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# Response to antiseptic agents of periodontal pathogens in *in vitro* biofilms on titanium and zirconium surfaces

M.C. Sánchez[a], E. Fernández[a], A. Llama-Palacios[a], E. Figuero[b], D. Herrera[b], M. Sanz[b,*]

[a] Laboratory of Dental Research, Faculty of Odontology, University Complutense, Madrid, Spain
[b] ETEP (Etiology and Therapy of Periodontal Diseases) Research Group, University Complutense, Madrid, Spain

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ABSTRACT

*Objective.* The aim of this study was to develop *in vitro* biofilms on SLA titanium (Ti-SLA) and zirconium oxide ($ZrO_2$) surfaces and to evaluate the effect of antiseptic agents on the number of putative periodontal pathogenic species.

*Methods.* An *in vitro* biofilm model was developed on sterile discs of Ti-SLA and $ZrO_2$. Three antiseptic agents [chlorhexidine and cetyl-pyridinium-chloride (CHX/CPC), essential oils (EEOOs) and cetyl-peridinium-chloride (CPC)] were applied to 72-h biofilms, immersing discs during 1 min in the antiseptic solution, either with or without mechanical disruption. Viable bacteria [colony forming units (CFU/mL)] were measured by quantitative polymerase chain reaction (qPCR) combined with propidium monoazide. A generalized lineal model was constructed to determine the effect of the agents on the viable bacterial counts of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* on each surface.

*Results.* The exposure to each antiseptic solution resulted in a statistically significant reductions in the number of viable target species included in the *in vitro* multi-species biofilm, on both Ti-SLA and $ZrO_2$ ($p < 0.001$) which was of up to 2 orders for *A. actinomycetemcomitans*, for *P. gingivalis* 2 orders on Ti-SLA and up to 3 orders on $ZrO_2$, and, for *F. nucleatum* up to 4 orders. No significant differences were found in counts of the tested bacteria between *in vitro* biofilms formed on both Ti-SLA and $ZrO_2$, after topically exposure to the antimicrobial agents whether the application was purely chemical or combined with mechanical disruption.

*Significance.* *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum* responded similarly to their exposure to antiseptics when grown in multispecies biofilms on titanium and zirconium surfaces, in spite of the described structural differences between these bacterial communities.



* *Corresponding author at*: Section of Periodontology, Faculty of Odontology, Plaza Ramón y Cajal s/n – Ciudad Universitaria, 28040 Madrid, Spain. Fax: +34 913941910.
E-mail address: marianosanz@odon.ucm.es (M. Sanz).

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## 1. Introduction

Biofilms are complex microbial communities developed on solid surfaces exposed to a wet environment [1]. In the oral cavity, different biofilms may be encountered attached to different solid oral surfaces, including teeth, prosthetic devices and dental implants [1–4]. The formation and maturation of biofilms on dental implant surfaces have been associated with the etiology of peri-implant mucositis and peri-implantitis, in a similar manner as the subgingival biofilm is associated with gingivitis and periodontitis [1–5].

Despite the similarities between biofilms on tooth and implant surfaces, some specific features might be attributed to the specific implant surface characteristics [4]. Previous *in vivo* and *in vitro* investigations have reported that surface characteristics such as roughness, surface free energy, wettability and degree of sterilization may affect biofilm formation and its bacterial three-dimensional distribution, although there is still controversy on the relevance of these differences. Recent studies evaluating biofilms on abutments, with different surface composition and topography, have shown that there is a correlation between surface roughness and viable biomass within the biofilm [6,7]. There is, however, controversy on which are the key factors guiding biofilm formation on implant surfaces, since in some studies using *in vitro* biofilm models, surface roughness seems to be the main factor [8–10], while in others, surface free energy, rather than roughness, seems to be the key factor determining initial bacterial adhesion [11,12]. Similarly, a positive correlation between surface roughness and bacterial colonization has been found in some models [13–15], while in others, certain titanium topographies seemed to inhibit bacteria adhesion together with the promotion of bone tissue formation [16,17]. Also, titanium purity, and not only surface topography, may influence early bacterial colonization [18]. Our research group, using an *in vitro* multibacterial species biofilm, has reported significant differences in biofilm thickness and three-dimensional structure, when comparing titanium and zirconium surfaces, with a higher number of initial and early colonizers (*Streptococcus oralis*, *Actinomyces naeslundii* and *Veillonella parvula*) on zirconium than on titanium surfaces [19]. These results are coincident with recent studies by de Avila et al. [20], reporting quantitative and qualitative differences between biofilms formed on titanium *versus* zirconium surfaces.

Antimicrobial agents, such as chlorhexidine (CHX), essential oils (EEOOs) or cetyl-pyridinium chloride (CPC), combined with mechanical debridement, are the gold standard therapy in the treatment of peri-implant mucositis and in the secondary prevention of peri-implantitis [21]. However, there is controversy whether implant micro-surface topography and chemistry influence the antimicrobial effect of these antimicrobial agents. This *in vitro* study was, therefore, aiming to assess whether the number of specific bacterial pathogens growing on *in vitro* biofilms over SLA titanium and zirconium oxide surfaces, were differentially affected when exposed to different antiseptic agents (alcohol-free EEOOSs, CPC and CHX/CPC).

## 2. Material and methods

### 2.1. Bacterial strains and culture conditions

Standard reference strains of *S. oralis* CECT 907T, *V. parvula* NCTC 11810, *A. naeslundii* ATCC 19039, *Fusobacterium nucleatum* DMSZ 20482, *Aggregatibacter actinomycetemcomitans* DSMZ 8324 and *Porphyromonas gingivalis* ATCC 33277 were used. These bacteria were grown on blood agar plates (Blood Agar Oxoid No 2; Oxoid, Basingstoke, UK), supplemented with 5% (v/v) sterile horse blood (Oxoid), 5.0 mg $L^{-1}$ hemin (Sigma, St. Louis, MO, USA) and 1.0 mg $L^{-1}$ menadione (Merck, Darmstadt, Germany) in anaerobic conditions (10% $H_2$, 10% $CO_2$, and balance $N_2$) at 37 °C for 24–72 h.

### 2.2. Material specimens

Sterile discs of 5 mm of diameter made of two different surface materials were used: (1) titanium with a SLA grade 2 surface (Ti-SLA) (Sand-blasted, Large grit, Acid-etched; Straumann; Institut Straumann AG, Basel, Switzerland), and (2) sterile zirconium oxide ($ZrO_2$), with a rough micro surface obtained after chemical treatment with a hot solution of hydrofluoric acid, according to a proprietary process of Institut Straumann AG (Institut Straumann AG, Basel, Switzerland). The resulting rough surface topography of $ZrO_2$ discs has a $S_a$ value of 0.55 mm (standard deviation, SD = 0.01) with a rough surface topography similar the Ti-SLA surface implants when evaluated with scanning electron microscopy (SEM), although zirconium surfaces seemed to have a flatter profile with less porosity [$S_a$ value of Ti-SLA surface of 1.17 mm (SD = 0.04)] [22].

### 2.3. Saliva preparation

Un-stimulated saliva was obtained from healthy volunteers in 10 mL aliquots at least 1.5 h after eating, drinking or tooth brushing. Each saliva sample was treated with 2.5 mmol $L^{-1}$ DL–Dithiothreitol (Sigma) for 10 min with continuous stirring in order to reduce salivary protein aggregation. It was then centrifuged (10 min, 4 °C and 12,000 rpm) and the obtained supernatant was diluted (1:1) with phosphate buffered saline (PBS; pH = 7.4). The sample was then filtered and sterilized through a 0.22 μm pore size Millex GV low-protein-binding filter X50 (Millipore, Millipore Corporation Bedford, USA) and stored at −20 °C. The efficacy of this protocol was validated by plating processed saliva samples onto supplemented blood agar plates for 72 h at 37 °C, when confirmed by lack of any bacterial growth on either aerobically or anaerobically incubated plates.

### 2.4. Biofilm development

Biofilms were generated using the method described by Sánchez et al. [23] with slightly different bacterial concentrations when preparing the bacterial suspension. Briefly, planktonic cultures of each bacteria were grown anaerobically in a protein-rich medium containing brain–heart infusion (BHI) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 2.5 g $L^{-1}$ mucin (Oxoid), 1.0 g $L^{-1}$