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Electrochemical decontamination of titanium dental implants. An in vitro biofilm model study

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Abstract

Objectives: The objective of this study is to study the effect of electrochemical treatment on biofilms developed on titanium dental implants, using a six-species in vitro model simulating subgingival oral biofilms.

Materials and Methods: Direct electrical current (DC) of 0.75 V, 1.5 V, and 3 V (anodic polarization, oxidation processes) and of −0.75 V, −1.5 V, and -3 V (cathodic polarization, reduction processes) was applied between the working and the reference electrodes for 5 min on titanium dental implants, which have been previously inoculated with a multispecies biofilm. This electrical application consisted of a three-electrode system where the implant was the working electrode, a platinum mesh was the counter electrode, and an Ag/AgCl electrode was the reference. The effect of the electrical application on the biofilm structure and bacterial composition was evaluated by scanning electron microscopy and quantitative polymerase chain reaction. A generalized linear model was applied to study the bactericidal effect of the proposed treatment.

Results: The electrochemical construct at 3 V and −3 V settings significantly reduced total bacterial counts ($p < .05$) from 3.15×10^6 to 1.85×10^5 and 2.92×10^4 live bacteria/mL, respectively. *Fusobacterium nucleatum* was the most affected species in terms of reduction in concentration. The 0.75 V and −0.75 V treatments had no effect on the biofilm.

Conclusion: Electrochemical treatments had a bactericidal effect on this multispecies subgingival in vitro biofilm model, being the reduction more effective than the oxidative treatment.

KEYWORDS
biofilm, dental implant, direct current, electrochemical, oxidation and reduction, peri-implantitis

Leire Virto and Verónica Odeh are contributed equally to the work.

1 | INTRODUCTION

Dental implants are commonly used medical devices for replacing missing teeth, and despite their proven long-term survival rates, they suffer from a high incidence of mechanical and biological complications (peri-implant diseases) (Derks & Tomasi, 2015; Jung et al., 2008; Rodrigo et al., 2018). These peri-implant diseases were recently defined as biofilm-associated pathological conditions affecting the tissues surrounding dental implants (Berglundh et al., 2018) and categorized as peri-implant mucositis and peri-implantitis, depending on the extent of the inflammatory process. Both share the presence of inflammation in the peri-implant mucosa, but only peri-implantitis is associated to loss of supporting bone (Renvert et al., 2007).

In spite of the important limitations on the existing epidemiological evidence on the prevalence of peri-implant diseases, due to the use of convenience population samples and different case definitions, current data range between 25%–65% and 15%–43% for the prevalence of peri-implant mucositis and peri-implantitis, respectively (Derks & Tomasi, 2015).

The treatment of these diseases is based on the elimination of the biofilms associated with the surfaces of implants and restorative components, using non-surgical and surgical strategies. Most commonly, these surface decontamination strategies are based on mechanical and/or chemical debridement, which can be supplemented with adjunctive measures, such as diode laser, photodynamic therapy, local or systemic antimicrobials, and probiotics, have also been proposed and tested (Renvert & Polyzois, 2018). These treatment protocols have been evaluated in different clinical studies, without anyone showing a consistent disease resolution, but many have been associated to significant reductions in clinical signs of inflammation and arrest in the bone destructive process (Figuero et al., 2014); (Liñares et al., 2019; Ramanauskaite et al., 2021).

A recent innovative treatment concept based on the use of electrical currents for implant surface decontamination has been proposed (Al-Hashedi et., 2016; Mohn et al., 2011). Its mechanism of action is based on either a direct effect of the electric current on the bacterial cell membrane, disrupting its integrity and causing bacterial inactivation, or through electrolysis resulting in production of oxidative substances (i.e., H_2O_2) also bacterial inactivation. Furthermore, the changes in temperature or pH caused by the electrical current may indirectly affect the molecules in the bacterial cell surface and also cause bacterial lysis (Al-Hashedi et., 2016). Recently, a device based on this electrochemical concept has been made commercially available for the treatment of peri-implantitis (GalvoSurge Dental AG, Widnau, Switzerland), although the evidence on its efficacy or safety for clinical use is still lacking. Results from an in vitro study have shown biofilm removal activity (Ratka et al., 2019), and the results from a prospective case series in peri-implantitis patients have shown the ability of this treatment to arrest clinical and radiological signs of disease (Schlee et al., 2021), although these studies do not demonstrate the efficacy of using electrical currents to treat peri-implantitis, given the (eminent) lack of a control group. It was, therefore, the purpose of the present in vitro investigation to evaluate the effect of a custom-designed electrochemical treatment model, using minimal amounts of direct currents on bacterial biofilms adhered to dental implant surfaces.

2 | MATERIALS AND METHODS

2.1 | In vitro multispecies biofilm model

Ethical approval was not required for this in vitro study. In brief, this model includes six species of oral bacteria (*Streptococcus oralis* CECT 907T, *Veillonella parvula* NCTC 11810, *Actinomyces naeslundii* ATCC 19039, *Fusobacterium nucleatum* DMSZ 20482, *Aggregatibacter actinomycetemcomitans* DSMZ 8324, and *Porphyromonas gingivalis* ATCC 33277), which are first grown on blood agar plates (Blood Agar Oxoid N° 2; Oxoid), supplemented with 5% (v/v) sterile horse blood (Oxoid), 5.0 mg L^{-1} hemin (Sigma) and 1.0 mg L^{-1} menadione (Merck) in anaerobic conditions (10% H_2, 10% CO_2, and balance N_2) at 37 ± 1°C for 24-72 h. Pure cultures of each bacterium were then grown anaerobically in a protein-rich medium containing brain heart infusion (BHI) (Becton, Dickinson and Company) supplemented with 2.5 g L^{-1} mucin (Oxoid), 1.0 g L^{-1} yeast extract (Oxoid), 0.1 g L^{-1} cysteine (Sigma–Aldrich), 2.0 g L^{-1} sodium bicarbonate (Merck), 5.0 mg L^{-1} hemin (Sigma–Aldrich) and 1.0 mg L^{-1} menadione (Merck), and 0.25% (v/v) glutamic acid (Sigma–Aldrich). The bacterial concentration was adjusted by spectrophotometry to obtain a solution in BHI medium containing 10^3 colony forming units (CFU)/mL for *S. oralis*, 10^5 CFU/mL for *V. parvula* and *A. naeslundii*, and 10^6 CFU/mL for *F. nucleatum*, *A. actinomycetemcomitans*, and *P. gingivalis*. Then, this pooled bacterial culture was added on to sterile titanium implants, 3.3 mm in diameter and 8 mm height (Roxolid SLA implant, Straumann) and incubated in anaerobic conditions (10% H_2, 10% CO_2 and balance N_2) at 37 ± 1 °C for up to 72 h (Bermejo et al., 2019). As negative controls, implant surfaces were bathed with culture medium without bacteria. The experimental design included three independent experiments, each with three replicas ($N = 9$).

2.2 | Electrolytic model

The electrochemical cell for electrical treatment was designed as a cylindrical box containing three-electrodes (Figure 1a), being the titanium implant (Straumann, S ø3.3 mm RN SLA® 10 mm Roxolid®, Loxim™) the working electrode (WE), a platinum mesh (Custom-built, ø 1.2 cm), the counter electrode (CE), and an Ag/AgCl the reference electrode (RE) used to control the electric potential at the working electrode during the treatment. As electrolyte, 15 mL of a potassium iodide and L lactic acid solution (KI-LA) (1 M pH = 6.4) (Schneider et al., 2018) was used. The applied direct current (DC) employed a polarization time of 5 min with a constant potential between WE and RE of 0.75 V, 1.5 V, 3 V (what created oxidant conditions with the implant as anode) and −0.75 V, −1.5 V, −3 V (what created reduction phenomena with the implant as cathode). As controls, we used

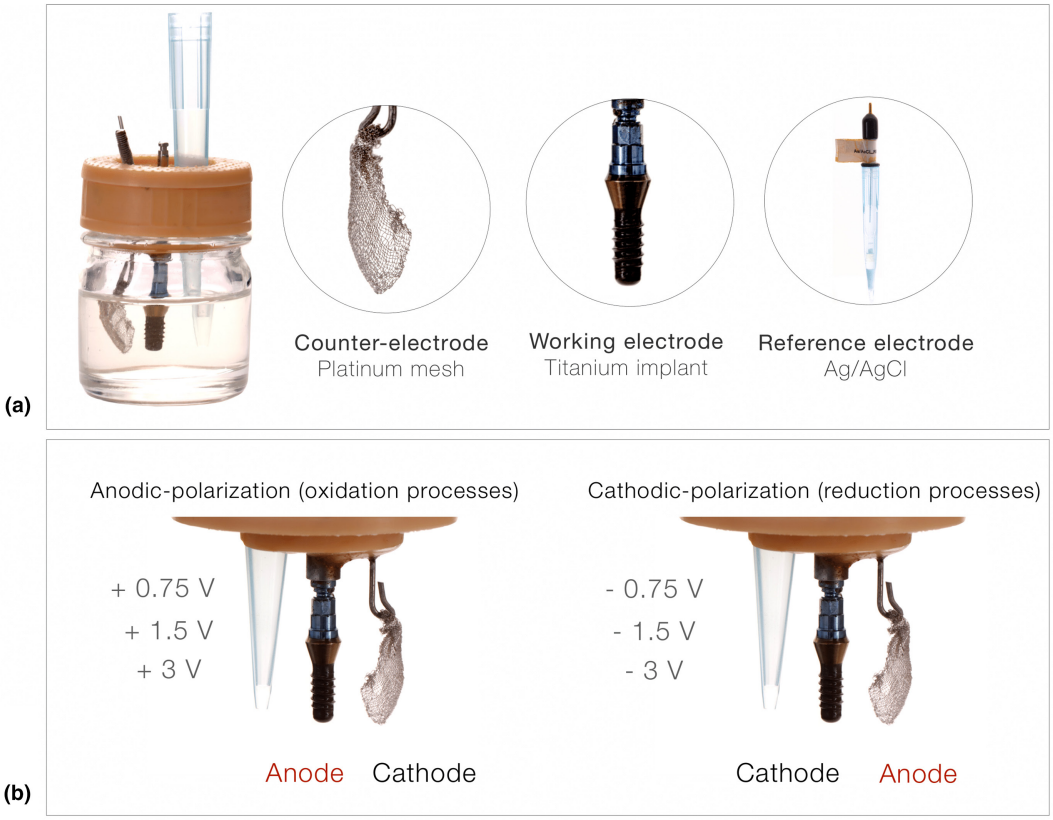

FIGURE 1 (a) Custom designed, three-electrode potentiostatic, electrochemical stimulation chamber. On the left side, it could be observed the three electrodes immersed in the electrolyte [lactic acid solution (KI-LA) (1 M pH = 6.4)]. An enlarged image of each of the electrodes (counter, working and reference electrode) used can be seen on the right. (b) Schematic representation showing in vitro electrical stimulation using a three-electrode system. The working electrode is the titanium implant on which a 72 h biofilm has been incubated, located between the Ag/AgCl electrode (reference) and the platinum electrode (counter). The assembly of the electrochemical cell was performed to apply direct current with cathodic (+0.75 V, +1.5 V, +3 V) or anodic polarization regimens (−0.75 V, −1.5 V, −3 V). In experiments with negative voltages (reduction) applied, the implant acts as an anode, and in experiments with positive voltages (oxidation) applied, the implant acts as a cathode.

the same construct immersed in the electrolyte for 5 min without the application of any potential (Figure 1b). The electrical polarization was applied with a dedicated potentiostat (VersaSTAT 3-200®; Princeton Applied Research).

In addition, internal resistance measurements were performed on the implants with and without biofilm formation using Alternating Current (AC) signals with multiple frequencies, often called simply "impedance spectroscopy measurement (EIS tests)".

2.3 | Outcomes

2.3.1 | Quantitative evaluation of live bacteria

After applying the electrochemical treatment, implants were removed from the electrolytic cell and placed in a tube containing 1 mL of phosphate buffered saline (PBS). Once the biofilm was disrupted by vortexing, a treatment with propidium monoazide (PMA) (Biotium Inc.,) was applied following a previous described protocol (Sanchez, Marin, et al., 2014.

After that bacterial DNA was isolated using a commercial kit (MolYsis Complete5. Molzym Gmbh & Co.KG.) following the manufacturer's instructions. The hydrolysis probe 5'nuclease assay, polymerase chain reaction (PCR) method, was used for detecting and quantifying the bacterial DNA from live cells. Primers and probes were obtained by Life Technologies Invitrogen (Carlsbad), Applied Biosystems (Carlsbad), and Roche (Roche Diagnostic GmbH) and were targeted against 16 S rRNA genes. The sequence and concentration of the primers, as well as the methodology used, have been described in previous studies (Marin et al., 2017, 2018).

2.3.2 | Scanning electron microscope (SEM) analyses

The specimens ($n = 6$) were fixed in a solution at 4% paraformaldehyde and 2.5% glutaraldehyde for 4 h, at 4°C. The implants were then washed once in PBS and another time in sterile water (immersion time per washed 10 min) and then dehydrated through a series of graded ethanol solutions (30%, 50%, 70%, 80%, 90%, and

100%; immersion time per series 10 min). After that, specimens were dried using the critical point method, sputter-coated with gold and observed by electron microscopy JSM 6400 (JSM6400; JEOL) with a back-scattered electron detector and an image resolution of 25 kV.

2.3.3 | Electrolyte

The electrolyte solution was assessed before and after the electrochemical treatment for changes in temperature (Digital Hand-held Temperature Thermometer, *MX-TDI2307, MX Onda, Madrid, Spain)* and pH (*Micro PH-metro-2001, CRISON, Barcelona, Spain*).

2.4 | Statistical analysis

An experiment-level analysis was performed for each study parameter ($n = 9$ for each parameter studied by qPCR). Shapiro-Wilk goodness-of-fit tests and distribution of data were used to assess normality. Data were expressed as means and standard deviation (SD) and 95% confidence intervals (CI). Analysis of variance (ANOVA) and post-hoc testing with Bonferroni's correction were used to determine differences among treatments setting the statistical significance at $p < .05$. A software package (IBM SPSS Statistics 27.0; IBM Corporation) was used for all data analyses.

3 | RESULTS

3.1 | Electrochemical parameters

During polarization (Figures 2a–f), the potentiostat recorded the current-time data showing two differentiated behaviors. After applying 0.75 V, 1.5 V and −0.75 V, −1.5 V, the absolute current increased sharply and later decreased exponentially until approaching zero, following the typical pattern of charges separation under potential step experiments. However, when applying 3 V and −3 V, the current-time data demonstrated a totally different behavior, in which the current did not decrease exponentially after the peak but instead tuned itself between $2.6{\cdot}10^{-3}$-$8.0{\cdot}10^{-3}$ mA/cm^2 and 88-167 mA/cm^2, respectively, suggesting a Faraday reaction mechanism much more intense under cathodic processes that could be also causing changes in the electric resistance of the set up. Accordingly, the amount of electrical charge during the experiments was very low for 0.75 V and 1.5 V pulses rating $1.5{\cdot}10^{-3}$ and $4.9{\cdot}10^{-3}$ mAh, respectively, in the anodic process, and for −0.75 V and −1.5 V with $0.9{\cdot}10^{-3}$ and $12.9{\cdot}10^{-3}$ mAh, respectively, under cathodic pulses, while much larger values; 0.516 and 12.9 mAh were recorded when applying 3 V and −3 V, respectively, again with more electrical charge in reduction treatments.

The results of EIS tests results are represented on a Nyquist plot (Figure 3).

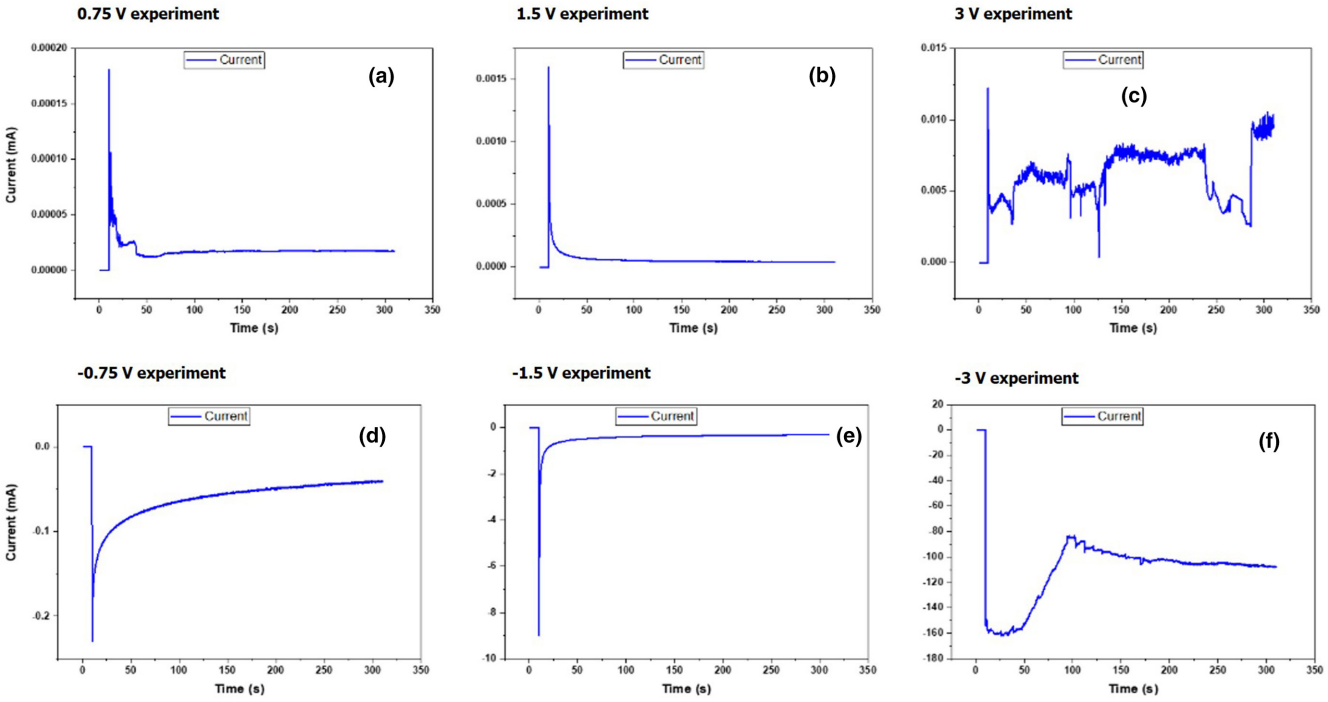

FIGURE 2 Current-time curves (current expressed as milliamperes and time expressed as seconds) registered in the potentiostat during the experiments from the six experimental groups (a) −0.75 V, (b) −1.5 V, (c) −3 V (d) 0.75 V, (e) 1.5 V and (f) 3 V.

3.2 | Effect of electrical treatment on the electrolyte: pH And temperature

The implants in the control group show no evidencing changes in the pH.

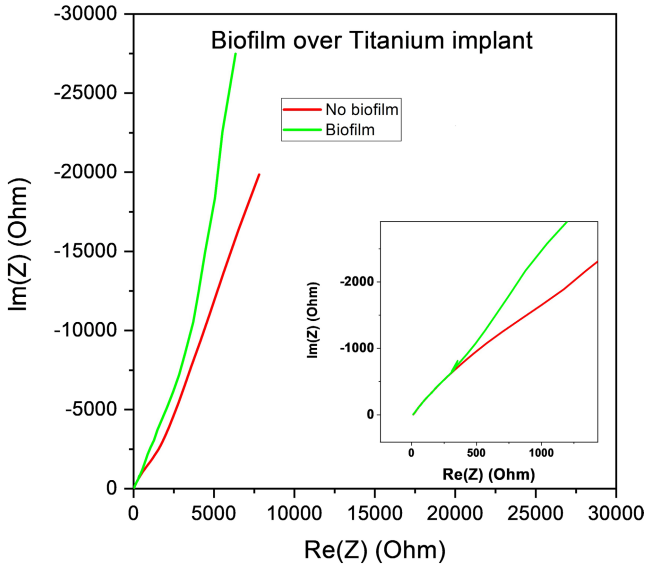

FIGURE 3 Nyquist plot. Impedance spectra of pure titanium electrodes at Open Circuit Potential (red) and impedance spectra of biofilms at titanium electrodes at Open Circuit Potential (green). 20 mV sinus amplitude, 100 mHz–10 kHz, WE: Ti, CE: Pt mesh, RE: Ag, cell type.

In the oxidation assays on the contaminated implants, statistically significant changes in pH were only observed for 3 V compared to 0.75 V ($p < .001$) and 1.5 V ($p < .001$), being the values for each group: [6.98 (SD = 0.89) for 0.75 V; 6.93 (SD = 0.47) for 1.5 V; and 8.13 (SD = 0.68) for 3 V]. Similarly, when applying 0.75 V and 1.5 V, there were no evident changes in the electrolyte, while after 3 V, the electrolyte turned to an orange hue, coming from the WE (the implant) (Figure 4a).

In the reduction assays, statistically significant changes in the initial pH of 6.4 were observed in the -3 V tests ($p < .001$), being the values for each group 6.50 (SD = 0.12) for −0.75 V, 6.65 (SD = 0.24) for −1.5 V, and 10.90 (SD = 0.31) for -3 V. When −0.75 V was applied, no changes were observed in the electrolyte, on the surface of the implant or the mesh. However, when −1.5 V was applied, a bubbling phenomenon was more clearly observed than in oxidation assays and affecting mainly the surface of the implant (cathode). These results were consolidated at −3 V (Figure 4b), where the reddish precipitate diffused in between the electrodes and almost covered the entire recipient after the 5 min of electrochemical treatment.

The most important temperature variation occurred in the −3 V experiments, producing an increase in 1.75°C (SD = 1.46), being significantly higher compared to the other groups [0.11°C (SD = 0.28) for 1.5 V; 0.21°C (SD = 0.42) for 3 V; 0.32°C (SD = 0.33) for −0.75 V; 0.38°C (SD = 0.28) for −1.5 V], except for the 0.75 V group [0.58°C (SD = 0.76)].

FIGURE 4 Electrochemical treatment for implant surface decontamination: (a) Corresponding photographs of the cell during and at the end of each oxidation electrochemical treatment (0.75 V, 1.5 V and 3 V). (b) Corresponding photographs of the cell during and at the end of each reduction electrochemical treatment (−0.75 V, −1.5 V and −3 V).

3.3 | Effect of electrical treatment on the biofilm vitality

Table 1 depicts the live bacteria remaining on the implant surface after the different electrochemical treatments. Statistically significant reductions in total live bacteria were observed in the groups at +3 V [1.85×10^5 (SD = 1.91×10^5) live bacteria/mL, $p = .044$] and -3 V [2.92×10^4 (SD = 2.56×10^4) live bacteria/mL, $p < .001$], when compared to the control group [3.15×10^6 (SD = 5.70×10^6) live bacteria/mL]. When calculating the net effect of the tested electrochemical treatments as: [(total number of bacteria in biofilms treated with the negative control − total number of viable bacteria in biofilms treated with electricity)/total number of bacteria in biofilms treated with negative control x100%], the decrease in total bacterial vitality was 94.12% with oxidation treatment (+3 V), compared to 99.07% with reduction treatment (−3 V), being these differences statistically significant.

When analyzing the results by specific bacteria, *F. nucleatum* was the most affected by the electrochemical treatment. All potentials, except 0.75 V and − 0.75 V, were able to significantly reduce the concentration of this species, compared with the control group. In contrast, concentrations of *S. oralis* were significantly reduced in both oxidation and reduction treatments at higher voltages of 3 V and −3 V, compared to the control group and the other test groups ($p < .001$) (decreasing in two orders of magnitude). No statistically significant differences were observed for *A. naeslundii* and *V. parvula* at any voltage, although reductions in one to two orders of magnitude occurred after the −3 V treatment. A similar tendency was noted for *P. gingivalis* and *A. actinomycetemcomitans*, although for the latter, statistically significant reductions ($p = .009$) occurred after applying −3 V [9.86×10^3 (SD = 2.40×10^4) live bacteria/mL], compared with the control group [5.40×10^6 (SD = 8.04×10^6) live bacteria/mL].

At the lowest electrical currents (0.75 V and −0.75 V), there was a paradoxical reaction, with a slight increase in counts of of *S. oralis*, *A. naeslundii*, *V. parvula*, and *P. gingivalis* after applying the current.

3.4 | Effect of electrochemical treatment on the biofilm structure

Figure 5 depicts the SEM images obtained at two levels of the implant surface, the peaks and the valleys of the threads, after the different electrochemical treatments. In the control group, clusters of cocci and elongated bacteria, corresponding to *F. nucleatum*, can be clearly observed. While in the test groups, after different electrochemical regimens, lower amounts of bacterial cells were identified, both at the thread peaks and within the valleys. It was observed a higher decrease of bacterial amount at implant surfaces treated with cathodic-oxidation conditions, compared with anodic-reduction conditions and with the control group. Similarly, in implants treated with −0.75 V, a greater number of rod shaped bacteria and accumulations of coccoid bacteria were observed, compared to the 0.75 V group. At the highest voltages (3 V, −3 V, and − 1.5 V), there was also a clear reduction in the presence of bacteria, especially at −3 V, with very few cocci left on or between the threads.

4 | DISCUSSION

This in vitro investigation using a multispecies biofilm model seeded on dental implant surfaces has demonstrated that the tested electrochemical treatments at higher voltages (3 V and −3 V groups) had a considerable anti-bacterial effect, with statistically significant reductions in the bacterial load quantified by qPCR, specifically affecting the species *F. nucleatum*, *S. oralis* and *A. actinomycetemcomitans* and a notable decrease in the amount of bacteria evidenced by SEM. Regarding the experimental set up, we selected a three-electrode system, with a reference electrode to control the voltage applied to the implant and a counter electrode to record the amount of current flowing through the system during the different treatments.

The results obtained in the EIS test depicting the beginning of a semi-circle at high frequencies, usually attributed to charge-transfer resistance process, overlapped with a vertical tail of the impedance in the middle, and ion diffusion problems in the implant-electrolyte interface at low frequencies. Both curves with and without biofilm had similar shape, demonstrating the high--electrical resistance of the system in the very high impedances (+1000 ohms). Within these fixed experimental conditions, the differences in the imaginary part of the curve at the high-frequency domain were only related to changes in the charge transfer resistance, while the growth of the biofilm on the implant surface limited the charge transfer processes.

Direct current with constant potential was applied for the implant surface decontamination using anodic (0.75 V, 1.5 V and 3 V) or cathodic (−0.75 V, −1.5 V, and −3 V) regimens. With this electrochemical treatment, the reduction was more effective than the oxidation protocols in reducing the biofilm bacteria, especially when applying higher potentials, resulting in percentage reductions of 94.12% (+3 V) and 25.39% (+1.5 V) in oxidation treatments, compared to 99.07% (−3 V) and 82.95% (−1.5 V) in reduction treatments, being these intragroup differences statistically significant. This might be explained by the hydrogen formation at the cathode that strongly promote the biofilm detachment, as opposed to the oxidant conditions, where essentially disinfection compounds are generated. However, when applying lower voltages (+0.75 or −0.75), no statistically significant effects were observed. These results were consistent with the obtained electrical response, because the highest cumulative charge of 12.93 mAh was registered for -3 V, compared to $1.5\cdot10^{-3}$–$4.9\cdot10^{-3}$ mAh for the other treatments. On the other hand, the current-time data recorded when the lower voltages of 0.75 V, 1.5 V and −0.75 V, −1.5 V were applied, corresponded with a mass transfer limited mechanism in which an interface between the implant and an unstirred solution was created, resulting in electrode oxidization or reduction of the nearby electroactive species. However, when applying 3 V and −3 V, the electrical behavior produced faradaic processes, more consistent with redox reactions,

TABLE 1 Effect of 5 min electrochemical treatments (0.75 V, 1.5 V and 3 V and −0.75 V, −1.5 V and −3 V) on the number of viable bacteria in the in vitro multi-species biofilm [live bacteria mL^{-1}, obtained by quantitative real-time polymerase chain reaction (qPCR)].

Bacteria	Treatment	Live bacteria/mL	Standard desviation	95% confidence interval for the mean	
				Lower bound	Upper bound
Streptococcus oralis	Control	1.93×10^5	1.08×10^5	1.24×10^5	2.62×10^5
	0.75 V	1.25×10^6	2.12×10^6	-3.88×10^5	2.88×10^6
	1.5 V	2.15×10^5	2.38×10^5	3.15×10^4	3.99×10^5
	3 V	$4.38 \times 10^{4a,b}$	8.71×10^4	-2.32×10^4	1.11×10^5
	−0.75 V	5.07×10^5	8.95×10^5	-1.81×10^5	1.20×10^6
	−1.5 V	1.10×10^5	1.08×10^5	2.66×10^4	1.93×10^5
	−3 V	$1.95 \times 10^{3a,b}$	1.94×10^3	4.51×10^2	3.45×10^3
Actinomyces naeslundii	Control	1.22×10^4	1.21×10^4	5.72×10^3	1.87×10^4
	0.75 V	8.04×10^3	6.09×10^3	2.40×10^3	1.37×10^4
	1.5 V	1.21×10^4	1.57×10^4	-6.79×10^1	2.42×10^4
	3 V	3.72×10^3	2.01×10^3	2.17×10^3	5.27×10^3
	−0.75 V	1.76×10^4	2.25×10^4	2.34×10^2	3.49×10^4
	−1.5 V	1.06×10^4	6.66×10^3	5.50×10^3	1.57×10^4
	−3 V	7.80×10^3	4.02×10^3	4.71×10^3	1.09×10^4
Veillonella parvula	Control	6.51×10^5	1.4×10^6	-1.62×10^5	1.46×10^6
	0.75 V	4.86×10^5	7.4×10^5	-1.99×10^5	1.17×10^6
	1.5 V	3.61×10^5	8.06×10^5	-2.59×10^5	9.81×10^5
	3 V	3.93×10^4	8.63×10^4	-2.71×10^4	1.06×10^5
	−0.75 V	9.07×10^5	2.10×10^6	-7.12×10^5	2.53×10^6
	−1.5 V	3.56×10^5	7.62×10^5	-2.31×10^5	9.42×10^5
	−3 V	8.98×10^3	9.03×10^3	2.04×10^3	1.59×10^4
Aggregatibacter actinomycetemcomitans	Control	5.40×10^6	8.04×10^6	1.12×10^6	9.69×10^6
	0.75 V	1.71×10^6	2.54×10^6	-2.45×10^5	3.66×10^6
	1.5 V	1.74×10^6	3.76×10^6	-1.15×10^6	4.64×10^6
	3 V	1.44×10^5	2.01×10^5	-7.37×10^2	2.88×10^5
	−0.75 V	1.84×10^6	2.74×10^6	-2.73×10^5	3.95×10^6
	−1.5 V	1.82×10^5	5.16×10^5	-2.15×10^5	5.80×10^5
	−3 V	$9.86 \times 10^{3a,b}$	2.40×10^4	-8.63×10^3	2.83×10^4
Porphyromonas gingivalis	Control	5.32×10^2	5.57×10^2	2.23×10^2	8.41×10^2
	0.75 V	3.70×10^2	4.79×10^2	-3.14×10^1	7.71×10^2
	1.5 V	3.78×10^2	4.92×10^2	-2.04×10^1	7.56×10^2
	3 V	2.40×10^2	3.15×10^2	-2.95×10^0	4.82×10^2
	−0.75 V	6.38×10^2	1.25×10^3	-4.09×10^2	1.68×10^3
	−1.5 V	9.94×10^2	2.43×10^3	-1.04×10^3	3.03×10^3
	−3 V	1.75×10^2	9.66×10^1	1.01×10^2	2.50×10^2
Fusobacterium nucleatum	Control	6.38×10^4	1.21×10^5	-1.05×10^3	1.29×10^5
	0.75 V	4.15×10^4	8.93×10^4	-3.32×10^4	1.16×10^5
	1.5 V	1.77×10^{4a}	3.13×10^4	-6.37×10^3	4.19×10^4
	3 V	$4.37 \times 10^{2a,b}$	5.41×10^2	2.09×10^1	8.53×10^2
	−0.75 V	1.50×10^4	2.13×10^4	-1.46×10^3	3.14×10^4
	−1.5 V	2.63×10^{3a}	2.74×10^3	3.36×10^2	4.93×10^3
	−3 V	$4.81 \times 10^{2a,b}$	8.98×10^2	-2.10×10^2	1.17×10^3

(Continues)

TABLE 1 (Continued)

Bacteria	Treatment	Live bacteria/mL	Standard desviation	95% confidence interval for the mean	
				Lower bound	Upper bound
Total bacterial counts	Control	3.15×10^6	5.70×10^6	-4.81×10^5	6.77×10^6
	0.75 V	2.78×10^6	2.45×10^6	5.09×10^5	5.05×10^6
	1.5 V	2.35×10^6	3.58×10^6	-4.09×10^5	5.10×10^6
	3 V	1.85×10^{6}[a,b]	1.91×10^5	3.79×10^4	3.32×10^5
	−0.75 V	3.08×10^6	5.86×10^6	-1.82×10^6	7.99×10^6
	−1.5 V	5.37×10^5	7.79×10^5	2.76×10^5	1.19×10^6
	−3 V	2.92×10^{4}[a,b]	2.56×10^4	9.48×10^3	4.90×10^4

Note: Data are expressed as mean ± standard deviation of the mean (SD). Analysis of variance (ANOVA) and post-hoc testing with Bonferroni's correction (alpha = .05) were used to determine differences among treatments.

[a]Statically significant differences when compared with the control group.

[b]Statically significant intragroup differences (differences between the three oxidation treatments among themselves (0.75 V, 1.5 V and 3 V) and among the three reduction treatments (−0.75 V, −1.5 V and −3 V)).

resulting in the loss and gain of electrons in the implant. This mechanism of action suggests that low potentials lead to charge separation on the implant surface, with discrete bactericidal activity, while high potentials involve faradaic reactions, with processes of gain and loss of electrons, what may be result in a higher reduction of the bacterial biomass.

The differences in bactericidal activity between reduction and oxidation processes may be explained by the electrical parameters recorded with the potentiostat. During the anodic polarization, the transferred charge was lower than expected, and negligible after applying 0.75 V and 1.5 V. Significant higher values in the charge exchanged were observed when cathodic conditions were forced in the implant, suggesting that under these conditions, the electrode surface acquires a reduction nature, thus resulting in higher bactericidal activity. This behaviour is consistent with reports from previous investigations (Schneider et al., 2018). Another explanation to the higher efficacy of reduction treatments could be due to the use of titanium (Ti) as supporting electrode material, as this metal has been widely used as a medical device in artificial prostheses and implants due to its biocompatibility, which results from the passive oxide film on its surface that spontaneously forms when exposed to air ($Ti + O_2 \rightarrow TiO_2$)or water ($Ti + H_2O \rightarrow TiO_2 + 4H^+ + 4e^-$) (Delgado-Ruiz & Romanos, 2018). In addition, it has been reported that the titanium surface properties may be altered during electrolysis by formation of thicker oxide barriers and porous structures on the anode surface (Sahrmann et al., 2014).

The antibacterial effect observed after applying 3 V and −3 V can be attributed to the electrolytically generated bubbles, which were mainly observed at −3 V, what may lead to physical detachment of the biofilm from the implant surface. This bubbling effect also allows the generation on the implant surface of hydrogen and highly reactive substances as I_2, H_2O_2, and hydroxyl radicals, with proven antimicrobial activity (Schneider et al., 2018; Wang et al., 2013); (Ehrensberger et al., 2015). Moreover, the measured changes in pH and temperature, especially after applying -3 V, may also explain the antibacterial effect, what agrees with previous studies correlating bacterial CFU reductions with changes in the microenvironment pH after electrical stimulation (del Pozo et al., 2009); (Mohn et al., 2011). In the present study, a potassium iodide and L lactic acid solution (KI-LA) was used as electrolyte. To avoid the acidity of the solution, its pH was neutralized (until reaching pH = 6.40) using a minimum amount of 10 M NaOH.

The results reported in this investigation, however, are difficult to compare within other similar investigations, due to the high variability in the systems used, in terms of the design of the electrolytic chamber, the application of either direct or alternating signals, the number and electrode materials, conductive medium, electrical parameters (voltage, current), and time of exposure. Moreover, the majority of previous studies have used bacteria in planktonic state (Canty et al., 2017; Nodzo et al., 2015) or mono-species biofilms of *E. coli* (Mohn et al., 2011; Schneider et al., 2018), *S. aureus* (Ehrensberger et al., 2015; Ercan et al., 2011) or *S. epidermidis* (Dauben et al., 2016), demonstrating bacterial load reductions of about 97%–99% within seconds, under direct electric current with an amplitude ranging from as low as 0.2 to 1.8 V and 2 to 10 mA. These in vitro models are very different from the environmental and microbiological conditions found in the oral cavity. In contrast, this investigation has used a validated in vitro multispecies biofilm model of six anaerobic bacteria. This biofilm model has been utilized and validated in multiple investigations studying biofilms formed on teeth or different implant surfaces. Furthermore, this model has been used to assess the effect of different mechanical and chemical methods to remove the biofilm from these surfaces (Fernández et al., 2017; Ribeiro-Vidal et al., 2020; Sánchez et al., 2017, 2019, 2020; Virto et al., 2022).

Under these conditions, low voltages (+0.75 V, −0.75 V, +1.5 V, −1.5 V) were not able to achieve significant bacterial reductions and only the application of high potentials of −3 V and 12.9 mAh during

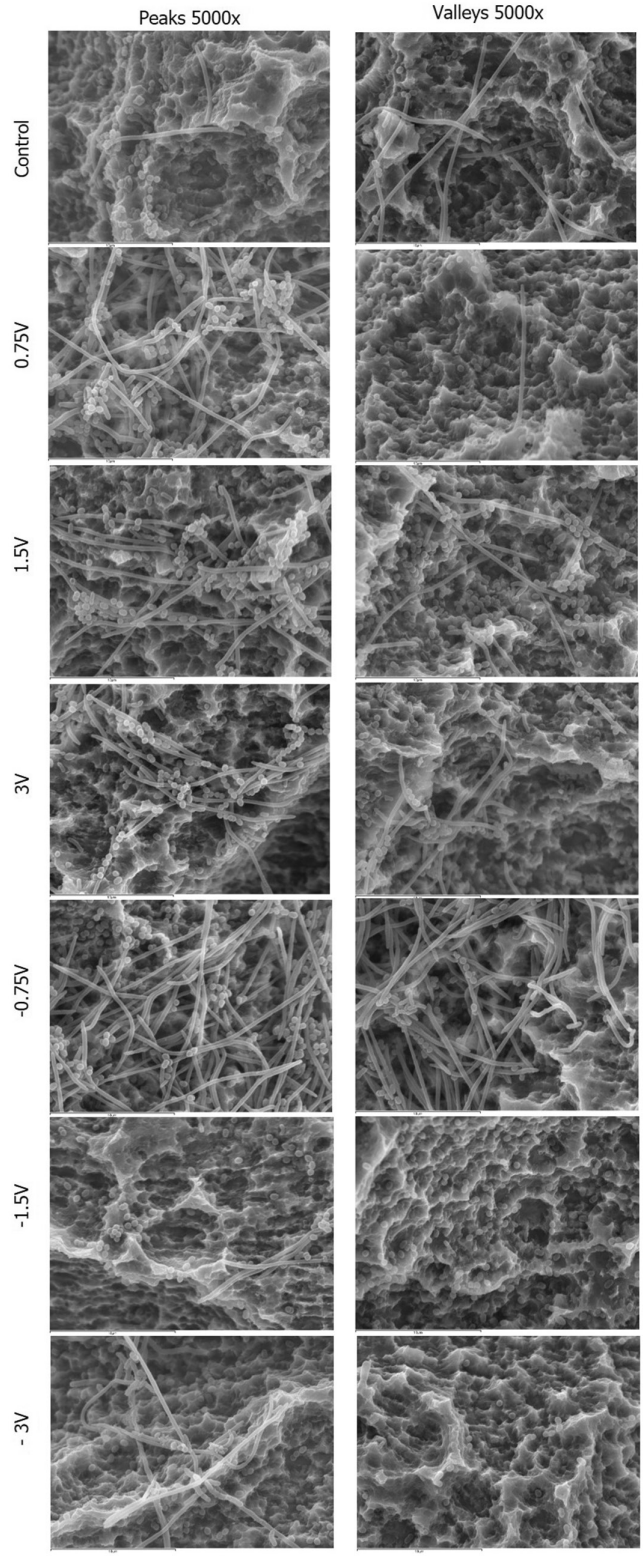

FIGURE 5 Scanning electron microscopy (SEM) of 72 h oral biofilms after the different electrochemical treatments applied. It can be observed the different bacterial disposition as well as the different three-dimensional structure of the biofilms in peaks and threads. Magnification: 5000×. Scale bar = 10 μm.

5 min resulted in significant bacterial reductions. There is only one previous investigation using a 72 h-multispecies biofilm with eight anaerobic species (*Actinomyces naeslundii*, *Streptoccocus oralis*, *Fusobacterium nucleatum and Porphyromonas gingivalis*) (Sharman et al., 2014). The results from this investigation were similar to our findings in regard to the relative antibacterial efficacy of reduction to oxidation processes but reporting higher reductions in viable bacterial counts, because all viable bacteria were eliminated. However, their experimental set up used titanium discs and higher voltages (11 V) with longer exposure times (10 min). In the present investigation, however, we used real implants with a macro/micro-surface topography that may have influenced the ability of the electrochemical treatments to reach less-accessible zones, and the use of lower voltages and reduced times (5 min), which may be more compatible with clinical practice and the viability of human cells. Although similar times have been tested in other investigations (Schneider et al., 2018), most of the other similar studies have used longer treatment times, such as 15 min (Mohn et al., 2011), 1 h (Ehrensberger et al., 2015; Nodzo et al., 2015), or even 1 day (Schmidt-Malan et al., 2015), which many not be suitable in the clinical practice.

The tested electrochemical treatment had a significant effect on *F. nucleatum*, an important biofilm structural bacterium that bridges early and late colonizers to the tooth surface (Kolenbrander & London, 1993) and favors the overgrowth of less aerotolerant and more pathogenic organisms, such as *P. gingivalis* (Bradshaw et al., 1998). When applying 0.75 V and −0.75 V treatments, there was a tendency for slight increases in live bacteria/mL of bacteria within the biofilms (*S. oralis*, *A. naeslundii*, *V. parvula* and *P. gingivalis*). This proliferative effect has been explained by the increase in bacterial metabolic activity and cell division at low-electric fields (Ueshima et al., 2002); Carvalho et al., 2019). These specific bacterial behaviors to the electrochemical treatments should be further explored with the appropriate experimental designs.

Electrochemical treatment as a method of decontamination seems promising, but further research is needed to become a true alternative to surgical treatment. The commercial method available with this technology requires a surgical intervention to reach the affected area (Schlee et al., 2021). Some authors developed a theory that low-electric currents could increased metabolic activity of sessile and persistent cells within mature biofilm, reducing the tolerance of bacteria to antimicrobials (Jass & Lappin-Scott, 1996; Jass et al., 1995). Both the elimination and the decrease of antimicrobial resistance of *F. nucleatum* could result in a change in biofilm dynamics that would allow greater penetrability of adjuvants of known pathogenicity, such as chlorhexidine or cetyl-peridinium-chloride (Sánchez et al., 2017). This scenario may open a novel line of research in which the electrochemical treatment could be used as a coadyuvant strategy to improve the performance of chemical components for surface decontamination in the treatment of peri-implant diseases. It is also important to further study the effects of

this method on the integrity of the cells and tissues adjacent to the treated implant (Kaiser et al., 2020).The results of the present investigation should be interpreted with caution, as the in vitro nature of the experimental model and the in vitro biofilm model used may lack the essential nutrients, physicochemical conditions (e.g., pH and redox potential), and the flow conditions found in the oral cavity. In the this study, the effects of electrochemical treatment on bacteria organized on the implant surface were evaluated, but these effects on the abutment chamber were not examined. Future studies should evaluate the impact of electrochemical treatments within that space. It is also important to recognize the limitations of the technique used for quantification of live bacteria, as it is determined by comparing the Ct (cycle threshold) values of the target gene template with a standard curve, what represents an estimate of the number of target bacteria present in the sample (Smith & Osborn, 2009). Furthermore, the electrical stimulation has been limited to direct current (DC), so the potential bactericidal activity of alternating current (AC) signals is still open.

In conclusion, and within the limitations acknowledged, the present investigation has evaluated the antibacterial effect of an innovative electrochemical treatment on a subgingival oral biofilm formed on titanium dental implants demonstrating its ability to affect the structure and vitality of a 72 h mature biofilm, using short time periods and without significant modifications of pH and temperature. This antibacterial activity was more significant after reduction, compared with oxidation treatments. The proposed electrochemical treatment demonstrated bactericidal effects, especially on *F. nucleatum*, a key bacterium for the formation and maintenance of subgingival dental biofilms. These results encourage further research on the potential use of electrical pulses to decontaminate affected implant surfaces within the treatment of peri-implantitis.

AUTHOR CONTRIBUTIONS

All authors conceived and planned the experiments. L.V., V.O., and E.G.Q carried out the biofilm experiments. L.V. and V.O. carried out the microbiological analysis. L.V. and V.O. carried out the microscopy analysis. L.V., V.O., and E.G.Q. performed the statistical analyses. L.V., V.O., E.G.Q. J.P, D.H. FT, and M.S. contributed to the interpretation of the results. L.V., V.O., E.G.Q., and M.S. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have stated explicitly that there are no conflicts of interest in connection with this work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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