particles, and this process may lead to cell necrosis.[11] Taken together, this complex inflammatory process seems to be responsible for periprosthetic osteolysis around implants.[12] The cytotoxic effect of Ti products in human cells seems to be dose-response dependent, both for ions[13] and particles.[11]

Bacterial accumulation around dental implants triggers inflammation and can lead to corrosion and release of Ti products on the surrounding tissues.[6] Although the effect of Ti products on human cells is widely recognized, their microbiological effect has only been tested in models using planktonic growth of few bacterial species.[14-16] The disadvantage of this type of methodology is not mimicking the complex structure/composition of the oral biofilm.

Since specific pathogens and Ti particles are both elevated in peri-implantitis and no previous studies have evaluated a possible interplay between them using an oral biofilm model, we designed this study to evaluate the dose-response effect of Ti particles and ions on the composition of biofilm formed on Ti surfaces, as well as the direct effect of Ti products on biofilm/cellular morphology.

2 | MATERIALS AND METHODS

2.1 | Ethical aspects and study population

The study protocol was approved by the local Research and Ethics Committee (protocol 55366416.0.0000.5418). Five volunteers were selected from Piracicaba Dental School, University of Campinas (Piracicaba, São Paulo, Brazil), and they signed an informed consent before the experimental phase.

The inclusion criteria were as follows: ≥18 years of age, good systemic and periodontal health, and normal stimulated salivary flow rate (>0.7 mL/min). The exclusion criteria were as follows: antibiotic therapy in the previous 2 months prior to entering the study, smoking, orthodontic treatment, and presence of periodontitis defined as the presence of ≥1 site with probing depth ≥4 mm with bleeding on probing.

2.2 | Experiment 1—in situ assay: effect of titanium products on oral biofilm composition

Five volunteers wore for 7 days a palatal appliance containing commercially pure titanium (cpTi) disks (8 × 2 mm). The cpTi disks (Conexão Ltd) were polished (sequential sandpaper—#320, #400, #600), cleaned, and degreased by immersion in isopropyl alcohol, ultrasound washing with liquid detergent and purified water.[17] Custom-made acrylic resin palatal appliances were made from plaster models of the upper arch of the volunteers. Slots (3 mm deep) were built in the appliance to accommodate Ti disks, and a plastic mesh was fixed on the slot entrance to assure biofilm accumulation.[17] To stimulate biofilm formation with higher levels of bacteria and elevated proportions of periodontal pathogens—mimicking a subgingival biofilm profile, the disks were treated extra orally with 20% sucrose solution, 4 times/d as previously described.[17] The volunteers used the appliances throughout the experimental phase, removing them only for sucrose treatment, for food and beverage intake and during oral hygiene procedures.

In the morning of the 8th day, the appliances of all volunteer were collected and disks with biofilms were carefully removed under sterile conditions and each disk was randomly assigned to one of the following groups using a computerized random number list: 24 hours of anaerobic incubation (37°C and 10% CO_2) in culture medium (BHI media) supplemented with (a) Ti particles (0.75% and 1%), (b) ions (10 and 20 ppm) or (c) with a combination of both products (1% particles + 20 ppm ions), and (d) pure culture medium (control). Subsequently, biofilms were collected using a modified cell scraper (TPP, Trasadingen, Switzerland—length, 240 mm) from a central area of disk, in one movement. The samples were inserted into a tube containing 150 μL of TE solution (Tris HCl 10 mM + ethylenediaminetetraacetic acid 1 mM, pH 7.6), and 100 μL of 0.5 M NaOH was added to each tube. Counts of 39 bacterial species were determined in each sample by checkerboard DNA-DNA hybridization.[18,19]

2.3 | Experiment 2—in vitro assay: effect of titanium products on biofilm/cellular morphology

To analyze the effect of Ti products on biofilm/cellular morphology and dispersion of particles in biofilm environment, an in vitro multispecies biofilm was grown and examined by transmission electron microscopy (TEM). Multispecies biofilms were formed in vitro for 7 days under anaerobic conditions using a pool of stimulated saliva from each of the five volunteers.

The bacterial inoculum of the multispecies in vitro biofilm was prepared from a pool of fresh stimulated human saliva from the same five volunteers from Experiment 1. This protocol led to a biofilm that simulates the oral microbiota diversity.[20] Volunteers did not eat or brush their teeth for at least 2 hours prior to saliva collection. Initially, to form the salivary pellicle, disks were immersed in 2 mL of ultrafiltered saliva on 24-well plates for 30 minutes at 37°C.[21] Saliva-coated disks were then transferred into new wells containing 1 mL of fresh BHI media supplemented with 1% sucrose and saliva as the bacterial inoculum (1:10 v/v) and incubated at 37°C, 10% CO_2. Culture media was changed every 24 hours, and the biofilms were formed for 7 days. The samples were then treated for 24 hours with Ti particles and ions, according to the four experimental groups described in Experiment 1 and submitted to TEM analysis. To perform the TEM analysis, biofilms were grown on resin slabs (n = 2/group) (Dr Spurr, Electron Microscopy Sciences) in the conditions previously described[22] and then stained using sodium periodate and osmium tetroxide. Samples were included in the same resin used to prepare the specimens, and ultrathin slices were done using diamond blade (Diatome 45°, Leica) in ultramicrotome (Ultracut E, Reichert). Obtained slices were placed in mesh grades and contrasted using

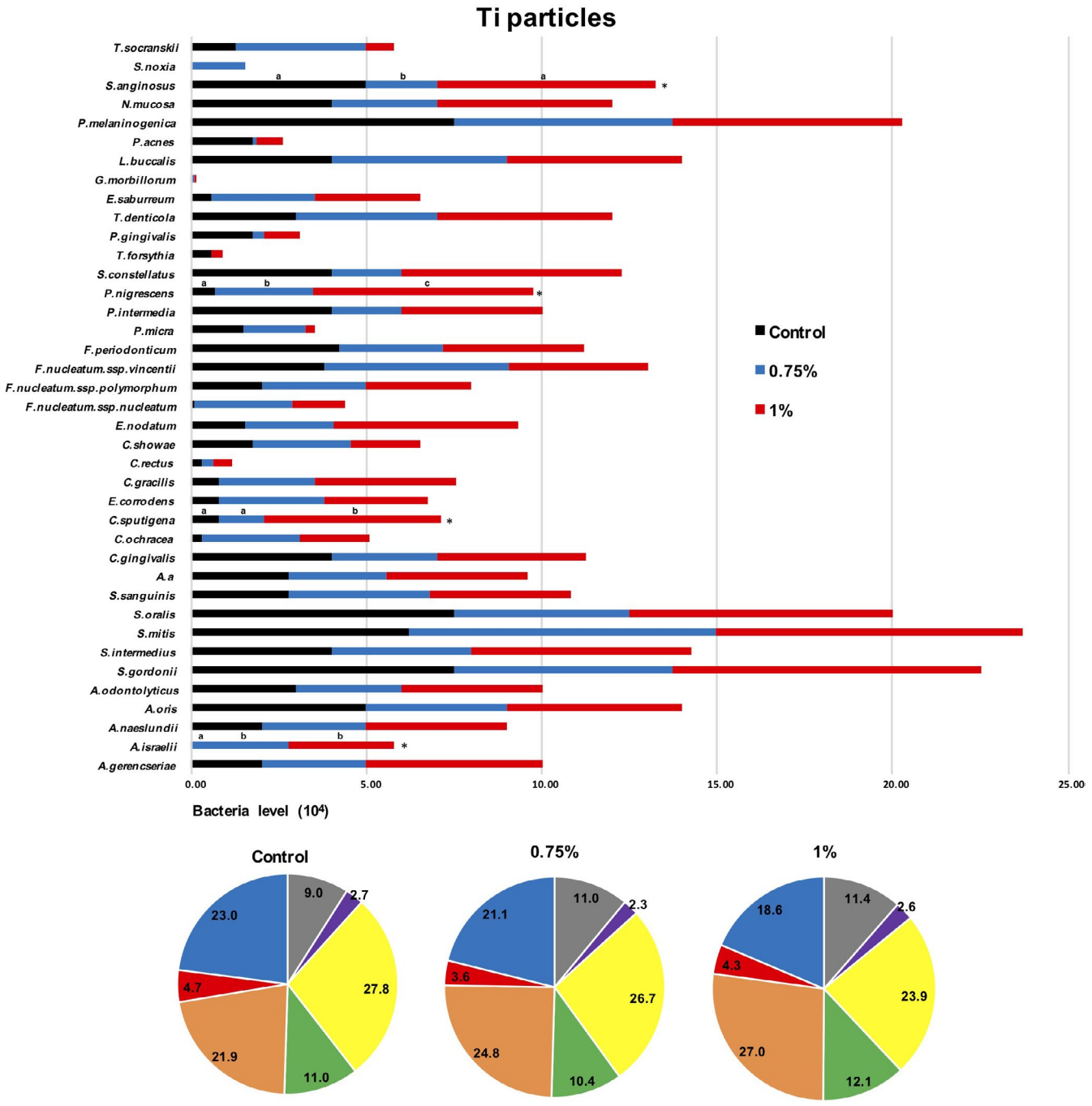

FIGURE 1 Profile of mean levels (×10^5) of 39 bacterial species and pie charts of the mean proportion of each microbial complex in biofilm samples according to particles treatment groups (control group, 0.75% and 1% of Ti particles). Levels of individual species were computed in each sample and then averaged in each group. The area of the pie charts was adjusted to reflect the mean of total levels of the species evaluated. * and different letters indicate statistically significant differences among the groups ($P < .05$)

uranyl acetate and lead citrate. Biofilms were visualized in transmission electron microscope Jeol JEM-1400 (Jeol Ltd.) at 80 kV.[23]

2.4 | Titanium products preparation

Ti particles treatment was conducted using 0.75% (7.5 mg/mL) and 1% (10 mg/mL) of particles concentration.[11] For Ti ions, 10 and 20 ppm of Ti solution were used to treat biofilms.[13] A combination of 1% of particle + 20 ppm of Ti solution was also used to test whether there was a synergistic effect of both Ti products on oral biofilms. Control biofilms were exposed only to culture medium. Ti (IV) oxide (TiO_2), mixture of rutile, and anatase nanoparticles (Sigma-Aldrich; <100 nm average particle size, purity 99.9% based on trace metals analysis) at specific concentrations (0.75 and 1%) were dispersed on brain heart infusion (BHI—Becton-Dickinson) media. Then,

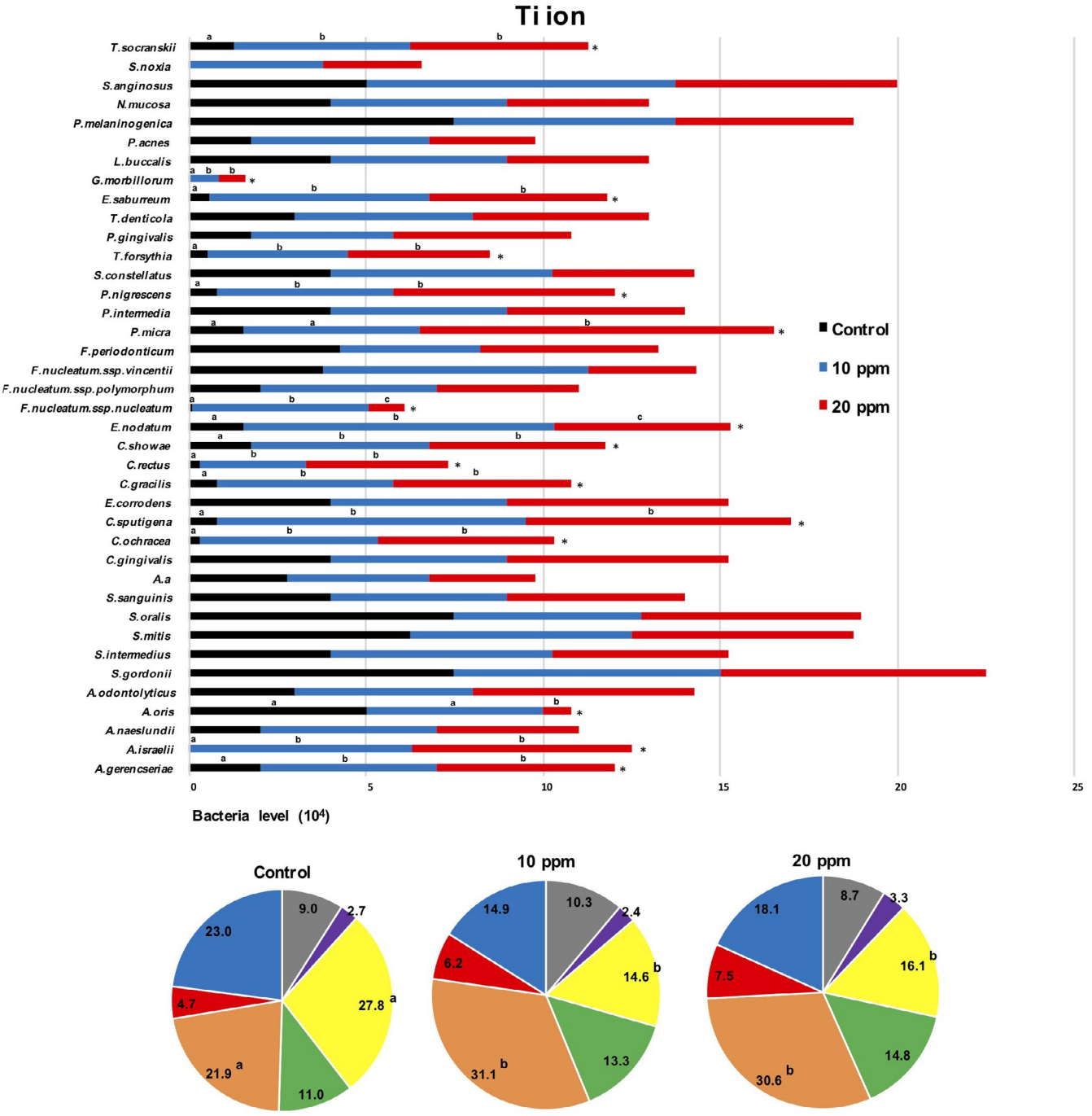

FIGURE 2 Profile of mean levels ($\times 10^5$) of 39 bacterial species and pie charts of the mean proportion of each microbial complex in biofilm samples according to ion treatment groups (control group, 10 and 20 ppm of Ti ion). Levels of individual species were computed in each sample and then averaged in each group. The area of the pie charts was adjusted to reflect the mean of total levels of the species evaluated. * and different letters indicate statistically significant differences among the groups ($P < .05$)

media-containing particles were ultrasonicated for 30 minutes at 360 W (UP400S, Hielscher) to allow homogenization.[11,24] Media-containing particles were sterilized in autoclave. To avoid particle aggregation in the culture medium, 100 μg/mL of BSA (Bovine serum albumin) was used to stabilize the particles.[24] Ti ions solution was prepared from a Ti standard solution—$(NH_4)_2TiF_6$ in H_2O (Merck)—at specific concentrations (10 and 20 ppm) in BHI medium and sterilized in autoclave.

2.5 | Data analysis

The mean levels of 39 individual species and mean percentage of the microbial complexes were determined in each sample for all experimental groups. The percentage of the total DNA probe counts was determined initially in each disk and then averaged across disks in the groups. The individual proportions of each species were added to determine the proportions of each microbial complex.[18,25] The

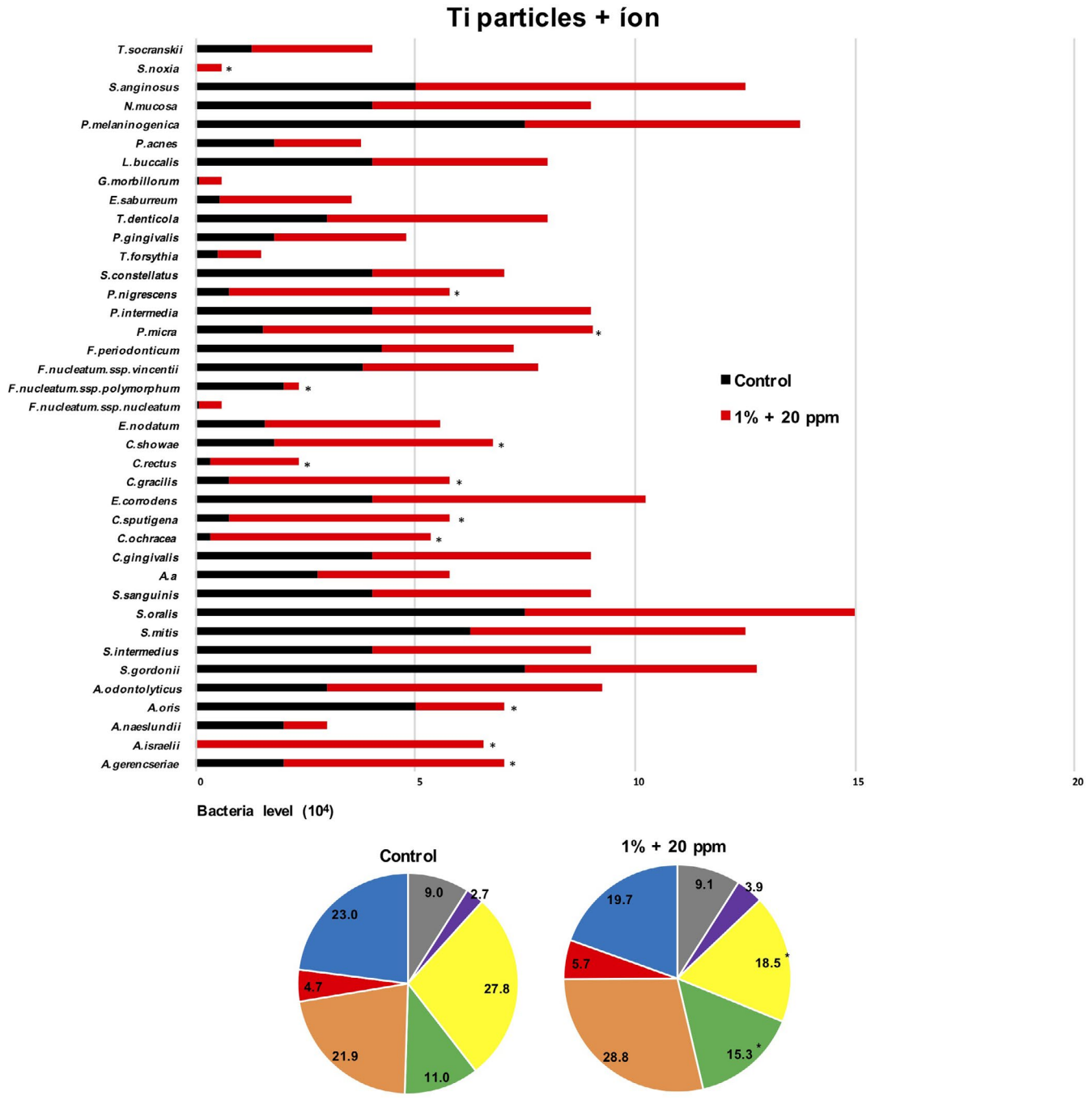

FIGURE 3 Profile of mean levels (×10^5 ± SD) of 39 bacterial species and pie charts of the mean proportion of each microbial complex in biofilm samples according to treatment groups (control group, and combination of 1% particle + 20 ppm ion). Levels of individual species were computed in each sample and then averaged in each group. The area of the pie charts was adjusted to reflect the mean of total levels of the species evaluated. * and different letters indicate statistically significant differences between the groups ($P < .05$)

significance of differences between two experimental groups was analyzed by the Mann-Whitney test or among groups by the Kruskal wallis test. SPSS software 21.0 (IBM) was used at a significance level of 5%.

3 | RESULTS

Figures 1, 2 and 3 show the levels of individual species and the proportions of the microbial complexes present in biofilms formed in situ and treated with different concentrations of Ti particles, ions, or particles + ions, respectively. In comparison with the controls, the levels of four bacterial species were elevated in the group exposed to Ti particles (*Streptococcus anginosus*, *Prevotella nigrescens*, *Capnocytophaga sputigena*, and *Actinomyces israelli*, $P < .05$; Figure 1), 16 species in the group exposed to Ti ions (*Treponema socranskii*, *Gemella morbillorum*, *Eubacterium saburreum*, *Tannerella forsythia*, *Prevotella nigrescens*, *Parvimonas micra*, *Fusobacterium nucleatum* ssp. *nucleatum*, *Eubacterium nodatum*, *Campylobacter showae*, *Campylobacter rectus*, *Campylobacter gracilis*, *C sputigena*, *Capnocytophaga ochracea*,

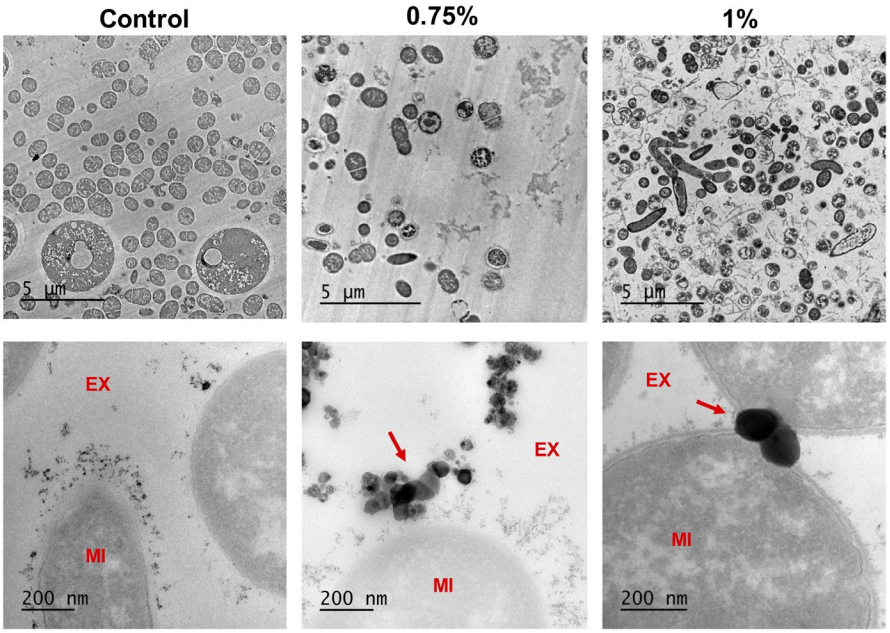

FIGURE 4 TEM images exhibiting organization of biofilms according to treatment groups. Biofilm images show a wide microbiological composition for all biofilms. Ti particles groups showed clusters of particles in extracellular environment (arrow) accumulated around microorganism cells. EX, extracellular environment; MI, microorganism

Actinomyces oris, *A israelli*, and *Actinomyces gerencseriae*; Figure 2), and ten bacterial species in the group exposed to Ti particles + ions (*S noxia*, *P nigrescens*, *P micra*, *C showae*, *C rectus*, *C gracilis*, *C sputigena*, *C ochracea*, *A israelli*, and *A gerencseriae*; Figure 3). Lower counts of *Actinomyces oris* were observed in the group treated with 20 ppm of Ti ions, in comparison with the control and the 10 ppm group (Figure 2). The majority of the significant changes in bacterial levels were observed when higher concentrations of particles or ions were used.

In terms of proportion of the microbial complex, orange complex was in significantly higher proportions and yellow complex in lower proportions in both Ti ions groups in comparison with the control (Figure 2). The proportions of the microbial complexes were not significantly affected by Ti particles (Figure 1). The combination of particles + ions increased green complex and reduced yellow complex proportions (Figure 3). Ti ion groups (107.4 ± 35.02, 207.5 ± 14.5, 187.3 ± 30.5, for control, 10 and 20 ppm groups, respectively) and combination of treatments (particle + ions; 107.4 ± 35.02, 158.9 ± 18.0, for control and 20 ppm + 1% particle group, respectively) increased total level of 39 species evaluated ($P < .05$), compared with control group.

TEM images suggested a dense biofilm, mainly formed by *phagocyte* spp., corroborating results found by checkerboard DNA-DNA hybridization technique. In the control group, the majority of coccal-shaped bacteria were going through division process, while the 1% Ti particles group seemed to harbor increased proportions of *Capnocytophaga* spp., based on the presence of long, spindle-shaped bacilli. Images of rod-shaped bacteria were also seen, suggesting the presence of *Actinomyces* and *Prevotella* spp. (Figure 4). Regarding the dispersion of Ti particles, both test groups showed clusters of particles only in the extracellular environment (biofilm matrix), suggesting no internalization of Ti particles by microorganisms. Moreover, images suggest that particles could be used as adhesion sites for bacterial coaggregation (Figure 4).

Both ion groups presented Ti ions precipitated within the biofilm matrix, with clusters in extracellular and intracellular sites (Figure 5). Moreover, Ti ions were also internalized by bacteria and could be seen inside the cells.

The combination of treatments (particle + ions) showed characteristics similar of those observed for Ti particles 1% and ions 20 ppm, with coccal-shaped and *Streptococcus* spp. and some fusiform- to rod-shaped bacteria, corroborating results found by checkerboard. Regarding the dispersion of Ti products, it is possible to observe that even though they were dispersed in the biofilm matrix, some Ti ions precipitated and agglomerated with Ti particles that were in contact with cells (Figure 6).

4 | DISCUSSION

The results of the present study showed that Ti products, specially Ti ions, can change the composition of oral biofilm formed in situ over Ti surfaces, and this effect seemed to be dose-response dependent. Even though Ti particles treatment led to an increase in the levels of only a few bacterial species, Ti ions led to a significant increase in the total level of biofilm formed and caused a dysbiotic shift in the microbial community. However, the combination of Ti particles and ions did not show a synergistic effect in changing the biofilm composition.

The few previous studies testing the microbiological effects of Ti products showed no major effects of these products in oral bacteria growth.[14,26,27] However, these studies have not tested a dose-response and used Ti incorporation in agar plates or planktonic microbial growth, conditions that do not necessarily reproduce the in vivo formation of biofilms. In addition, the results of the present study showed that Ti particles favored the growth of four species,

including *P nigrescens*, an anaerobic microorganism. Although Ti particles did not profoundly change the biofilm composition, the particles were used as substrate to bacteria adhesion and coaggregation, favoring biofilm growth. Interestingly, although Ti particles have shown to be cytotoxic for eukaryotic cells,[7,9,11] this effect was not observed in the bacterial species from the biofilms analyzed in this study.

On the other hand, Ti ions showed a striking effect on microbial load. Sixteen bacterial species increased after incubation with Ti ions for 24 hours. Several known periodontal/peri-implant pathogens such as *T forsythia*, *T socranskii*, *E nodatum*, *P nigrescens*, and *Campilobacter* spp showed an increase in levels after biofilm treatment with Ti ions. Interestingly, many of these changes seemed to be dose-dependent. *T forsythia* has been associated with peri-implantitis in clinical studies[28] and in a recent systematic review.[29] T *socranskii*, *P nigrescens*, *P acnes*, and *Campylobacter* spp. have also been found elevated in supra or subgingival biofilm samples of peri-implantitis in comparison with healthy implants.[28,30,31] Ti ions also caused a biofilm dysbiosis, characterized by a significant increase in the proportions of the putative pathogens from the orange complex and a reduction in the host-compatible *Streptococcus* spp from the yellow complex. Also, although not statistically significant, as one goes from no Ti ions exposure (control) to 10 and 20 ppm Ti ions there is a progressive increase in the proportions of periodontal pathogens from the red complex. Taken together, these data suggest that these Ti ions commonly released by dental implants may favor the growth of periodontal/peri-implant-pathogenic species. These findings corroborate the results of studies with planktonic cultures and showed a specie-specific effect on bacteria growth in a dose-dependent manner.[15] Moreover, biofilm can lead to implant corrosion and even decontamination methods can affect products releasing[2]; therefore, a synergistic effect is expected between Ti products concentration and biofilm virulence, since Ti products can increase bacteria levels and biofilm formation can enhance implant degradation.

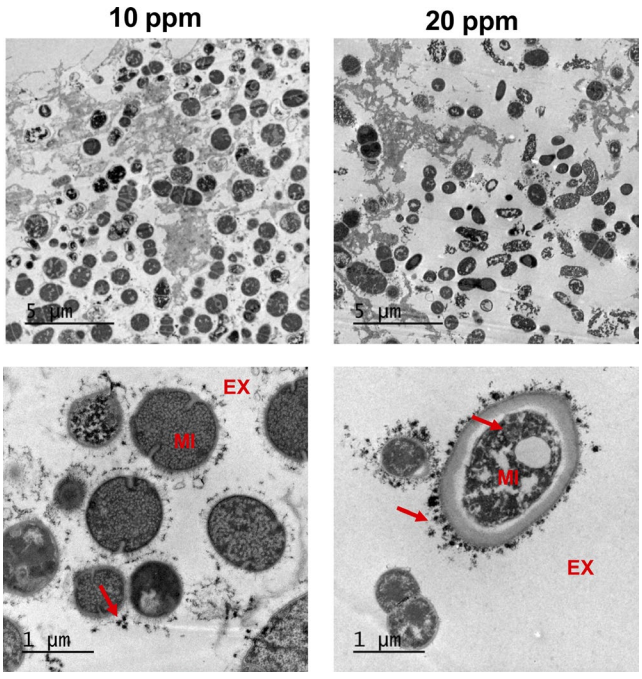

FIGURE 5 TEM images exhibiting organization of biofilms according to treatment groups. Ti ion groups showed clusters of material precipitated in extracellular environment (arrow) accumulated around microorganism cells, suggesting an intimate contact with cells; and also suggesting an effect in intracellular environment. EX, extracellular environment; MI, microorganism

The lack of synergism of Ti particles and ions combination in changing biofilm composition could be explained by (a) lower ions precipitation in biofilm in combination group (as showed by TEM images), since extracellular medium may became saturated due to particles presence and it reduces Ti ions precipitation; (b) an increase of anaerobic bacteria in the biofilm was found only for the Ti ions group, but not for Ti particles.

The mechanisms by which Ti particle and ions increase bacteria growth are still unknown. It has been suggested that the different charges between Ti and bacterial cell wall may act as an ionic bonding coaggregation, as cell membranes are essentially composed by negatively charged lipids[32] and TiO_2 layer formed on Ti material implants is positively charged.[33] Coupled with that, the dose-dependent effect from Ti ions on the significant increase of anaerobic periodontal pathogens suggests a direct effect from Ti ions high oxygen vacancies, whose excess electrons transitions from Ti^{3+} excited states to the occupied reduced TiO_2 states,[34] thus reducing the O_2 availability in the biofilm microenvironment and favoring the shift of the microbial community toward specific anaerobic bacteria species. These mechanisms may explain significant effect of excited Ti ions on bacterial dysbiosis when compared with reduced Ti particles, which is confirmed by the TEM images showing the close contact of Ti products around bacterial

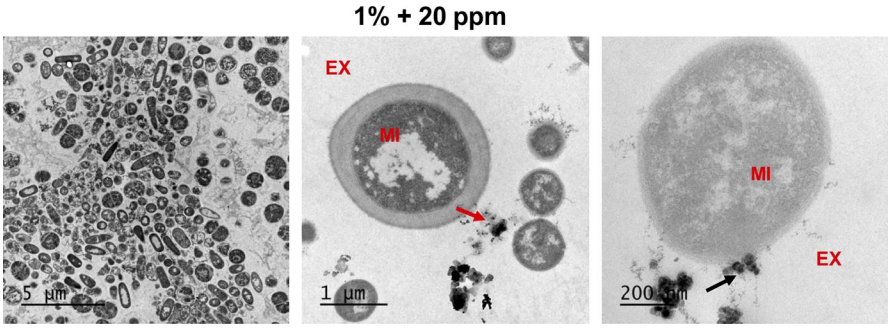

FIGURE 6 TEM images exhibiting organization of biofilms according to treatment groups. Same biofilms showing both materials: Ti ion precipitated in extracellular environment (red arrow) accumulated around microorganism cells. Clusters of Ti particles accumulated in extracellular environment (black arrow). EX, extracellular environment; MI, microorganism

cells, homogeneously dispersed in the extracellular environment, not affecting bacterial viability, as showed by checkerboard DNA-DNA hybridization technique with increasing bacteria levels at the test groups, compared with control.

The main strength of this study is to be the first one to provide the experimental evidence on the effect of Ti products in modulating an in situ biofilm to a dysbiotic state. These results may guide future studies in the field, as they suggest a possible role for Ti products in the etiopathogenesis of peri-implantitis. We were able to show a direct effect of Ti products in changing the biofilm composition toward a profile similar to that observed in peri-implantitis.[17] Increasing concentrations of Ti products or multiple Ti products exposures could be tested by other studies in order to verify if these products could change the biofilm during different phases of growth, as well as a possible effect on bacterial adhesion and bacterial interactions. One limitation of this study was the morphological characterization. Since the TEM procedure needs to be performed using samples of small thickness and low hardness, the biofilm was formed on Spurr resin slices up to 1 mm in thickness. Although physical-chemical properties of surface could affect bacterial adhesion, it is not expected any striking difference in the microbial composition biofilm after 7 days of growth.

In conclusion, the results of this study showed that Ti products, especially Ti ions, have the potential to change the microbiological composition of in situ biofilms formed on titanium surfaces. These findings suggest that the presence of Ti products around dental implants may contribute to the development of peri-implantitis.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this study.

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