Received: 10 May 2022 | Revised: 8 September 2022 | Accepted: 15 November 2022

DOI: 10.1111/jre.13076

Journal of PERIODONTAL RESEARCH WILEY

ORIGINAL ARTICLE

Weak direct current exerts synergistic effect with antibiotics and reduces the antibiotic resistance: An in vitro subgingival plaque biofilm model

Peihui Zou | Yanfeng Wang | Pei Cao | Peng Li | Jia Liu | Qingxian Luan

Department of Periodontology, Peking University School and Hospital of Stomatology & National Center of Stomatology & National Clinical Research Center for Oral Diseases & National Engineering Research Center of Oral Biomaterials and Digital Medical Devices, Peking University, Beijing, China

Correspondence
Qingxian Luan, Department of Periodontology, School and Hospital of Stomatology & National Center of Stomatology & National Clinical Research Center for Oral Diseases & National Engineering Research Center of Oral Biomaterials and Digital Medical Devices, Peking University, No. 22, Zhongguancun South Avenue, Haidian District, Beijing 100081, China.
Email: kqluanqx@126.com

Abstract

Background and Objective: Weak direct current (DC) exerts killing effect and synergistic killing effect with antibiotics in some specific bacteria biofilms. However, the potential of weak DC alone or combined with periodontal antibiotics in controlling periodontal pathogens and plaque biofilms remains unclear. The objective of this study was to investigate whether weak DC could exert the anti-biofilm effect or enhance the killing effect of metronidazole (MTZ) and/or amoxicillin–clavulanate potassium (AMC) on subgingival plaque biofilms, by constructing an in vitro subgingival plaque biofilm model.

Methods: The pooled subgingival plaque and saliva of patients with periodontitis (n = 10) were collected and cultured anaerobically on hydroxyapatite disks in vitro for 48 h to construct the subgingival plaque biofilm model. Then such models were stimulated with 0 μA DC alone (20 min/12 h), 1000 μA DC alone (20 min/12 h), 16 μg/ml MTZ, 16 μg/ml AMC or their combination, respectively. Through viable bacteria counting, metabolic activity assay, quantitative real-time PCR absolute quantification and 16S rDNA sequencing analysis, the anti-biofilm effect of 1000 μA DC and enhanced killing effects of 1000 μA DC combined with antibiotics (MTZ, AMC or MTZ+AMC) were explored.

Results: The old subgingival plaque model (48 h) had no significant difference in total bacterial loads from subgingival plaque in situ, which achieved a similarity of 80%. The 1000 μA DC plus MTZ or AMC for 12 h showed a stronger synergistic killing effect than the same combination for 20 min. The metabolic activity was reduced to the lowest by DC plus MTZ+AMC, as 37.4% of that in the control group, while average synergistic killing effect reached 1.06 log units and average total bacterial loads decreased to 0.87 log units. Furthermore, the relative abundance of the genera *Porphyromonas*, *Prevotella*, *Treponema*_2, and *Tannerella* were decreased significantly.

Conclusion: The presence of weak DC (1000 μA) improved the killing effect of antibiotics on subgingival plaque biofilms, which might provide a novel strategy to reduce their antibiotic resistance.

KEYWORDS
amoxicillin–clavulanate potassium, direct current, killing effect, metronidazole, subgingival plaque biofilm

1 | INTRODUCTION

Severe periodontitis is the 6th most common disease in humans with a global prevalence of 11.2%, resulting in multiple tooth loss and bringing a large healthcare burden worldwide.[1,2] As an inflammatory disease, periodontitis is caused by the multispecies subgingival plaque biofilms.[3] To eradicate the plaque biofilms, mechanical therapy is basic and necessary for the treatment of periodontitis.[4] For patients suffering from of severe chronic periodontitis or aggressive periodontitis,[4] using antibiotics as adjunctive therapy was confirmed to be effective.[5] Clinically, the most commonly used antibiotics are metronidazole, amoxicillin, amoxicillin–clavulanate potassium (AMC) or their combination.[5,6] Unfortunately, many factors limit the access, diffusion, and killing effect of antibiotics on subgingival plaque biofilms, such as the composition and organization of biofilms, the presence of antimicrobial destroying enzymes and quorum sensing and signaling systems, the existence of persistent bacteria and the exchange of genes, thus leading to biofilm resistance.[7–9] For complex biofilms, the eradication of bacteria requires higher antibiotic concentrations than that of planktonic bacteria.[6] However, such high doses are impractical clinically due to the risk of side effects and development of antibiotic-resistant bacteria.[10] To date, the emergence of resistant strains in plaque biofilms has been widely reported.[10–12] Therefore, it is necessary to develop a novel anti-biofilm method or approach that can reduce the antibiotic dose and improve antibiotic efficacy to effectively kill biofilms.[13]

At present, the main methods against plaque biofilms include physical control, chemical intervention, and biological control.[14] In addition to the mechanical and antibiotic therapy, using ultrasound to disrupt biofilms and specific photosensitizers to perform photodynamic therapy is helpful in killing bacteria in plaque biofilms.[15,16] However, their clinical long-term outcomes are conflicting in the treatment of periodontitis.[5,16] Previous studies have demonstrated that low-intensity electric fields acting on the biofilms continuously could improve the efficacy of antimicrobials, which is referred to as the bioelectric effect (BE).[17,18] Electric current and field, as a physicotherapeutic approach, provides a novel approach to overcome the antibiotic resistance of plaque biofilms effectively.[19,20] Earlier studies in our laboratory showed that 1000 μA DC not only suppressed the formation of *Porphyromonas gingivalis* biofilms but also enhance the killing efficacy of MTZ/AMC on them.[21] However, there are limited studies about whether the electric current and low-dose common periodontal antibiotics have synergistic benefits against the complex subgingival plaque biofilms.

Taken together, the aim of this study was to evaluate whether the weak direct current (1000 μA DC) can enhance the efficacy of MTZ and AMC (alone or in combination) against the model of subgingival plaque biofilm cultured in vitro and to explore the shifts of microbial flora after DC or/and antibiotic treatment via 16S ribosomal DNA (16S rDNA) sequencing analysis.[22]

2 | MATERIALS AND METHODS

2.1 | Subject recruitment

The present study was registered with the Chinese Clinical Trial Registry (ChiCTR; ChiCTR2100048436). The subject protocol was approved by the Peking University Biomedical Ethics Committee (Beijing, China). All patients enrolled at the Peking University School and Hospital of Stomatology agreed with written informed consent ($n = 10$).

2.2 | Diagnosis, inclusion criteria, exclusion criteria, and sample collection

The diagnostic criteria for periodontitis were in accordance with the 1999 Classification for Periodontal Diseases of International Workshop.[23] All included patients were diagnosed with severe chronic periodontitis or aggressive periodontitis. They did not receive any surgical/nonsurgical periodontal therapy during the previous 6 months ranging in age from 20 to 65 years. Patients were recruited if the examined periodontal pockets were no <6 mm depth in at least 3 non-adjacent sites. The exclusion criteria were as follows: (a) Patients suffering from any systemic diseases. (b) Pregnant and lactating women. (c) Patients who received antibiotic therapy in the past 3 months. (d) Patients in smoking status (ex-smoker or current smoker). The clinical characteristics of all included patients are shown in Table S1.

One week after completion of supragingival scaling, periodontal parameters of patients were recorded and included when eligible. Subsequently, all the subgingival plaque samples of every patient were collected and pooled from three different periodontal pockets (probing depth ≥6 mm) by using sterile Gracey curettes.[24] Saliva (3 to 5 ml) was collected from patients during resting states (no stimulation). The diagram of the entire experimental design is shown in Figure 1.

2.3 | Establishment of the subgingival plaque model

The pooled saliva samples were centrifuged at 3000*g* for 20 min, sterile filtered (Steriflip 0.22 μm, Millipore), and then diluted (1:10) with sterile PBS. Subsequently, the sterile ceramic calcium

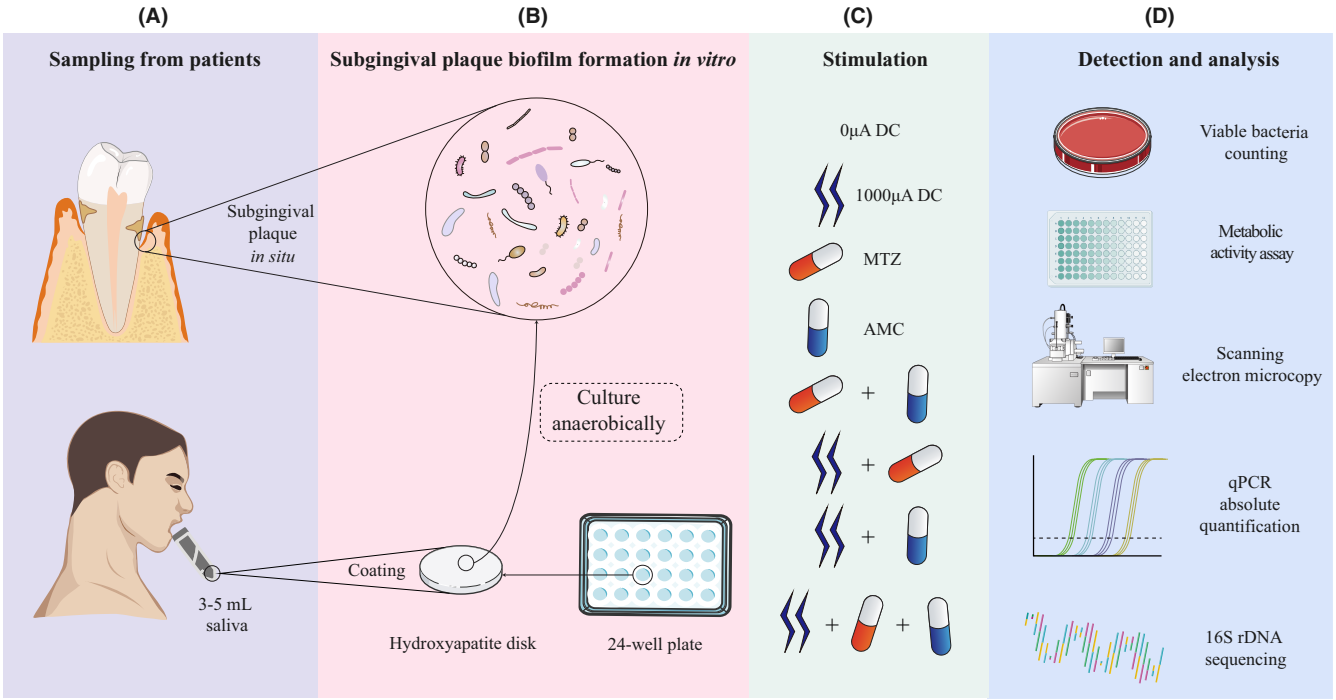

FIGURE 1 Schematic diagram of the whole experiment design. (A) Sampling of subgingival plaque and saliva from patients with severe chronic periodontitis or aggressive periodontitis. (B) Establishment of the subgingival plaque model in vitro. (C) Stimulation of the model with 0 μA DC, 1000 μA DC, 16 μg/ml MTZ, 16 μg/ml AMC separately or their combination. (D) Detection and analysis by viable bacteria counting, metabolic activity assay, quantitative real-time PCR absolute quantification, and 16S rDNA sequencing analysis.

hydroxyapatite (HA) discs (Clarkson Chromatography Products, Williamsport, PA), 10-mm diameter and 2-mm thickness, were coated with diluted saliva overnight at room temperature and placed in the wells of a 24-well tissue culture plate containing brain–heart infusion broth supplemented with 5 μg/ml hemin and 1 μg/ml vitamin K1 (BHIS).

Thirty subgingival plaque samples were collected in Eppendorf (EP) tubes containing 1 ml BHIS solution. Then, they were mixed and divided into three independent pooled plaque samples. Every sample was dispersed by vortexing (1 min) and sonication (1 min). And each well in 24-well plates containing HA disks was inoculated with 100 μl dispersed subgingival plaque and 900 μl BHIS solution. The discs were incubated in an anaerobic chamber (80% N_2, 20% CO_2) by using AnaeroPack (Mitsubishi Gas Chemical Company, Japan) at 37°C for 2 days. Subsequently, the subgingival plaque model was established (Figure 1B).[25,26]

2.4 | Verification and treatment of the subgingival plaque model

To verify the feasibility of this model compared with subgingival plaque in situ, absolute quantification in real-time quantitative reverse transcriptase polymerase chain reaction (qPCR), scanning electron microscopy (SEM) observation, and 16S RNA sequencing analysis was conducted.

After verification, the model was stimulated by 0 μA DC alone (control group), 16 μg/ml MTZ or 16 μg/ml AMC alone, 1000 μA DC alone or their combination for 20 min or 12 h. The DC stimulation apparatus used was identical to that used for our previous study, which is composed of one 5 voltage lithium battery, one constant DC output chip and two carbon electrodes.[29] A schematic diagram of the whole process steps is shown in Figure 1.

2.5 | Antibiotic resistance of subgingival plaque model in vitro

The model was stimulated with 128 μg/ml MTZ, 128 μg/ml AMC or their combination for 24 h anaerobically. The antibiotic resistance of subgingival plaque model was assessed by CFU assay and biofilm metabolic activity.

2.6 | Viable bacteria counting of subgingival plaque

CFU assay was performed to quantify the viable bacteria amount of subgingival plaque biofilm. The treated biofilms in HA disks were harvested by vortex (1 min) and sonication (100 W, 1 min). After 10-fold serial dilution in BHIS medium, the bacteria in biofilms were plated on blood agar for further counting by using Easyspiral Pro® (Interscience, France). The killing effect was calculated by the reduction of viable bacteria cultivated compared with the control group. The synergistic effect of DC was calculated by the subtraction of killing effect of DC alone and antibiotics alone from killing effect of DC combined with antibiotics.

2.7 | Biofilm metabolic activity

Biofilm metabolic activity was assessed through the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) reduction assay. The washed biofilms were incubated for 2 h in 100 μl of MTT solution (5 mg/ml in PBS) at 37°C. After incubation, 50 μl mixed solution was transferred to 96-well plates, and the unbound MTT was replaced with 150 μl DMSO for 15 min with shaking. The absorbance

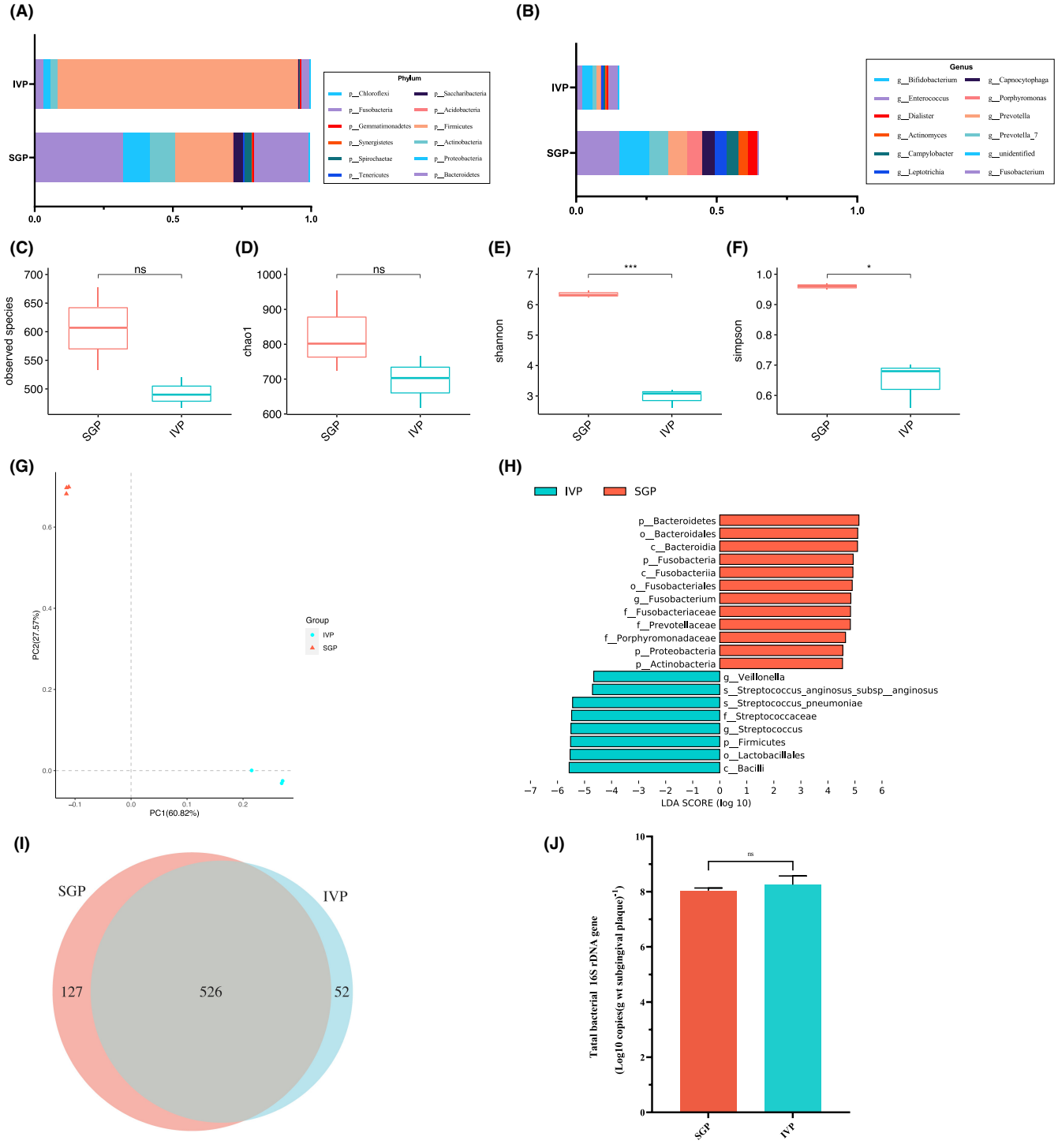

FIGURE 2 Comparisons of subgingival plaque in vitro (IVP) and in situ (SGP). The top 10 dominant phylum (A) and genus (B). The alpha-diversity comparisons included observed species (C), chao1 index (D), shannon index (E), and simpson index (F). The beta-diversity comparisons: the principal component analysis (G). Histogram of LDA scores (H). Venn diagram of species similarities (I). Quantitative real-time PCR absolute quantification of 16S rRNA gene copy number (J). *, **, ***, **** denote significant differences of $p < .05$, $p < .01$, $p < .001$, $p < .0001$, respectively. "ns" denote $p \geq .05$.

of 100 μl treated samples was measured using a microplate reader (Enspire, Germany) at 490 nm. In all assays, the control group with no intervention was set as the negative control with 100% metabolic activity.

2.8 | Absolute quantification of total bacterial loads of subgingival plaque

The total bacterial loads of subgingival biofilm samples were determined by qPCR using the absolute quantification method. To quantify the 16S copy number, a standard curve was generated from the purified PCR product of a segment between V3 and V4 of the 16S rDNA gene 338 F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806 R (5′-GGACTACHVGGGTWTCTAAT-3′) primers. The calibrator plasmid was used as the external standard for the quantification of genomic DNA samples by fluorometry. The reaction mixtures were performed in duplicates using 5 μl of SYBR No-ROX mastermix, 0.5 μM of forward and reverse primer, 25 ng of DNA and ultrapure water to complete a final volume of 20 μl. The cycling conditions were as follows: 95°C for 30 s, followed by 40 cycles for 5 s at 95°C and 40 s at 60°C.

2.9 | SEM observation of subgingival biofilms

The treated subgingival biofilms were washed twice in PBS and fixed in 2.5% glutaraldehyde for 30 min at 4°C. The postfixed samples were then dehydrated in a series of graded alcohols (50%, 60%, 70%, 80%, 90%, and 100%) and finally dried at temperature for 30 min. After gold coating, plaque biofilm samples were observed at magnifications of 5000× under SEM (Hitachi, Japan).

2.10 | Microbial DNA extraction, 16S rRNA gene library preparation, and pyrosequencing

DNA from plaque samples was extracted using the MoBio PowerSoil DNA Isolation Kit (100) (MoBio Laboratories, USA) according to the protocol. The extracted DNA was amplified by PCR using primers targeting the bacterial 16S V3-V4 rRNA gene. All products of the samples were then sequenced on the Illumina MiSeq PE250 platform at Beijing Allwegene Technology (Allwegene Technology Co., China).

After quality filtering and removal of primers, short sequences and chimeras, high-quality sequences were obtained. All the clean tags were sorted and clustered into operational taxonomic units (OTUs) using QIIME (Version 1.8.0) with an identity threshold of 97% by UPARSE29. Taxonomies assignments for OTUs were annotated by Human Oral Microbiome Database (HOMD, version 15.1). For diversity analysis, alpha diversity was estimated by the observed species, chao1 index, Shannon index, and Simpson index. To determine the dissimilarity between samples of every group, beta diversity analysis was conducted by principal component analysis (PCA) based on the distance matrix, and linear discriminant analysis (LDA) was conducted to identify key species in abundance differences.

2.11 | Absolute quantitative reverse transcriptase polymerase chain reaction (q-PCR)

The subgingival plaque samples in situ and plaque biofilms cultured in vitro were collected for q-PCR. DNA was isolated from treated plaque samples (750 μl) using a soil genomic DNA extraction kit (Tiangen Biotec, China) according to the manufacturer instructions. The 16S rDNA V3-V4 region was amplified and ligated using the 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) primers to design and obtain the plasmid. DNA was quantified using SYBR® Premix Ex Taq™ (ROX Plus) kit (Takara, Japan) and the 7500 Real-Time PCR System (Applied Biosystems, USA). 2 μl isolated DNA was added to 18 μl of master mix, resulting in a 20-μl reaction mixture. The plasmid standard was diluted 10-fold from 10^1 to 10^5 to establish the standard curve. The copy numbers of plaque biofilm samples were determined by standard curve of the plasmid standard. The PCR settings were as follows: 95°C for 30 s and 40 cycles of 95°C for 9 s with 60°C at 40 s. The cycle threshold (CT) was determined automatically.

2.12 | Statistical analysis

The data are presented as the mean ± SD, and the viable bacterial amount were converted to logarithms. For the viable bacterial amount, 6 replicates were made in every group. For other assays, 3 replicates were made in every group. Comparisons between two or multiple groups were made with Student's *t*-test or one-way ANOVA, in which post hoc analysis was performed with the Holm–Sidak test. Data were analyzed by GraphPad Prism 9.0 software (GraphPad Software Inc., USA). The raw sequencing data were analyzed using (principally) the pipeline tools MOTHUR v.1.33, and QIIME v1.8.0. LDA was performed with an alpha value <0.05 and score >3.5. Student's *t*-test was used to compare alpha and beta diversities. *p*-values $<.05$ were considered to indicate statistical significance.

3 | RESULTS

3.1 | Comparisons of the subgingival plaque model in vitro and subgingival plaque in situ

In the groups of subgingival plaque in vitro (IVP) and in situ (SGP), the dominant phyla and genera were different. In the SGP group, the most abundant phyla were *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Proteobacteria*, and *Actinobacteria*, the most abundant genera were Fusobacterium, unidentified, *Prevotella_7*, *Prevotella*, and *Porphyromonas* (Figure 2A,B). In the IVP group, the

most abundant phyla were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Actinobacteria*, and the most abundant genera were *Streptococcus*, *Veillonella*, *unidentified*, *Enterococcus*, and *Fusobacterium* (Figure 2A,B).

Regarding the alpha diversity, there were no significant differences in the observed species and chao1 index between the two groups. However, the Shannon index, and Simpson index of IVP group were significantly lower than those of SGP group (Figure 2C–F). For the beta diversity, PCA showed that the contribution rate of PC1 and PC2 reached 60.82% and 27.57%, respectively (Figure 2G). The LDA showed the relative abundances of the top 20 differentially abundant species (based on the LDA score) in the two groups, of which *Streptococcaceae*, *Streptococcus*, *Firmicutes* and *Lactobacillales* and *Bacilli* were the most enriched (Figure 2H). The Venn diagram showed that the species similarity of two groups reached 80.5% (Figure 2I).

For the total bacterial loads, the absolute quantification showed that there were no significant differences in the 16S rRNA gene copy number between two groups (Figure 2J).

3.2 | Antibiotic resistance of the subgingival plaque model in vitro

Viable bacteria counting showed that 128 μg/ml MTZ, 128 μg/ml AMC and their combination exerted significant killing effect of 1.47, 2.11, and 4.29 $\log_{10}$ CFU/ml on subgingival plaque biofilms in vitro, respectively (Figure 3A). In addition, 128 μg/ml AMC showed a stronger killing effect than that of 128 μg/ml MTZ.

The MTT assay showed that 128 μg/ml MTZ, 128 μg/ml AMC and their combination could significantly decrease the metabolic activity of plaque biofilms (Figure 3B), which reached 38.09%, 29.74%, and 15.64% of the control group, respectively. In addition, 128 μg/ml AMC showed a stronger killing effect than that of 128 μg/ml MTZ.

3.3 | The synergistic effect of 1000 μA DC and MTZ alone, AMC alone, or MTZ combined with AMC in killing subgingival plaque in vitro

Viable bacteria counting showed that 20 min DC had no antimicrobial effect; conversely, 12 h DC, MTZ, AMC and MTZ combined with AMC showed killing effects of 0.77, 0.13, 0.24, and 0.94 $\log_{10}$ CFU/ml on subgingival plaque biofilms in vitro, respectively (Figure 4D). Besides, DC treatment for 20 min and 12 h had synergistic effect with MTZ/AMC/MTZ combined AMC. The synergistic effect of 20 min DC treatment with MTZ/AMC/MTZ combined AMC reached 0.20, 0.24 and 0.32 $\log_{10}$ CFU/ml, respectively (Figure 4A,D). The synergistic effect of 12 h DC treatment with MTZ/AMC/MTZ combined with AMC were stronger regardless of antibiotics, reaching 0.73, 0.78, and 1.06 $\log_{10}$ CFU/ml, respectively (Figure 4G).

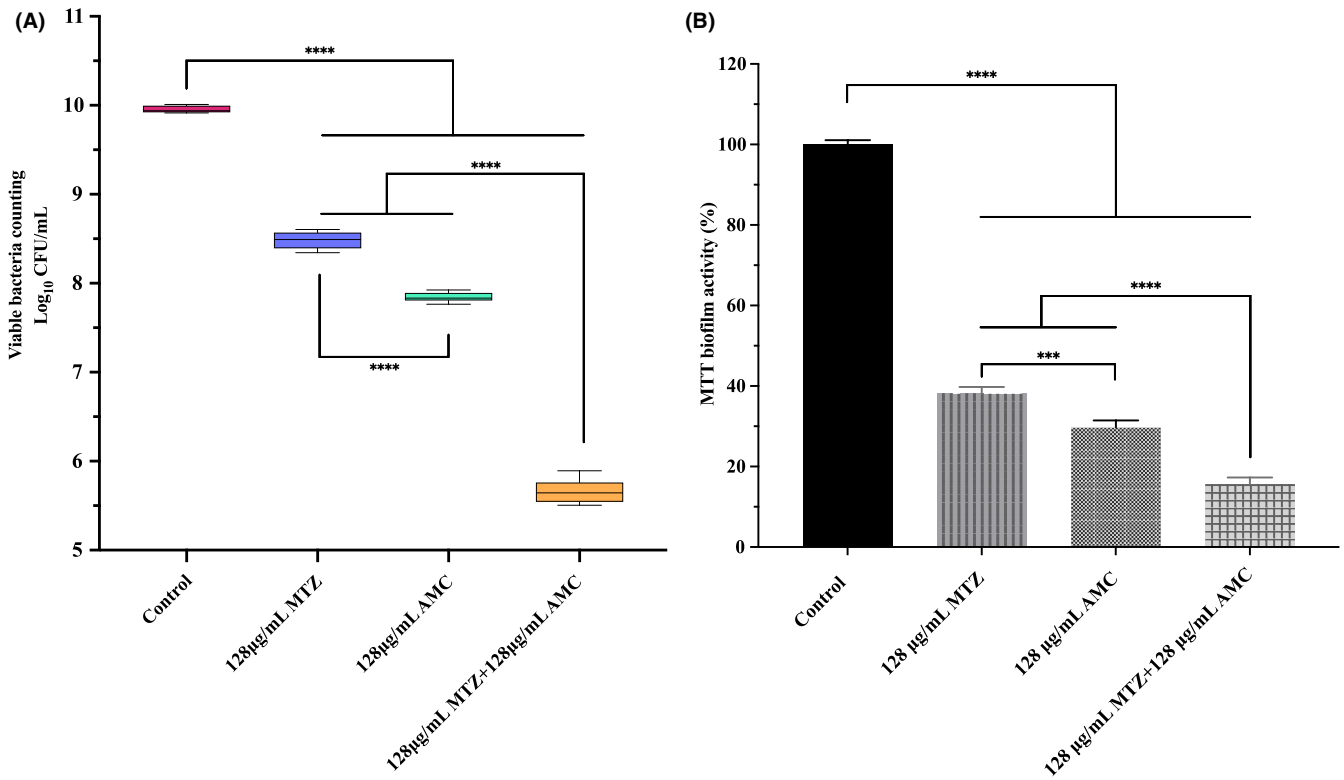

FIGURE 3 Antibiotic resistance of subgingival plaque model in vitro. The subgingival plaque biofilms (48 h) were stimulated anaerobically by 128 μg/ml MTZ, 128 μg/ml AMC or their combination for 24 h. (A) Viable bacteria counting showed non-complete killing effect of MTZ and AMC ($n = 6$). (B) The MTT assay showed MTZ and AMC caused an incomplete reduction in biofilm metabolic activity ($n = 3$). ***, **** denote significant differences of $p < .001$ and $p < .0001$, respectively.

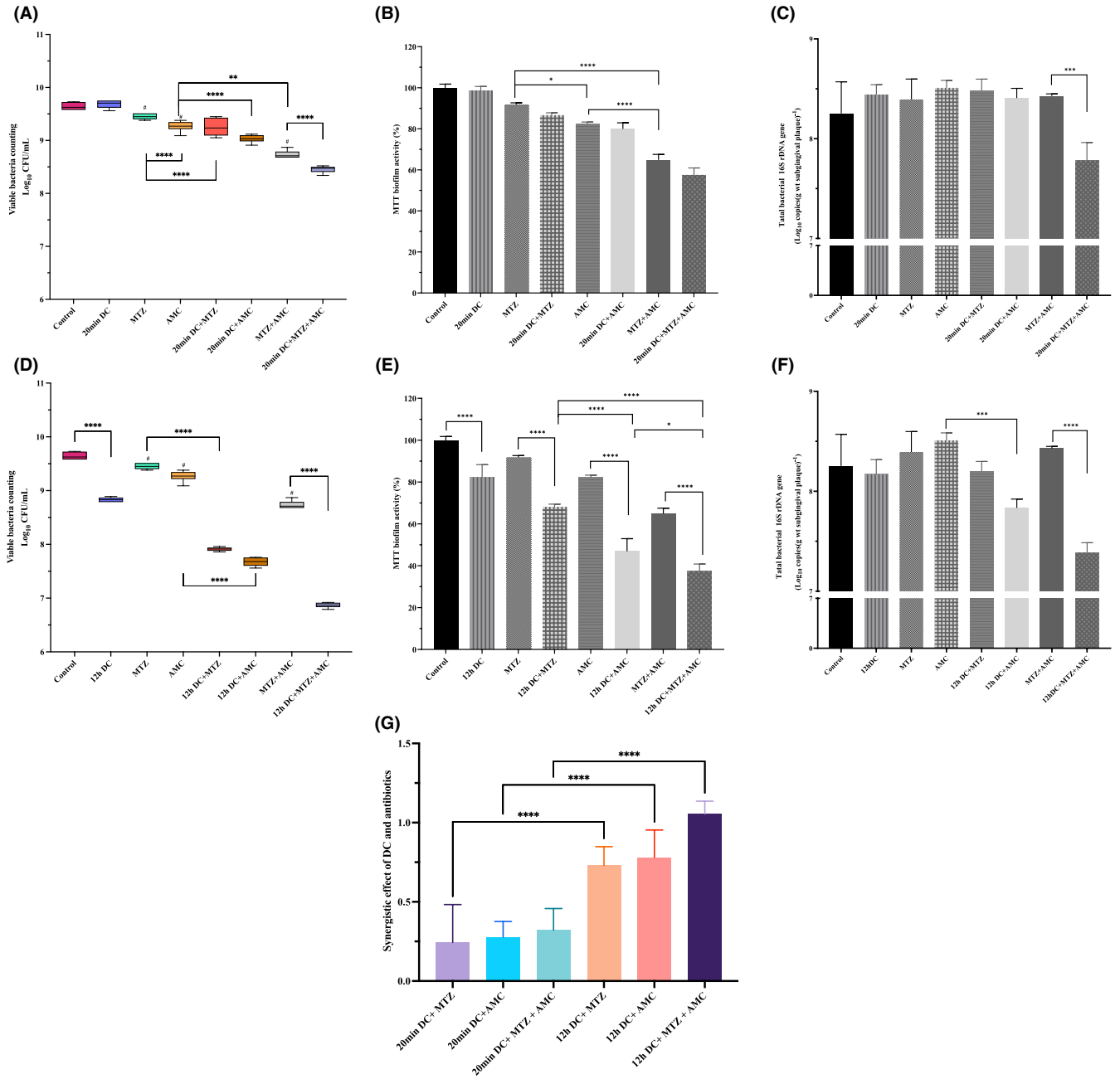

FIGURE 4 Killing effect and synergistic effect with two antibiotics of short-term DC and long-term DC on the subgingival plaque models. The models were treated with 0 μA DC, 1000 μA DC (20 min or 12 h), 16 μg/ml MTZ, 16 μg/ml AMC, or their combination, and subjected to analyses of viable bacteria counting (1000 μA DC for 20 min in A and 12 h in D, $n = 6$), metabolic activity assay (1000 μA DC for 20 min in B and 12 h in E, $n = 3$), quantitative real-time PCR absolute quantification (1000 μA DC for 20 min in C and 12 h in F, $n = 3$), and the synergistic effect of DC combined with two antibiotics (G). *, **, ***, **** denote significant difference of $p < .05$, $p < .01$, $p < .001$, and $p < .0001$, respectively. # denote significant difference of p-value <.001 compared with the control group.

The MTT assay showed 12 h DC, MTZ, AMC, and MTZ combined with AMC could significantly decrease the metabolic activity of plaque biofilms (Figure 4B,F). In contrast, the metabolic activity of AMC group was the lowest and MTZ group was the highest. 20 min DC had no impact on the metabolic activity (Figure 4B). Moreover, in the group of 12 h DC treatment with MTZ combined AMC, the metabolic activity exhibited the lowest metabolic activity that reached 37.39% of the control group (Figure 4E).

The total bacterial loads assay showed DC treatment for 20 min or 12 h, MTZ/AMC/MTZ combined with AMC had no impact on 16S rRNA gene copy number compared to the control group (Figure 4C,F). In contrast, when MTZ combined with AMC and DC treatment for 20 min or 12 h were administered in combination, the genes copy number strikingly increased (Figure 4C,F). Besides, 12 h DC had synergistic effect with AMC alone on reducing the bacterial loads (Figure 4F).

As shown in Figure 5, compared with the subgingival plaque model, the biofilm density of control group and all other treatment groups were increased. Besides, the group of DC treatment for 12 h with MTZ combined with AMC showed less biofilm density than that of the MTZ combined with AMC group.

3.4 | Comparisons of dominant species, dominant genera, and relative abundance of common periodontal pathogens after treatment with 1000 μA DC, MTZ, and AMC

The alpha-diversity analysis showed the observed species, chao1 index, Shannon index, and Simpson index of all treatment groups were lower than the control group (Figure 6). After treatment, the most visible changes of phylumn in relative abundance were *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria* (Figure 7A), and the most visible changes in genera in relative abundance were *Streptococcus*, *Veillonella*m, *Lactobacillus*, *Enterococcus*, *and Klebsiella* (Figure 7B). Notably, compared to the control group, the relative abundance of *Porphyromonas*, *Treponema_2*, *Tannerella*, and *Prevotella* in DC combined MTZ and AMC group were obviously lower (Figure 7C).

4 | DISCUSSION

The present study firstly showed that DC (1000 μA) had antimicrobial effect and synergistic effect with two antibiotics (alone or in combination) on subgingival plaque biofilms in vitro. In this study, we found the 1000 μA DC for 12 h showed <1 log antibiofilm effect and approximately 0.74–1.44 log synergistic effect with MTZ/AMC/

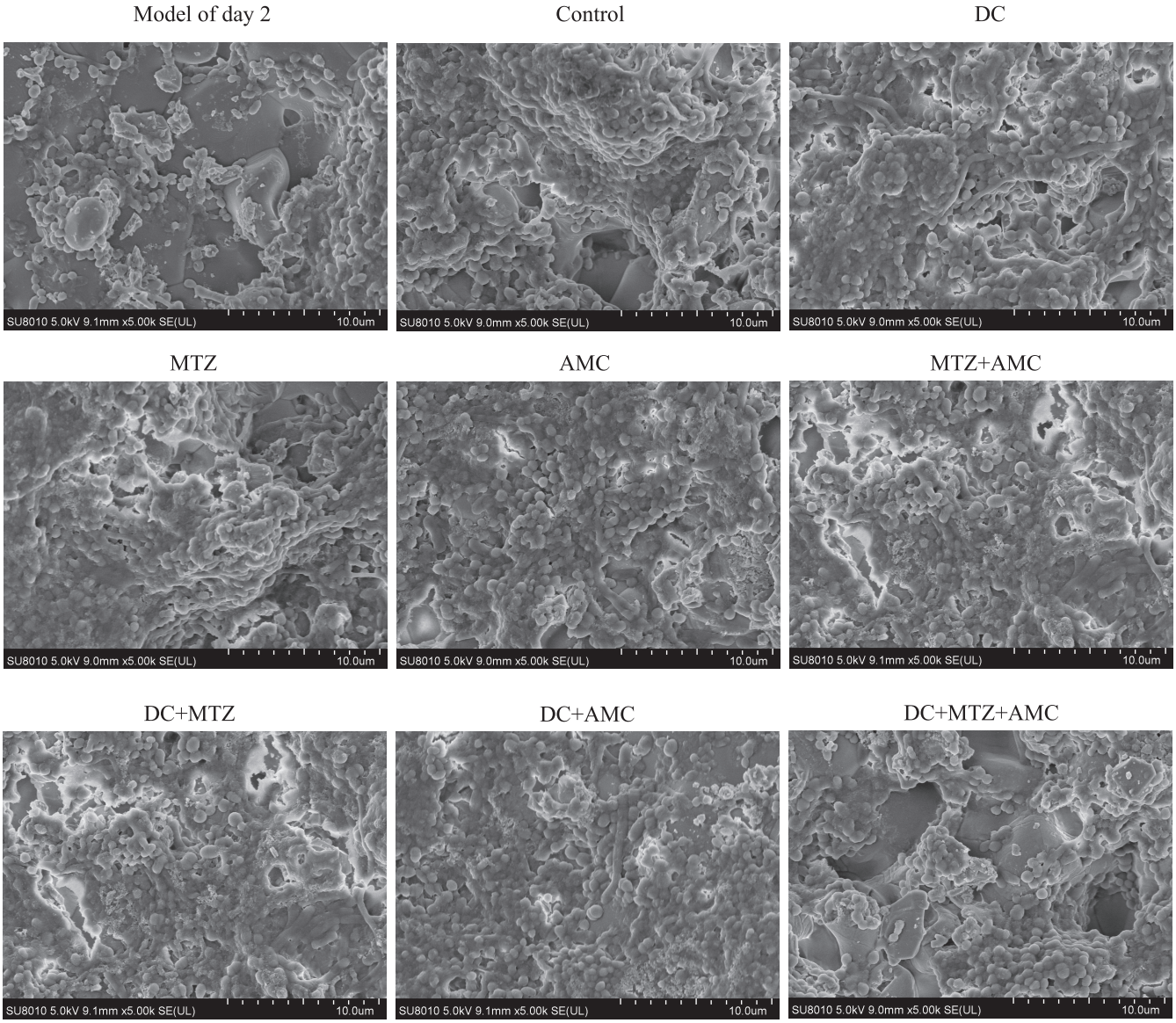

FIGURE 5 Biofilm density of the subgingival plaque model and plaque biofilms after treatment of 0 μA DC, 1000 μA DC, MTZ, AMC, or their combination for 12 h by scanning electron microscopy under 5000× magnification ($n = 3$).

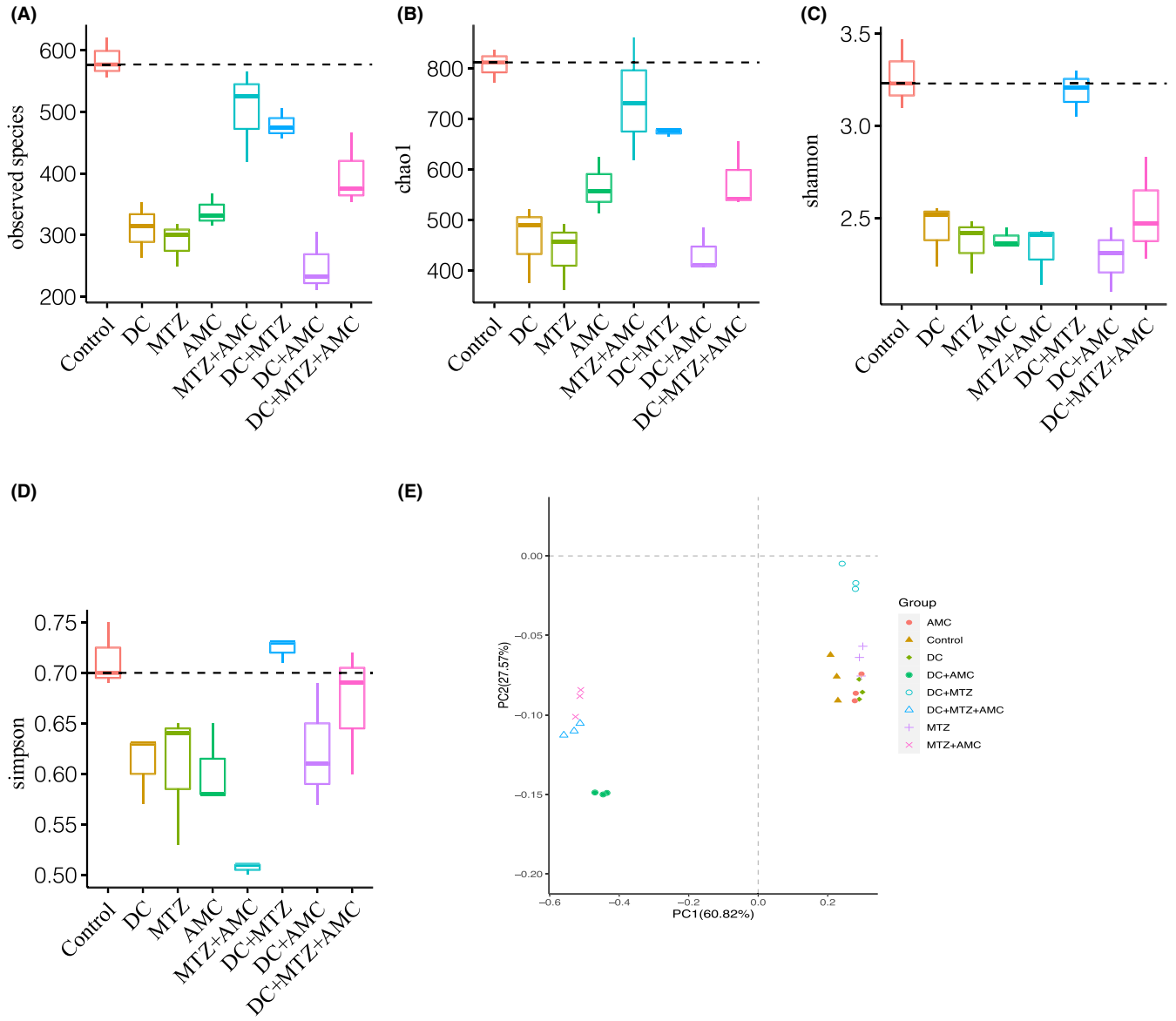

FIGURE 6 Alpha-diversity and beta-diversity comparisons of groups treated by 0 μA DC, 1000 μA DC (12 h), 16 μg/ml MTZ, 16 μg/ml AMC, or their combination, which included observed species (A), chao1 index (B), Shannon index (C) and Simpson index (D), and the principal component analysis (E).

MTZ+AMC, which suggested that weak DC might be a novel method of effective management of subgingival plaque biofilms.

As for the subgingival plaque biofilm model, we found that the Shannon index and Simpson index of this model were significantly lower than those obtained from the plaque in situ, which demonstrated that the bacterial richness and diversity in the model decreased when cultured in vitro. These results were consistent with previous studies.[8,25] Notably, the *Streptococcus* and *Veillonella* were the most abundant genus in the plaque model, and the relative abundance of the red and orange complex was strikingly lower than that in the plaque in situ. Due to the limitation of culture conditions and the antagonistic effect between microorganisms, a considerable part of oral microorganisms based on HOMD cannot be cultured, whose proportion reaches 20% to 60%.[30] Considering that the model was established on the surface of the HA disk, the dominant bacteria are mainly initially colonized *Streptococcus* and gradually increased *Actinomycetes*, *Bacteroides*, and *Campylobacter* later,[31] and the colonized time of periodontal pathogens were relatively late.[8] The culture time of model in our study was limited to 48 to 60 h, which may result in simpler structure, lower maturity, and lower abundance of red and orange complexes in the model. Nonetheless, the plaque biofilms in vitro showed resistance to high concentrations (128 μg/ml) of antibiotics such as MTZ and AMC, and there were high similarities in observed species and the same bacterial loads between the model and plaque in situ, which supported the feasibility of this model for further studies.

Based on this model, we evaluated the killing effect of weak DC alone, antibiotics or in combination on plaque biofilms. It has been assumed that the pulsed electromagnetic field had antimicrobial effects on polymicrobial subgingival biofilms and weak electric field could enhance doxycycline or doxycycline and in

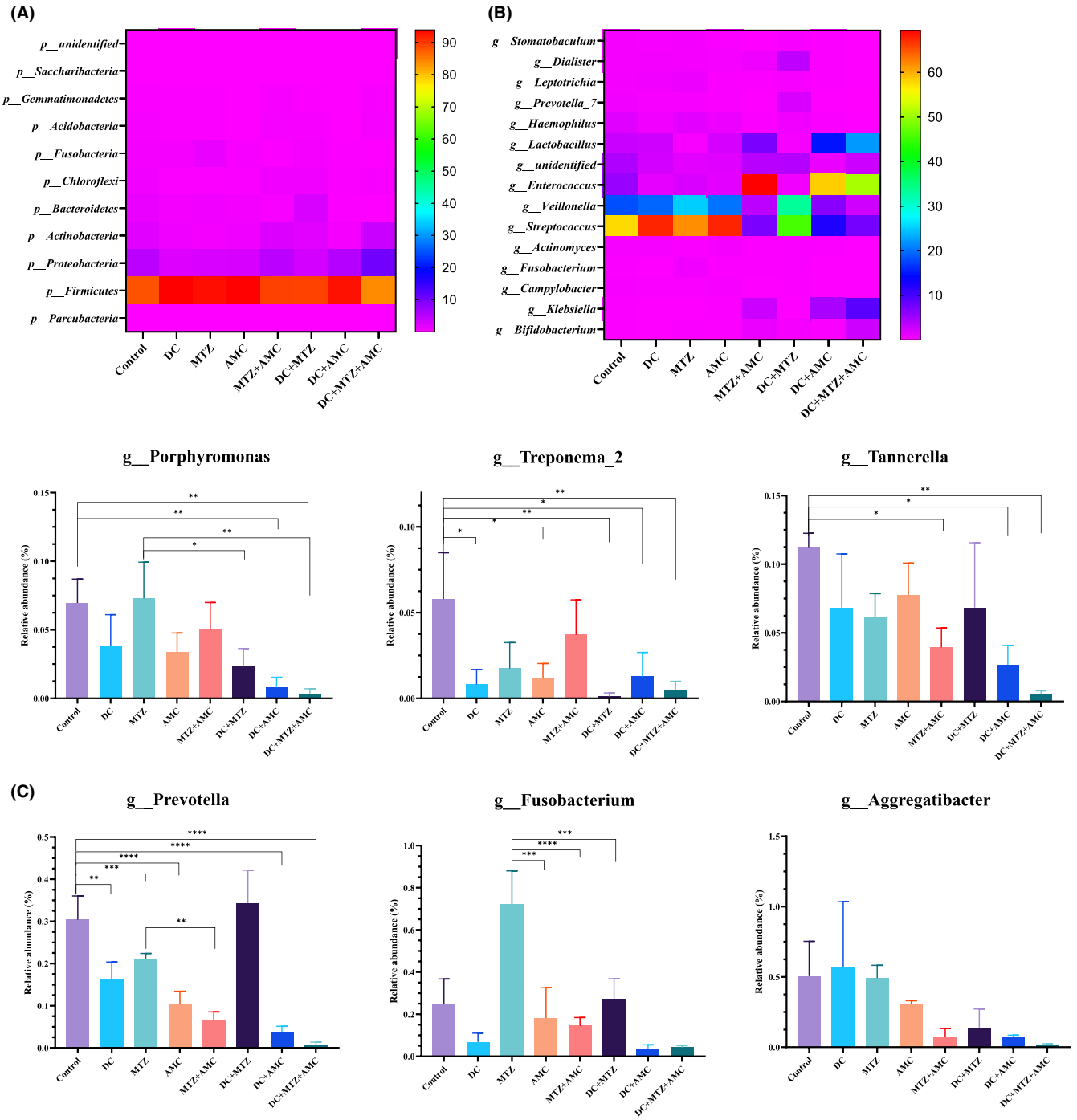

FIGURE 7 Top 10 dominant phyla (A) and genera (B) and the relative abundance of six common periodontal pathogens genus (C) of groups treated with 0 μA DC, 1000 μA DC (12 h), 16 μg/ml MTZ, 16 μg/ml AMC, or their combination. *, **, ***, **** denote significant differences of $p < .05$, $p < .01$, $p < .001$, and $p < .0001$, respectively.

inhibiting polymicrobial biofilm.[32–34] Similarly, we found that weak DC (1000 μA) exerted antimicrobial effect by itself and synergistic effect with MTZ/AMC on killing the cultured subgingival plaque in vitro. To further confirm the specific impact of DC on the model, we examined the microflora composition and abundance by 16S rRNA sequencing. We found that the diversities of biofilms were simplified after whether DC treatment or antibiotic treatment, *Firmicutes* and *Streptococcus* were the most influenced taxa, and the relative abundance of *Porphyromonas*, *Treponema*_2, *Tannerella*, and *Prevotella* in DC combined MTZ and AMC group were significantly decreased. This is an analysis that has been lacking in most of other studies related to electric current or antibiotics.[35,36] Given these findings, hereby, we revealed weak DC as a potential means to reduce antibiotic resistance of biofilm and control plaque biofilms.

Interestingly, the killing effect of 16 μg/ml MTZ alone, 16 μg/ml AMC alone and their combination were not exceeded 1 log. In

addition, AMC showed a stronger killing effect than MTZ, and two antibiotics exhibited weak synergistic effect. These results were consistent with previous studies,[6] which provided a strong proof that antibiotic administration alone is not sufficient enough to replace mechanical therapy to completely remove plaque biofilms.[5] Notably, long-term (12 h) DC treatment showed stronger synergistic effect with MTZ/AMC/MTZ combined with AMC than those of short-term (20 min) DC treatment, which partly demonstrated the strength of BE depended on the stimulation time of DC. These findings were in accordance with previous studies,[20] which pointed out the longer stimulation time of DC was, the stronger the enhanced killing efficacy by gentamicin was. In addition, under the condition of the same current density and stimulation time, DC combined with AMC showed stronger viable bacteria killing and more bacterial loads decrease than DC combined with MTZ. This may be attributed to the narrow spectrum and limited antimicrobial efficacy of MTZ when used alone in multi-species biofilms.[37,38]

The specific mechanism of BE has not been elucidated up to now. The synergistic killing effect may contribute to the reduction in biofilm capacity for binding antibiotics, increased antibiotics transport and permeabilization, and enhanced susceptibility to antibiotics and electrochemical generation of reactive oxygen species after stimulation of electric current.[17,18,35,39] Most likely, multiple factors participate in this synergistic effect, which makes it difficult to study and reconcile. For the subgingival plaque biofilms, polysaccharide–protein complexes limit the penetration of antibiotics, low metabolic rates reduce the susceptibility of bacteria to antibiotics and complex symbiotic and antagonistic relationships provide protection to internal bacteria.[6] Although our study did not aim to explore the exact mechanism responsible for the synergistic killing effect, we confirmed the time-dependent character between DC and MTZ/AMC, further studies may need focus on the most suitable DC intensity and stimulation time with antibiotics to achieve the ideal killing effect.

There are several limitations in this model and methodology. Firstly, as for the viable bacteria counting and metabolic activity assay, the anaerobic condition and specific liquid medium in vitro limited the survival and cultivation of some oral microorganisms, which may lead to inherent systemic bias. Secondly, limited sample size and single in vitro experiment may weaken the reliability of such conclusions, future studies with larger number of samples, animal study and relevant human study are needed to validate our findings and the application of DC in human body. Finally, the recommended clinical intensity restriction, voltage restriction, and time restriction of DC is 1000 μA, 5 V and 20 min, respectively. DC intensity and voltage in our study were safe in humans,[40] however, the stimulation for 12 h is not practical, a lack of the suitable DC output device and safe oral stimulation patterns with the best stimulation time may limit the application of DC in the adjunctive treatment of periodontitis.

Overall, this study provides the first evidence of the synergistic killing effect and microbiota composition profiles of DC and MTZ/AMC/MTZ combined with AMC in the subgingival plaque model cultured in vitro. The presence of 1000 μA DC could not only exert antimicrobial effect but also enhance MTZ/AMC/MTZ combined with AMC to kill internal bacteria, reduce total bacterial loads and the relative abundance of common periodontal pathogens in the subgingival plaque model cultured in vitro. However, due to the complex biochemical effect and application limitation, further mechanistic and translational medical studies are required to address the underlying mechanisms and safe range of stimulation time and intensity when applied in the oral environment. As such, our results reveal a means to reduce drug resistance of plaque biofilms and a possibility of DC to be applied in enhancing antibiotic efficacy and controlling plaque biofilms.

AUTHOR CONTRIBUTIONS

Peihui Zou and Qingxian Luan conceived and designed the experiments. Peihui Zou, Pei Cao, and Yanfeng Wang performed the experiments. Peihui Zou and Yanfeng Wang analyzed the data. Peihui Zou wrote the manuscript with contributions from the other authors. Peihui Zou, Peng Li, Jia Liu and Qingxian Luan revised the manuscript.

ACKNOWLEDGEMENTS

The authors are grateful to Lilian Li (KuShi Medical Technology Co., Ltd., Shanghai, China) for technical assistance.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data supporting the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Peihui Zou https://orcid.org/0000-0003-4055-1694
Yanfeng Wang https://orcid.org/0000-0002-9655-7924
Jia Liu https://orcid.org/0000-0001-5336-8712

REFERENCES

1. Kassebaum NJ, Bernabe E, Dahiya M, Bhandari B, Murray CJL, Marcenes W. Global burden of severe periodontitis in 1990–2010: a systematic review and meta-regression. *J Dent Res*. 2014;93(11):1045-1053.
2. Jiao J, Jing W, Si Y, et al. The prevalence and severity of periodontal disease in mainland China: data from the fourth National Oral Health Survey (2015–2016). *J Clin Periodontol*. 2021;48(2):168-179.
3. Curtis MA, Diaz PI, Van Dyke TE. The role of the microbiota in periodontal disease. *Periodontol 2000*. 2020;83(1):14-25.
4. Keestra JAJ, Grosjean I, Coucke W, Quirynen M, Teughels W. Non-surgical periodontal therapy with systemic antibiotics in patients with untreated chronic periodontitis: a systematic review and meta-analysis. *J Periodontal Res*. 2015;50(3):294-314.
5. Abdallaoui-Maan L, Bouziane A. Effects of timing of adjunctive systemic antibiotics on the clinical outcome of periodontal therapy: a systematic review. *J Clin Exp Dent*. 2020;12(3):e300-e309.
6. Belibasakis GN, Thurnheer T. Validation of antibiotic efficacy on In vitro subgingival biofilms. *J Periodontol*. 2014;85(2):343-348.

7. Smith AW. Biofilms and antibiotic therapy: is there a role for combating bacterial resistance by the use of novel drug delivery systems? *Adv Drug Deliv Rev*. 2005;57(10):1539-1550.
8. Hope CK, Wilson M. Biofilm structure and cell vitality in a laboratory model of subgingival plaque. *J Microbiol Methods*. 2006;66(3):390-398.
9. Mah TFC, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol*. 2001;9(1):34-39.
10. Veloo ACM, Seme K, Raangs E, et al. Antibiotic susceptibility profiles of oral pathogens. *Int J Antimicrob Agents*. 2012;40(5):450-454.
11. Mínguez M, Ennibi OK, Perdiguero P, et al. Antimicrobial susceptibilities of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* strains from periodontitis patients in Morocco. *Clin Oral Investig*. 2019;23(3):1161-1170.
12. Ardila CM, Granada MI, Guzman IC. Antibiotic resistance of subgingival species in chronic periodontitis patients. *J Periodontal Res*. 2010;45(4):557-563.
13. Del Pozo JL. Biofilm-related disease. *Expert Rev Anti Infect Ther*. 2018;16(1):51-65.
14. Slots J. Low-cost periodontal therapy. *Periodontol 2000*. 2012;60:110-137.
15. Chambrone L, Wang HL, Romanos GE. Antimicrobial photodynamic therapy for the treatment of periodontitis and peri-implantitis: an American Academy of Periodontology best evidence review. *J Periodontol*. 2018;89(7):783-803.
16. Krishna R, De Stefano JA. Ultrasonic vs. hand instrumentation in periodontal therapy: clinical outcomes. *Periodontol 2000*. 2016;71(1):113-127.
17. Blenkinsopp SA, Khoury AE, Costerton JW. Electrical enhancement of biocide efficacy against *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol*. 1992;58(11):3770-3773.
18. Costerton JW, Ellis B, Lam K, Johnson F, Khoury AE. Mechanism of electrical enhancement of efficacy of antibiotics in killing biofilm bacteria. *Antimicrob Agents Chemother*. 1994;38(12):2803-2809.
19. Freebairn D, Linton D, Harkin-Jones E, Jones DS, Gilmore BF, Gorman SP. Electrical methods of controlling bacterial adhesion and biofilm on device surfaces. *Expert Rev Med Devices*. 2013;10(1):85-103.
20. Zhang J, Neoh KG, Hu X, Kang E-T. Mechanistic insights into response of *Staphylococcus aureus* to bioelectric effect on polypyrrole/chitosan film. *Biomaterials*. 2014;35(27):7690-7698.
21. Zou P, Li P, Liu J, Cao P, Luan Q. Direct current exerts electricidal and bioelectric effects on *Porphyromonas gingivalis* biofilms partially via promoting oxidative stress and antibiotic transport. *J Microbiol*. 2022;60(1):70-78.
22. Liu G, Luan Q, Chen F, Chen Z, Zhang Q, Yu X. Shift in the subgingival microbiome following scaling and root planing in generalized aggressive periodontitis. *J Clin Periodontol*. 2018;45(4):440-452.
23. Armitage GC. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol*. 1999;4(1):1-6.
24. Jervøe-Storm P-M, AlAhdab H, Koltzscher M, Fimmers R, Jepsen S. Comparison of curet and paper point sampling of subgingival bacteria as analyzed by real-time polymerase chain reaction. *J Periodontol*. 2007;78(5):909-917.
25. Walker C, Sedlacek MJ. An in vitro biofilm model of subgingival plaque. *Oral Microbiol Immunol*. 2007;22(3):152-161.
26. Sedlacek MJ, Walker C. Antibiotic resistance in an in vitro subgingival biofilm model. *Oral Microbiol Immunol*. 2007;22(5):333-339.
27. Tenenbaum H, Jehl F, Gallion C, Dahan M. Amoxicillin and clavulanic acid concentrations in gingival crevicular fluid. *J Clin Periodontol*. 1997;24(11):804-807.
28. Pahkla ER, Koppel T, Saag M, Pahkla R. Metronidazole concentrations in plasma, saliva and periodontal pockets in patients with periodontitis. *J Clin Periodontol*. 2005;32(2):163-166.
29. Zou P, Cao P, Liu J, Li P, Luan Q. Comparisons of the killing effect of direct current partially mediated by reactive oxygen species on *Porphyromonas gingivalis* and *Prevotella intermedia* in planktonic state and biofilm state - an in vitro study. *J Dent Sci*. 2022;17(1):459-467.
30. Deng ZL, Szafrański SP, Jarek M, Bhuju S, Wagner-Döbler I. Dysbiosis in chronic periodontitis: key microbial players and interactions with the human host. *Sci Rep*. 2017;7(1):3703.
31. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol*. 1998;25(2):134-144.
32. Hari P, Kacharaju KR, Anumala N, Pathakota KR, Avula J. Application of bioelectric effect to reduce the antibiotic resistance of subgingival plaque biofilm: an in vitro study. *J Indian Soc Periodontol*. 2018;22(2):133-139.
33. Lasserre JF, Leprince JG, Toma S, Brecx MC. Electrical enhancement of chlorhexidine efficacy against the periodontal pathogen *Porphyromonas gingivalis* within a biofilm. *New Microbiol*. 2015;38(4):511-519.
34. del Pozo JL, Rouse MS, Mandrekar JN, Steckelberg JM, Patel R. The electricidal effect: reduction of staphylococcus and pseudomonas biofilms by prolonged exposure to low-intensity electrical current. *Antimicrob Agents Chemother*. 2009;53(1):41-45.
35. Sultana ST, Babauta JT, Beyenal H. Electrochemical biofilm control: a review. *Biofouling*. 2015;31(9–10):745-758.
36. Del Pozo JL, Rouse MS, Patel R. Bioelectric effect and bacterial biofilms. A systematic review. *Int J Artif Organs*. 2008;31(9):786-795.
37. Soares GMS, Mendes JAV, Silva MP, et al. Metronidazole alone or with amoxicillin as adjuncts to non-surgical treatment of chronic periodontitis: a secondary analysis of microbiological results from a randomized clinical trial. *J Clin Periodontol*. 2014;41(4):366-376.
38. Li P, Fung YE, Yin X, Seneviratne CJ, Che CM, Jin L. Controlled cellular redox, repressive hemin utilization and adaptive stress responses are crucial to metronidazole tolerance of *Porphyromonas gingivalis* persisters. *J Clin Periodontol*. 2018;45(10):1211-1221.
39. Sandvik EL, McLeod BR, Parker AE, Stewart PS. Direct electric current treatment under physiologic saline conditions kills *Staphylococcus epidermidis* biofilms via electrolytic generation of hypochlorous acid. *PLoS ONE*. 2013;8(2):e55118.
40. Prasad D, Sharma AK, Sharma HC. Electric shock and human body. *Int J Electr Power Eng*. 2010;4(3):177-181.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Zou P, Wang Y, Cao P, Li P, Liu J, Luan Q. Weak direct current exerts synergistic effect with antibiotics and reduces the antibiotic resistance: An in vitro subgingival plaque biofilm model. *J Periodont Res*. 2023;58:143-154. doi:10.1111/jre.13076