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Antimicrobial photodynamic therapy alone or in combination with antibiotic local administration against biofilms of *Fusobacterium nucleatum* and *Porphyromonas gingivalis*

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ABSTRACT

Antimicrobial photodynamic therapy (aPDT) kills several planktonic pathogens. However, the susceptibility of biofilm-derived anaerobic bacteria to aPDT is poorly characterized. Here, we evaluated the effect of Photodithazine (PDZ)-mediated aPDT on *Fusobacterium nucleatum* and *Porphyromonas gingivalis* biofilms. In addition, aPDT was tested with metronidazole (MTZ) to explore the potential antimicrobial effect of the treatment. The minimum inhibitory concentration (MIC) of MTZ was defined for each bacterial species. Single-species biofilms of each species were grown on polystyrene plates under anaerobic conditions for five days. aPDT was performed by applying PDZ at concentrations of 50, 75 and 100 mg/L, followed by exposure to 50 J/cm^2 LED light (660 nm) with or without MTZ. aPDT exhibited a significant reduction in bacterial viability at a PDZ concentration of 100 mg/L, with 1.12 $\log_{10}$ and 2.66 $\log_{10}$ reductions for *F. nucleatum* and *P. gingivalis* in biofilms, respectively. However, the antimicrobial effect against *F. nucleatum* was achieved only when aPDT was combined with MTZ at 100x MIC. Regarding *P. gingivalis*, the combination of PDZ-mediated aPDT at 100 mg/L with MTZ 100x MIC resulted in a 5 $\log_{10}$ reduction in the bacterial population. The potential antimicrobial effects of aPDT in combination with MTZ for both single pathogenic biofilms were confirmed by live/dead staining. These results suggest that localized antibiotic administration may be an adjuvant to aPDT to control *F. nucleatum* and *P. gingivalis* biofilms.

Keywords: photodynamic therapy, combined modality therapy, metronidazole, anaerobic bacteria

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1. Introduction

The ability of microbial cells to interact with abiotic and biotic surfaces [2-4] enables the formation of a complex microbial community surrounded by an extracellular matrix of polymeric substances [5, 6]. This organized structure, a biofilm, promotes an imbalance between microorganisms of the normal flora and opportunistic pathogens and is considered the precursor to the initiation of the inflammatory response by the host [7, 8]. Among bacteria involved in this process, Gram-negative species have virulence factors directly related to several aggressive oral infections, such as periodontal and peri-implant diseases [9, 10].

The microbiota in periodontal and peri-implant pockets include both *P. gingivalis* and *F. nucleatum* [11-14]. *P. gingivalis* is a significant pathogenic species directly related to the progression of both diseases [15] because of the numerous toxic enzymes produced by most of its strains [16] and its communication with host cells that triggers a strong inflammatory response [17, 18]. *F. nucleatum* is another critical organism strongly associated with periodontal and peri-implant diseases due to congregation with other species (including *P. gingivalis*) via its high number of multivalent adhesins onto cell surfaces [19-22]. In addition, this bacterium can generate a capnophilic environment that enables the growth of anaerobic pathogenic bacteria, including *P. gingivalis* [23-29]. Finally, the interaction between *F. nucleatum* and host cells enhances the invasion of *P. gingivalis* into host cells [30]. Considering bacterial aggressiveness and possible sequelae provoked by disease progression (tooth/implant loss), biofilm control on oral substrates is critical for controlling the inflammatory response.

From a clinical point of view, nonsurgical treatment directed toward mechanical removal of subgingival biofilms is mainly limited by the depth of periodontal/peri-implant pockets and surface irregularities [31]. In consideration of the limited effects of

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mechanical debridement [32], the further benefits of antibiotic therapy must be weighed against concerns regarding increases in antibiotic resistance [33]. The antibiotic resistance crisis, attributed to the systemic overuse and misuse of these medications, has pressured researchers to investigate new strategies to avoid dental or implant damage as a consequence of infection progression [33-35]. Thus, antimicrobial photodynamic therapy (aPDT) has been introduced as a potential alternative approach for the bacterial decontamination of tooth or implants surfaces [36, 37].

The success of aPDT requires that a photosensitizer (PS) be adsorbed by microbial cells and become adsorbed by the cell wall with subsequent activation by light irradiation. As the desired mechanism of action, singlet oxygen and free radicals produced by the irradiated PS will interact with cell structures, thereby damaging the cytoplasmic membrane and DNA, which leads to cell death [38, 39]. Among the commercially available PSs, Photodithazine (PDZ), which is based on chlorine e6 (Ce6), is a second-generation PS with potential antimicrobial effects against pathogenic microorganisms and low toxicity at appropriate concentrations [40]. Our previous *in vitro* [41, 42] and *in vivo* [40, 43] outcomes have demonstrated the successful inactivation of fungal *Candida* spp. biofilms by aPDT with PDZ. However, information on the effects of PDZ-mediated aPDT on anaerobic bacteria species is lacking.

Therefore, here, we evaluated the effects of PDZ-mediated aPDT on *F. nucleatum* and *P. gingivalis* biofilms. Furthermore, we investigated the association of this approach with an antibiotic commonly prescribed for periodontal and peri-implant diseases.

2. Materials and Methods

2.1 Human saliva preparation

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Human saliva collection was carried out after approval by the Institutional Ethical Committee (CAAE 26142014.0.0000.5416). Unstimulated saliva was obtained from three healthy male adults aged between 25 and 30 years. The inclusion criteria were as follows: no active caries or periodontal disease, no systemic disease and no antibiotic-related therapy for at least three months before the study. After collection, saliva was mixed and clarified by centrifugation at 10,000 g for 15 minutes at 4°C [44]. Immediately after centrifugation, the clarified saliva was filtered with a Millipore® (Merck Millipore Group, MA, USA) membrane 0.22-µm pore size filter and stored at -80°C until use [45, 46].

2.2 Bacterial strains and inoculum preparation

F. nucleatum (National Collection of Type Cultures (NCTC) 11326) and *P. gingivalis* (American Type Culture Collection (ATCC) 32277) were grown on Brucella agar (HiMedia) with 5% defibrinated sheep blood (Microlab) at 37°C under anaerobic conditions (85% N_2, 10% de H_2, and 5% CO_2) (Don Whitley Scientific, England). After 48 hours, bacterial colonies were transferred to 10 mL Brain Heart Infusion (BHI–Difco Laboratories Inc, MI, USA) broth medium supplemented with hemin (10 mg/mL), menadione (5 mg/mL) and yeast extract (6 g/L), followed by incubation at 37°C and anaerobiosis for 24 hours. Subsequently, 500 µL bacterial cells was suspended in 9.5 mL fresh BHI broth medium and incubated under anaerobic conditions until reaching the midexponential phase, according to the following established growth curves: 15 hours for *P. gingivalis* and 5 hours for *F. nucleatum* [47]. Finally, the bacterial cell concentrations were set at 10^7 colony-forming units (CFU)/mL for each species with a spectrophotometer (Spectrum–SP 2000 UV) at a wavelength of 600 nm.

2.3 Antibacterial susceptibility testing

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Planktonic susceptibility tests were performed following the Clinical and Laboratory Standards Institute guidelines (M07-A9, 2012) [48]. Briefly, 95 µL metronidazole (MTZ–Sigma-Aldrich, St. Louis, MO, USA) was serially diluted from 32 to 0.015 µg/mL in BHI broth medium in a 96-well plate. Then, five µL of each bacterial species was added at 1×10^7 CFU/mL into each well, bringing the final volume to 100 µL after the inoculation. Bacteria were also inoculated directly into polystyrene plates to serve as growth controls, while medium without bacterial inoculation served as a negative control for growth. Plates were incubated under anaerobic conditions at 37°C for 24 hours. Next, the bacterial growth in the plate was measured at 595 nm with a spectrophotometer (Spectrum–SP 2000 UV, Mettler-Toledo Ind. and Com. Ltda, SP, Brazil). The minimum inhibitory concentration [1] was considered the lowest antibiotic concentration capable of inhibiting at least 100% of bacterial growth compared to the growth controls and media without bacterial inoculation.

2.4 Biofilm formation

The single-species biofilms of *F. nucleatum* and *P. gingivalis* were formed as previously described [47]. Briefly, a conditioned saliva-derived film (i.e., salivary pellicle) was developed for the attachment of the initial pathogenic biofilm. Fifty µL sterilized saliva was added to each well of a 96-well plate (Corning Costar cell culture plates; Fisher Scientific, NY, USA) and kept in an orbital shaker (75 rpm) at 37°C [49]. After 4 hours of incubation, the saliva was removed, and the wells were gently washed twice with 100 µL sterile phosphate-buffered saline (PBS). A 150 µL aliquot of each bacterial species at $1x10^7$ cells/mL was individually inoculated onto the acquired salivary pellicle, and the plates were incubated at 37°C under anaerobic conditions. Following 24 hours of the adhesion phase, unbound cells were removed by gentle washing with 200 µL PBS, and

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150 µL fresh BHI-supplemented broth medium was added to promote biofilm formation. Plates were statically incubated at 37°C under anaerobic conditions for five days, and the culture medium was replaced every 24 hours. Bacterial species individually cultured in the polystyrene plate served as a positive control for biofilm formation. For all experiments, the wells were washed twice with 200 µL PBS at the end of the incubation period before further analyses.

2.5 PDZ-mediated aPDT: photosensitizer and light source properties

Stock solutions of PDZ (VETA-GRAND Company, Moscow, Russia) diluted in saline solution were freshly prepared at concentrations of 50, 75 and 100 mg/L and stored in the dark at room temperature until use. Red light-emitting diode (LED) light irradiation was used with a constant dose of 50 J/cm^2 at a 660 nm excitation wavelength with a power density of 71.7 mW/cm² for 28 minutes.

2.6 Effect of PDZ-mediated aPDT on biofilms

To understand the effect of PDZ on bacterial cell viability, we incubated single-species biofilms of *F. nucleatum* and *P. gingivalis* with PDZ at the following concentrations: 50 mg/L, 75 mg/L and 100 mg/L. PDZ activity without light irradiation was compared to PDZ-mediated aPDT activity.

After an incubation period for the formation of biofilms, wells were washed twice with PBS, and 200 µL aliquots of PDZ were added to the experimental samples, aPDT and PDZ. For controls, biofilms were incubated with 200 µL PBS with and without light irradiation (L^+ and L^-, respectively). All samples were incubated at room temperature for 10 minutes in darkness, as a preirradiation phase. In sequence, a 96-well plate containing PBS and PDZ groups was submitted to LED irradiation for 28 minutes and defined as

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follows: aPDT 50 mg/L, aPDT 75 mg/L and aPDT 100 mg/L. Concomitantly, another 96-well plate containing PBS and PDZ was kept in the dark with the groups PDZ 50 mg/L, PDZ 75 mg/L and PDZ 100 mg/L. After treatments, 200 μL PBS was added to all of the wells, and bacterial cells were harvested from the polystyrene wells by scraping with a sterile pipette tip. In sequence, bacterial culture was re-suspended several times using a pipette tip and a vortex to disperse the cells before serial dilution procedure. Then, 25 μL of ten-fold, serially diluted samples was plated on Brucella agar. The plates were incubated at 37°C under anaerobic conditions for seven days. Colony counts were then obtained with a digital colony counter and expressed as CFU/mL. The experiments were conducted in triplicate on three distinct experimental occasions.

2.7 Effect of PDZ-mediated aPDT on biofilms in comparison with MTZ

The effect of aPDT on biofilms was compared with that of MTZ. For each experiment, biofilm samples were formed in two different 96-well plates, corresponding to the irradiated and nonirradiated groups. For the PDZ-mediated aPDT groups, the procedures were performed as previously described (see 2.6). For the antibiotic-treated groups, 5-day-old biofilm samples were washed twice with PBS and then incubated with 200 μL MTZ at the following concentrations: MIC (MTZ MIC), MIC 50x (MTZ 50x) and MIC 100x (MTZ 100x) for 24 hours. Next, 25 μL of PBS serially diluted samples was plated on Brucella agar, and the plates were incubated at 37°C to obtain CFU/mL values after 7 days. The experiments were performed in triplicate and on three different occasions.

2.8 Potential effect of combination therapy on biofilms

Another approach to overcome *F. nucleatum* and *P. gingivalis* biofilms was to combine aPDT with local antibiotic administration. Here, 5-day-old biofilm samples were

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subjected to aPDT 50 mg/L, aPDT 75 mg/L and aPDT 100 mg/L and in sequence, incubated with 200 µL MTZ at the following concentrations: MTZ MIC, MTZ 50x and MTZ 100x, for 24 hours. At the end of the incubation period, biofilms were harvested in PBS, and 25 µL serially diluted cultures was plated on Brucella agar to obtain viable colonies after seven days. All experiments were performed in triplicate with three repetitions to ensure biological reproducibility.

For each experiment, biofilm samples were assessed in two different plates corresponding to red LED light exposure (aPDT) and a dark room. Additionally, biofilm samples submitted to PDZ-mediated aPDT or treated with MTZ, as well as bacterial cell concentrations inoculated on polystyrene, served as controls.

2.9 Effect of PDZ-mediated aPDT/MTZ on biofilms under confocal analyses

Biofilms were grown and treated as described in sections 2.4 and 2.5. The biofilm thickness and bacterial cell viability were evaluated via fluorescent labeling with a LIVE/DEAD BacLightTM Bacterial Viability staining kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. PBS-washed biofilm samples were visualized with a PASCAL LSM5 confocal laser-scanning microscope (Zeiss, Jena, Germany). Images were taken through 20x dry (Plan NeoFluar NA 0.3 air) objectives. Excitation wavelengths of 488 nm (Ar laser) and 561 nm (HeNe laser) were employed to reveal the effect of L^+, aPDT 100 mg/L, MTZ 100x, L^+ MTZ 100x and aPDT 100 mg/L MTZ 100x on the distribution of live and dead bacterial cells, as well as their accumulated biomass.

2.10 Statistical analyses

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A schematic was designed to better represent the groups and methods used to conduct the investigation (Fig. 1). All experiments were performed in triplicate and repeated three times for each bacterial species (n = 9) except for confocal analyses, which were performed in duplicate and repeated twice to ensure the reproducibility of the experiment. The data normality distribution was assessed using the D'Agostino-Pearson omnibus test. Normally distributed data were analyzed using one-way analysis of variance (ANOVA) with Tukey's post hoc test ($p < 0.05$). Nonnormally distributed data were analyzed using the Kruskal-Wallis test, followed by Dunn's multiple comparison test. The data presented are plotted as the mean ± standard deviation (SD) in a normal distribution. Nonnormally distributed data are plotted as the median ± SD. Analyses were performed using GraphPad Prism version 5.0c; $p < 0.05$ was considered statistically significant.

3. Results

3.1 Minimum inhibitory concentration

MIC was considered the lowest concentration of MTZ that completely inhibited 100% of bacterial growth after 24 hours. The concentration was confirmed by cells plated on Brucella agar. The MIC values of MTZ were 1.0 μg/mL for *F. nucleatum* and 0.125 μg/mL for *P. gingivalis*.

3.2 PDZ-mediated aPDT on biofilms

The number of viable colonies from single-species biofilms after PDZ incubation was compared to that after PDZ followed by light application (aPDT) to confirm the effect of the PS on bacterial cell reduction. Based on the data, for *F. nucleatum*, combining PDZ at the highest concentrations (75 and 100 mg/L) with red LED light irradiation is crucial to reduce the number of bacterial cells (Fig. 2A). However, different outcomes were

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observed for *P. gingivalis* biofilms since red LED light affected cell viability (L^-) even in the absence of PDZ. Moreover, the CFU/mL reduction was higher when 100 mg/L PDZ was combined with red LED light (aPDT 100 mg/L) (Fig. 2B).

3.3 PDZ-mediated aPDT compared to antibiotics (MTZ) applied to biofilms

The following experiments were performed to compare the effect of MTZ alone on biofilm growth reduction with that of PDZ-mediated aPDT. *F. nucleatum* biofilm samples submitted to aPDT with 75 (aPDT 75 mg/L) and 100 mg/L (aPDT 100 mg/L) PDZ revealed significant reductions in CFU/mL (approximately 1 $\log_{10}$ reduction). Light irradiation did not alter the effect of MTZ on *F. nucleatum* biofilms. Regardless of the presence of light, the highest concentrations of antibiotic significantly reduced the log of CFU/mL, corresponding to 2.22 and 2.14 $\log_{10}$ reductions for the L^- MTZ 100x and L^+ MTZ 100x groups, respectively, compared to that for the control groups (P^-L^- group) (Fig. 3A).

For *P. gingivalis* biofilms, no difference in CFU/mL reduction was observed between aPDT with 100 mg/L PDZ and MTZ 100x without light (L^- MTZ 100x) compared to the control (L^-) (2.59 $\log_{10}$ and 2.53 $\log_{10}$, respectively). A significant antimicrobial effect was achieved only when MTZ 100x was associated with red LED light (3.12 $\log_{10}$) and compared with the positive control (L^-) (Fig. 3B).

3.4 Effect of PDZ-mediated aPDT in association with MTZ on biofilms

Since a substantial reduction in biofilm viability was observed with the highest concentration of aPDT and with MTZ, the potential effect of their association was also investigated.

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For *F. nucleatum* biofilms, aPDT 75 mg/L PDZ plus MTZ 100x MIC (aPDT 75 mg/L MTZ 100x) and aPDT 100 mg/L PDZ plus MTZ at concentrations of 50x MIC and 100x MIC (aPDT 100 mg/L MTZ 50x and aPDT 100 mg/L MTZ 100x) significantly reduced the viability by 2.99, 2.9 and 3.94 $\log_{10}$, respectively. Compared with the control, P^-L^-, the combination of MTZ 100x MIC with aPDT 100 mg/L (aPDT 100 mg/L–MTZ 100x) yielded pronounced antimicrobial activity by decreasing CFU by more than 3 $\log_{10}$ CFU (Fig. 4A).

For *P. gingivalis* biofilms, aPDT promoted a significant reduction in cell viability when combined with MTZ 100x, independent of the PDZ concentration. However, an extensive 5 $\log_{10}$ reduction was obtained with aPDT 100 mg/L PDZ in association with MTZ 100x MIC (aPDT 100 mg/L–MTZ 100x). In fact, light irradiation alone affected cell viability in *P. gingivalis* biofilms, as demonstrated by lower CFU/mL after light exposure in the (L^+) groups compared with that in the L^- groups (Fig. 4B).

3.5 Three-dimensional visualization of L^+, PDZ, MTZ, PDZ-mediated aPDT in association with or without MTZ on biofilms

The bacterial microcolony distribution and biofilm thickness was evaluated via confocal visualization to confirm the CFU/mL results obtained for L^+, aPDT 100 mg/L PDZ, MTZ 100x MIC, L^+ MTZ 100x MIC, and aPDT 100 mg/L PDZ plus MTZ 100x MIC. Interestingly, compared with the positive control (L^-), light alone did not affect *F. nucleatum* viability but interfered with biofilm thickness and cell distribution (Fig. 5A-B). Similar effects were also observed for aPDT. Specifically, aPDT contributed to *F. nucleatum* biofilm disturbances (Fig. 5C) since bacterial microcolonies from *F. nucleatum* biofilms were generally less abundant than those from *F. nucleatum* biofilms without light (Fig. 5A). Alternatively, MTZ alone did not seem to affect the *F. nucleatum*

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biofilm structure because there was only a slight difference in overall green fluorescence intensity caused by application of MTZ to *F. nucleatum* biofilms (Fig. 5D) compared to that caused application of the control (Fig. 5A). The potential effect of MTZ was also investigated in combination with light or aPDT (Fig. 5E-F). Consistent with the observed difference in the number of *F. nucleatum* viable cells, fluorescent live/dead staining revealed a bactericidal effect when MTZ was applied in combination with L^+ or aPDT (Fig. 5E-F). Light irradiation appeared to have a significant impact on the action of MTZ by disturbing bacterial cells on *F. nucleatum* biofilms (Fig. 5E-F).

For *P. gingivalis*, a slight antibacterial effect was observed after biofilm exposure to light (L^+) alone, with a marked reduction in biofilm thickness compared with that after exposure to the control (Fig. 6A-B). In the other evaluated groups, very few cells were nonviable (red color; Fig. 6C-E). In contrast to the controls, MTZ, aPDT and L^+ MTZ did not show markedly distinct effects on the spatial structure of *P. gingivalis* biofilms. The biofilms treated with aPDT in combination with MTZ (Fig. 6F) exhibited a vast number of dead bacterial cells without a significant detectable change in the biofilm thickness.

4. Discussion

aPDT has previously been demonstrated to kill planktonic periodontal pathogens [41, 50-52]. However, the susceptibility of microorganisms to aPDT is considerably reduced when they are organized into biofilms. To gain new insight into the effect of PDZ-mediated aPDT on anaerobic bacteria, we investigated the impact of different PDZ concentrations with or without light on *F. nucleatum* and *P. gingivalis* biofilms. Although PDZ-mediated aPDT promoted a significant viability reduction in both bacteria after therapeutic application, a potentiated antimicrobial effect was achieved only in combination with local MTZ antibiotic administration.

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Bacteria organized in biofilms are highly resistant to conventional antimicrobial treatments [53]. Strong evidence indicates that *F. nucleatum* and *P. gingivalis* are two anaerobic species involved in the initiation and progression of periodontal and peri-implant diseases [54]. The possibility of tooth and implant loss because of disease progression has encouraged the search for new strategies to disrupt pathogenic biofilms and disease progression with treatment. Considering the successful preliminary data obtained from PDZ-mediated aPDT by our research group [40, 43, 55], we moved forward and evaluated the effect of aPDT treatment against *F. nucleatum* and *P. gingivalis* biofilms. For *F. nucleatum*, the CFU/mL reduction was directly related to the association between the highest PDZ concentrations and light. Our data revealed that 75 mg/L and 100 mg/L PDZ-mediated aPDT promoted 0.97 and 1.12 $\log_{10}$ reductions in CFU/mL, respectively. In contrast to our outcomes, a previous study demonstrated a significant effect against *F. nucleatum* ATCC 25586, with more than a 3 $\log_{10}$ reduction when aPDT with visible light (vis) and water infiltrated infrared A (wIRA) were combined with Ce6 [50]. The strong antimicrobial effect of aPDT against *F. nucleatum* cultures was also found in another recent study where 99.7% of the bacterial population died. However, these promising data cannot be compared with our data since the experiments were carried out in planktonic cultures [56]. Remarkably, the authors indicated a slight reduction in *F. nucleatum* cells even after light irradiation alone [56], which is consistent with our fluorescence information. However, in both reported studies [50, 56], the treatment was performed on planktonic bacterial species; this approach was inconsistent with our experimental approach in which we developed biofilms that presented a complex tridimensional architecture. Thus, the difference in microbial culture type can explain why the effect of aPDT was lower in our studies than in previous studies [50, 56].

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For *P. gingivalis*, the effectiveness of aPDT was achieved only when PDZ was applied at the highest concentration (100 mg/L), resulting in a 2.66 $\log_{10}$ bacterial reduction. Despite the efficacy of aPDT against biofilm viability, an antimicrobial effect was not observed to ensure antibacterial properties since we obtained less than a 3 $\log_{10}$ CFU reduction [57]. In contrast, an antimicrobial effect of aPDT using vis+wIRA combined with Ce6 was recently reported against different periodontal pathogens in a pool of subgingival oral biofilms with disruption of their tridimensional structures before the application of aPDT [50]. This disruption in the architecture of a biofilm can potentiate the effects of antimicrobial strategies. Here, biofilms with intact architecture were subjected to distinct antimicrobial approaches. Thus, the substantial differences in bacterial CFU reduction between this study and the previous study [38[50] can be explained by the different methodologies applied. In the previous study [50], subgingival oral biofilms were developed on *in situ* devices, but the biofilm samples were pooled, centrifuged and resuspended in saline solution [38]. Therefore, in that study, the treatment was applied to planktonic bacteria cells arising from biofilms; in contrast, in our study, aPDT was directly applied to a complex, organized pathogenic structure.

In the absence of PDZ, exposure to light irradiation affected the viability of *P. gingivalis* cells [58]. The endogenous porphyrin production in the cell wall of *P. gingivalis* can explain this finding as porphyrin acts as a natural PS [59-62]. The susceptibility of some oral black-pigmented bacterial species to light irradiation in the absence of PS has been previously demonstrated in laboratory research and *in vivo* experiments [63]. Porphyrin is chemically excited by light of specific wavelengths [64] and promotes the generation of reactive oxygen species (ROS) capable of reacting and affecting biological systems [65]. Thus, *P. gingivalis* viability in culture medium is easily reduced by over 90% by exposure to 70 mW/cm² with a broadband light ranging from

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380-520 nm, which includes blue light [63]. Using a similar power density of 71.7 mW/cm², our results indicated a 1.33 $\log_{10}$ CFU/mL reduction in *P. gingivalis* after 28 minutes of red light exposure at 660 nm. The notable difference in overall bacterial viability observed in our study may be explained by the, organized biofilm that we developed since the biofilm extracellular matrix can directly interfere with light diffusion [66, 67]. Surprisingly, the live/dead viability assay confirmed a slight effect of light on *P. gingivalis* biofilms, with red fluorescence emitted by dye-labeled dead cells and a marked reduction in biofilm thickness. Another interesting result was demonstrated by confocal imaging of *F. nucleatum* biofilms. Although CFU/mL counts were similar to those of *F. nucleatum* viable cells after L^+ and aPDT compared with those after the control (L^-), fluorescence analyses revealed a disturbed biofilm with sparse green areas and reduced pixel intensity in L^+ and aPDT samples, respectively. The presence of red LED light (L^+) seems to interfere with biofilm stability.

Although PDZ-mediated aPDT significantly reduced the number of cultivable bacteria within biofilms, the effect of this therapy remains far below the microbial reduction rates required to be considered as an effective antimicrobial approach against *F. nucleatum* and *P. gingivalis* biofilms. To date, a major limitation of aPDT involves the poor uptake of PS. The idea of combining aPDT and local antibiotic delivery was undertaken in an attempt to widen the possibility of therapy application against pathogenic biofilms [68-70]. It is important to empathize that the dramatically question involving antibiotic is due to their indiscriminate systemic administration. Although, antibiotic resistance represents one of the biggest threats to global health and economic burden [71], systemic antibiotic prescription to fight periodontal and peri-implant disease still is strongly recommended [72]. From an overall healthcare perspective, microbial resistance and the development of superinfection as potential risks caused by antibiotic

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therapy tackling the threatening possibility of bone resorption and subsequent tooth and/or dental implant loss [73]. Because antibiotics still are essential to treat infectious diseases, the local antibiotic administration has emerged as a possible coadjutant in reducing microbial resistance [74] and controlling inflammation in diseased periodontal sites [75-79]. Based on this principle, we first investigated the effect of different MTZ concentrations on the anaerobic biofilms of two bacterial strains. In agreement with the literature [80], we found that low concentrations of MTZ, specifically, 0.125 μg/mL and 1 μg/mL, are sufficient to inhibit *P. gingivalis* and *F. nucleatum* growth in planktonic cells, respectively. MTZ possesses the ability of covalently binding to DNA, inhibiting bacterial nucleic acid synthesis and killing the cell. However, a different process is expected when MTZ is applied to biofilms [81]. The findings arising from controlled experiments have reported that biofilms are up to 1000 times more resistant to antimicrobial treatments than planktonic cells [82-84]. In our study, MTZ MIC, MTZ 50x and MTZ 100x showed no antimicrobial activity against either *F. nucleatum* or *P. gingivali*s biofilms.

Our outcomes demonstrated that although aPDT did not display antimicrobial activity of 3 log (previously defined by [57]), the treatment with this approach significantly reduced the number of pathogenic bacteria inside the biofilm structure. Similar results were also observed to MTZ -treated groups, regardless antibiotic concentration. However, either aPDT neither MTZ was capable of destroying the organized structure and conferring the desired antimicrobial effect. Thus, to explore the potential upshot of the treatment we combined an antimicrobial agent that has excellent activity against strict anaerobic bacteria[85] after bacteria cells from biofilm being disturbed by aPDT. Based on the experimental outcomes from this study, MTZ seems to make biofilm cells more sensitive to PDZ penetration [69, 70], and antimicrobial activity

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was strongly influenced by this association. One hundred mg/L PDZ-mediated aPDT associated with 100x MIC MTZ resulted in a significant 3.94 $\log_{10}$ CFU/mL reduction in bacterial load for *F. nucleatum* biofilms. Comparable antimicrobial activity was demonstrated for *P. gingivalis* grown as a biofilm since PDZ-mediated aPDT combined with 100x MIC MTZ promoted more than a 3 $\log_{10}$ reduction in CFU, regardless of the PDZ concentration. The remarkable effect of a 5 $\log_{10}$ *P.* gingivalis CFU reduction was achieved with 100 mg/L of PDZ-mediated aPDT combined with 100x MIC MTZ. Underlining the influence of light on porphyrins from the *P. gingivalis* cell wall, the higher efficacy of 100x MIC MTZ in the biofilm was obtained only after light exposure, leading to a 3.07 $\log_{10}$ CFU/mL reduction in bacterial viability. However, the desired antimicrobial properties were achieved only when MTZ was applied as an adjunctive therapy to inactivate *F. nucleatum* and *P. gingivalis* bacteria. The significant bactericidal activity against both single pathogenic biofilms was confirmed by live/dead viability assays via confocal microscopy.

Indeed, aPDT remains under development. Although, PDZ-mediated aPDT has already demonstrated effectiveness against *Candida albicans*, for the management of denture stomatitis [86], the questions raised by our outcomes highlight the limitation of PDZ-mediated aPDT alone against anaerobic biofilms and the contribution of antibiotics to its success as an antimicrobial approach. From a clinical perspective, PDZ gel can be easily applied on the sick site for 20 minutes, followed by a light irradiation period of 4 minutes (50 J/cm2) [86]. Further, the subsequent local delivery of antibiotics in the infected sites has already been descripted by the scientific community and can be carried out by using different approaches: fibers, films, microparticles and gels [87-90]. However, although we used a robust anaerobic biofilm model with human saliva to simulate an oral environment [47], polystyrene substrates are not natural surfaces on

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which periodontal biofilm formation occurs. Therefore, the results obtained do not reflect the clinical setting. Furthermore, as our study was the first to investigate the use of PDZ-mediated aPDT against anaerobic biofilms, we opted to grow bacteria in single-species cultures to reduce the number of variables and gain a better understanding of the benefits of aPDT against resistant infections. Additional *in vitro* experiments involving multispecies biofilm and tooth/implant substrates followed by *in vivo* research are necessary to determine the aPDT-antibiotic combination approach as a valuable prospect for future clinical implementation.

Conflicts of Interest

The authors declare no conflicts of interest.

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Figure Legends

Fig. 1. A schematic illustration of the experimental setup performed to conduct this investigation. L^+: under light irradiation; L^-: without light irradiation; PDZ: photodithazine; 50 mg/L, 75 mg/L and 100 mg/L: photodithazine's concentrations, aPDT: antimicrobial photodynamic therapy, MTZ: metronidazole, MIC, 50x and 100x: metronidazole's concentrations.

Fig. 2. (A) Effect of light and different concentrations of PDZ and aPDT on *F. nucleatum* biofilms. One-way analysis of variance (ANOVA) was employed with Tukey's *post hoc* test. **(B)** Effect of light and different concentration of PDZ and aPDT on *P. gingivalis* biofilms. The Kruskal-Wallis test was employed, followed by Dunn's multiple comparison test. Bacterial counts after each treatment are expressed in logarithm of colony-forming units (CFU) per milliliter. Statistically significant differences are indicated as: $^{*, +, **, \#}$ $p<0.0001$. Data represent the median ± SD of three biological replicates from three independent experiments by using GraphPad Prism version 5.0c.

Fig. 3. Effect of MTZ in the presence and absence of light irradiation compared to that of aPDT on **(A)** *F. nucleatum* and **(B)** *P. gingivalis* biofilms. One-way analysis of variance (ANOVA) was employed with Tukey's *post hoc* test. Bacterial counts after each treatment are expressed in logarithm of colony-forming units (CFU) per milliliter. Statistically significant differences are indicated as: $^{*, +, **, ***, \#}$ $p<0.0001$. Data represent

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the mean ± SD of three biological replicates from three independent experiments by using GraphPad Prism version 5.0c.

Fig. 4. Effect of aPDT in combination with MTZ at different concentrations on (A) *F. nucleatum* and (B) *P. gingivalis* biofilms. **Gray lines:** bacterial growth controls; **Blue and Red lines:** experimental groups disclosing antimicrobial effect. One-way analysis of variance (ANOVA) was employed with Tukey's *post hoc* test. Bacterial counts after each treatment are expressed in logarithm of colony-forming units (CFU) per milliliter. Statistically significant differences are indicated as: $^{*, +, **, \#}$ $p<0.0001$. Data represent the mean ± SD of three biological replicates from three independent experiments, by using GraphPad Prism version 5.0c.

Fig. 5. Representative images generated by confocal laser-scanning microscopy (z-axis stack) illustrating the effect of **(B)** L^+, **(C)** aPDT 100 mg/L PDZ, **(D)** MTZ 100x MIC, **(E)** L^+ MTZ 100x MIC, and **(F)** aPDT 100 mg/L PDZ plus MTZ 100x MIC on *F. nucleatum* biofilms. The panels demonstrate the live and dead bacterial populations as well as microcolony distribution and biofilm thickness compared to those of the untreated *F. nucleatum* biofilm control group **(A).** The multiple Z sections in panels A to F were generated by sectioning at 50 μm.

Fig. 6. Representative images generated by confocal laser-scanning microscopy (z-axis stack) images illustrating the effect of **(B)** L^+, **(C)** aPDT 100 mg/L PDZ, **(D)** MTZ 100x MIC, **(E)** L^+ MTZ 100x MIC, and **(F)** aPDT 100 mg/L PDZ plus MTZ 100x MIC on *P. gingivalis* biofilms. The panels demonstrate the live and dead bacterial populations as well as microcolony distribution and biofilm thickness compared to those of the untreated *P. gingivalis* biofilm control group **(A).** The multiple Z sections in panels A to F were generated by sectioning at 50 μm.

Graphical Abstract

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Highlights

- *F. nucleatum* and *P. gingivalis* are involved in the progression of oral diseases
- PDZ-mediated aPDT reduces the number of bacteria within mature biofilms
- MTZ seems to make biofilm cells more sensitive to PDZ penetration
- Desired antimicrobial properties are achieved when MTZ is combined with PDZ-mediated aPDT

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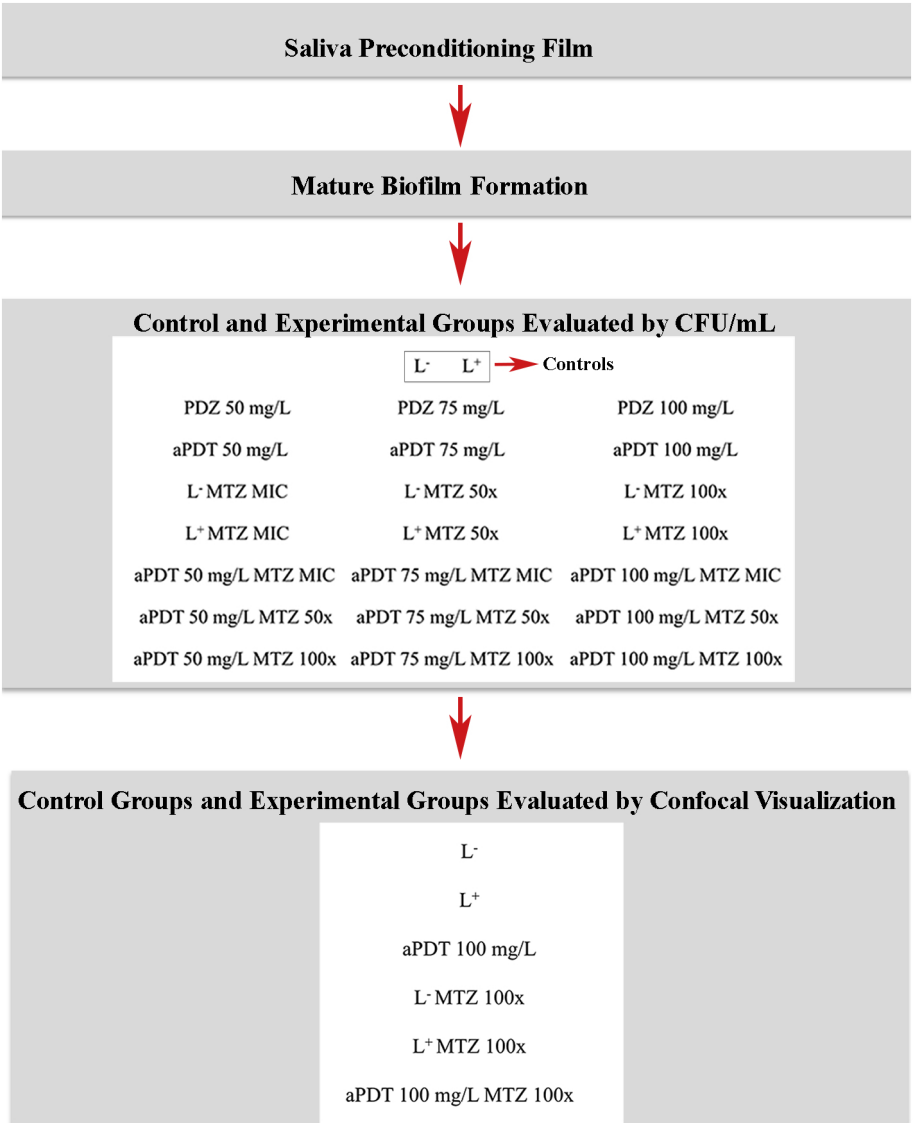
Saliva Preconditioning Film
Mature Biofilm Formation
Control and Experimental Groups Evaluated by CFU/mL
L- L+ Controls
PDZ 50 mg/L
PDZ 75 mg/L
PDZ 100 mg/L
aPDT 50 mg/L
aPDT 75 mg/L
aPDT 100 mg/L
L- MTZ MIC
L- MTZ 50x
L- MTZ 100x
L+ MTZ MIC
L+ MTZ 50x
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aPDT 100 mg/L MTZ 100x
Control Groups and Experimental Groups Evaluated by Confocal Visualization
L-
L+
aPDT 100 mg/L
L- MTZ 100x
L+ MTZ 100x
aPDT 100 mg/L MTZ 100x

Figure 1

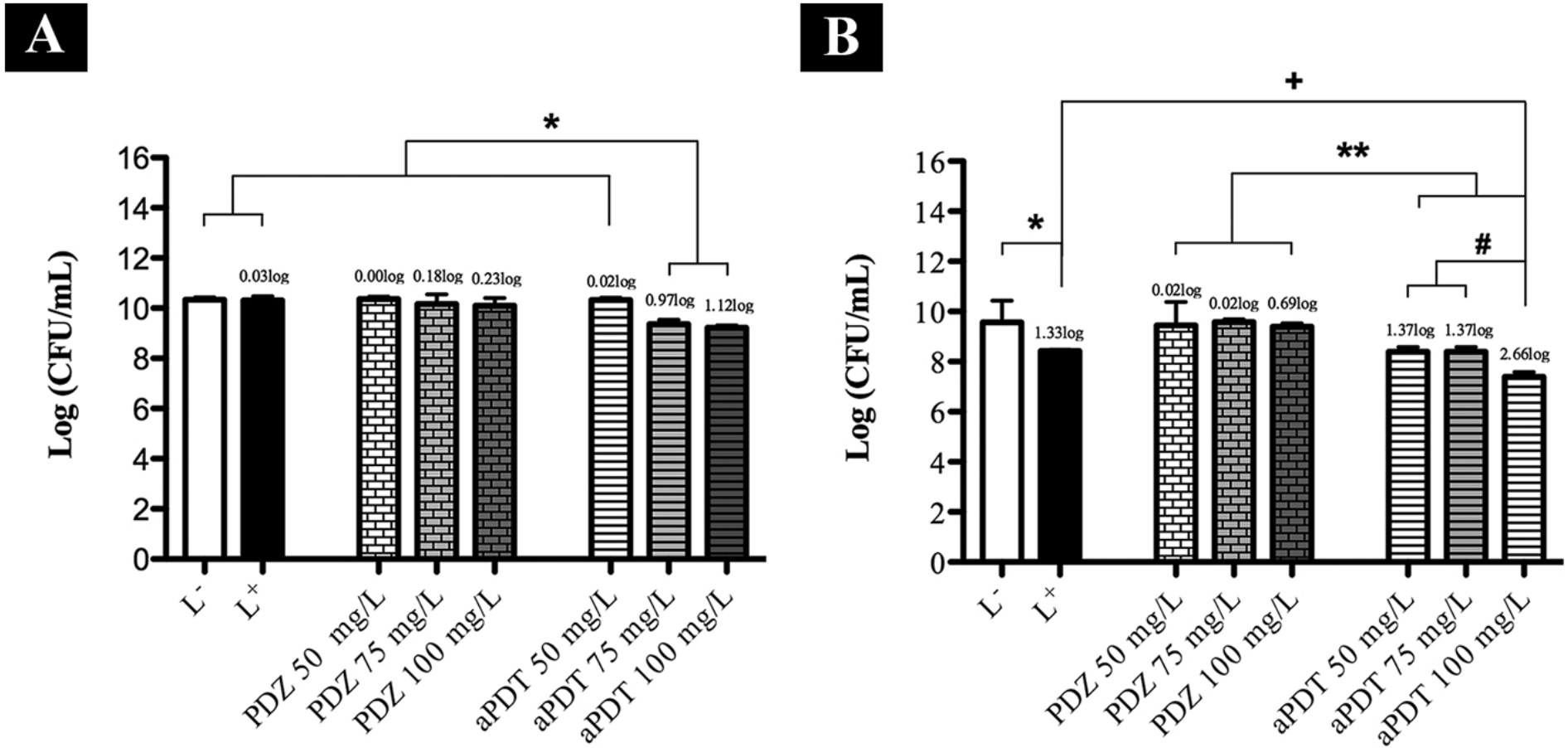
A
Log (CFU/mL)
16
14
12
10
8
6
4
2
0
*
0.03log
0.00log
0.18log
0.23log
0.02log
0.97log
1.12log
L-
L+
PDZ 50 mg/L
PDZ 75 mg/L
PDZ 100 mg/L
aPDT 50 mg/L
aPDT 75 mg/L
aPDT 100 mg/L
B
Log (CFU/mL)
+
**
*
#
1.33log
0.02log
0.02log
0.69log
1.37log
1.37log
2.66log

Figure 2

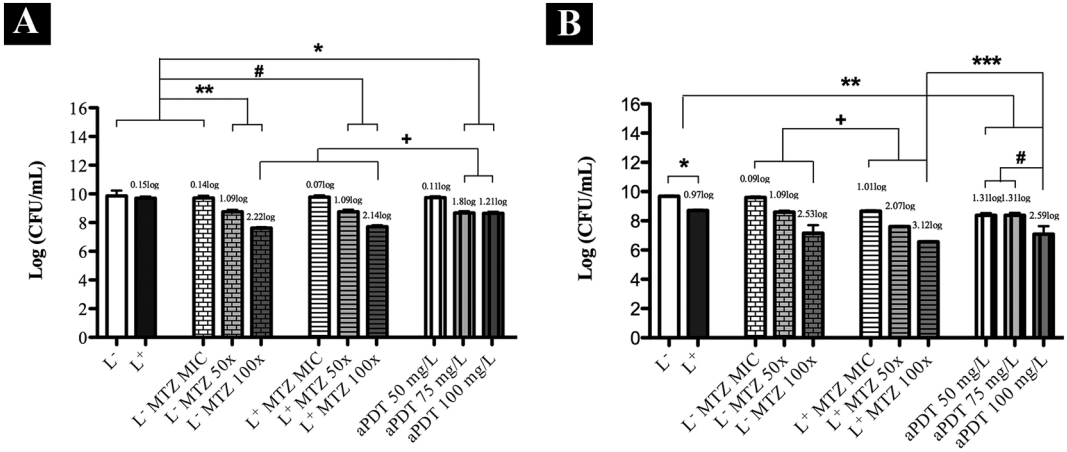
A
B
Log (CFU/mL)
0.15log
0.14log
1.09log
2.22log
0.07log
1.09log
2.14log
0.11log
1.8log
1.21log
0.97log
0.09log
1.09log
2.53log
1.01log
2.07log
3.12log
1.31log
1.31log
2.59log
L-
L+
L- MTZ MIC
L- MTZ 50x
L- MTZ 100x
L+ MTZ MIC
L+ MTZ 50x
L+ MTZ 100x
aPDT 50 mg/L
aPDT 75 mg/L
aPDT 100 mg/L

Figure 3

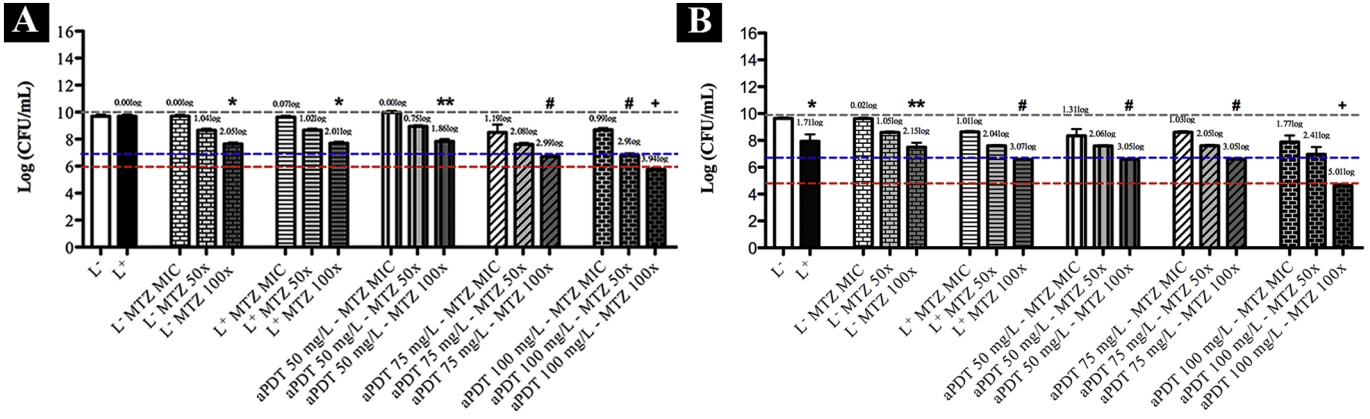
A
Log (CFU/mL)
0
2
4
6
8
10
12
14
16
0.00log
0.00log
1.04log
2.05log
0.07log
1.02log
2.01log
0.00log
0.75log
1.86log
1.19log
2.08log
2.99log
0.99log
2.9log
3.94log
*
*
**
#
#
+
L-
L+
L- MTZ MIC
L- MTZ 50x
L- MTZ 100x
L+ MTZ MIC
L+ MTZ 50x
L+ MTZ 100x
aPDT 50 mg/L - MTZ MIC
aPDT 50 mg/L - MTZ 50x
aPDT 50 mg/L - MTZ 100x
aPDT 75 mg/L - MTZ MIC
aPDT 75 mg/L - MTZ 50x
aPDT 75 mg/L - MTZ 100x
aPDT 100 mg/L - MTZ MIC
aPDT 100 mg/L - MTZ 50x
aPDT 100 mg/L - MTZ 100x
B
Log (CFU/mL)
0
2
4
6
8
10
12
14
16
1.71log
0.02log
1.05log
2.15log
1.01log
2.04log
3.07log
1.31log
2.06log
3.05log
1.05log
2.05log
3.05log
1.77log
2.41log
5.01log
*
**
#
#
#
+
L-
L+
L- MTZ MIC
L- MTZ 50x
L- MTZ 100x
L+ MTZ MIC
L+ MTZ 50x
L+ MTZ 100x
aPDT 50 mg/L - MTZ MIC
aPDT 50 mg/L - MTZ 50x
aPDT 50 mg/L - MTZ 100x
aPDT 75 mg/L - MTZ MIC
aPDT 75 mg/L - MTZ 50x
aPDT 75 mg/L - MTZ 100x
aPDT 100 mg/L - MTZ MIC
aPDT 100 mg/L - MTZ 50x
aPDT 100 mg/L - MTZ 100x

Figure 4

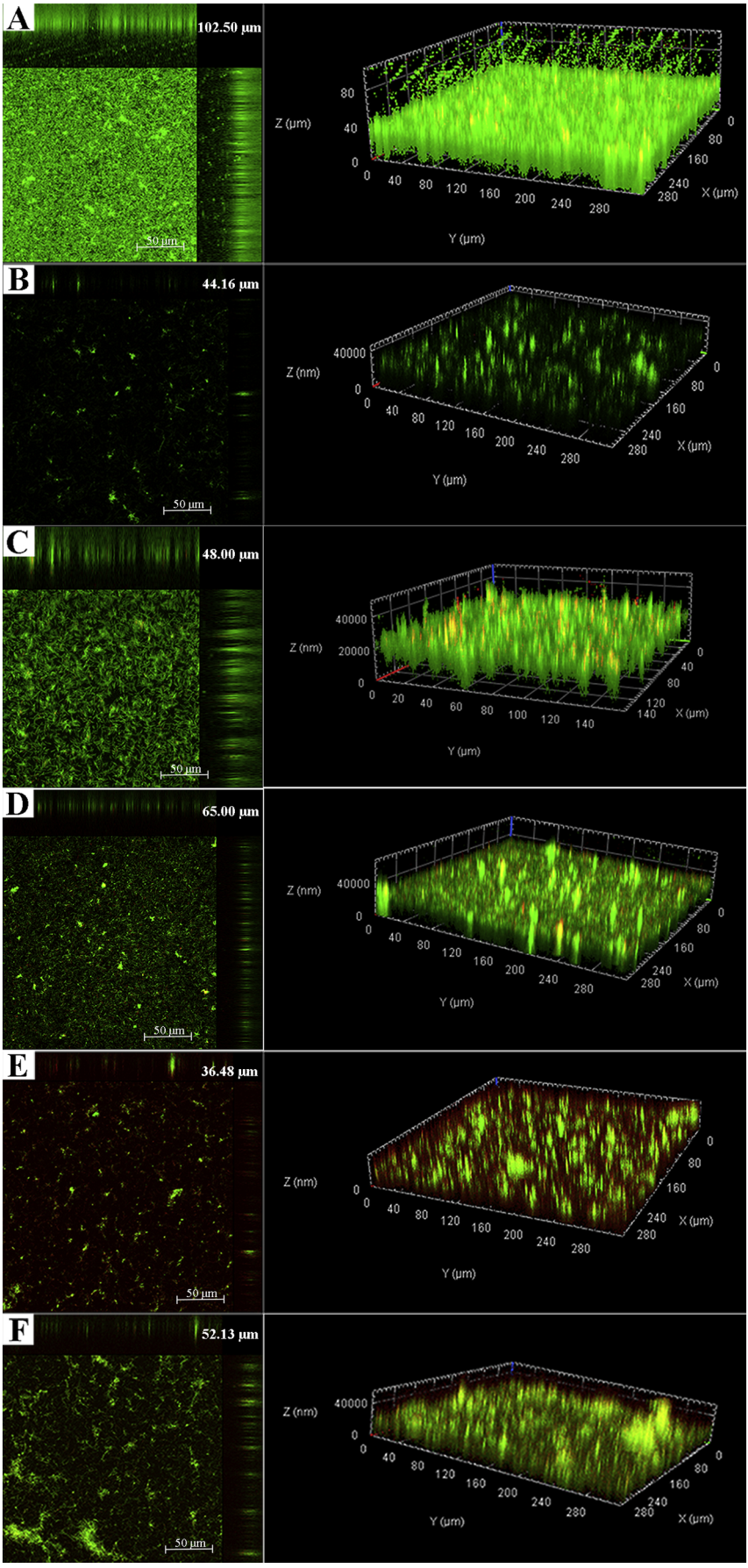
A
102.50 µm
50 µm
Z (µm)
Y (µm)
X (µm)
B
44.16 µm
50 µm
Z (nm)
Y (µm)
X (µm)
C
48.00 µm
50 µm
Z (nm)
Y (µm)
X (µm)
D
65.00 µm
50 µm
Z (nm)
Y (µm)
X (µm)
E
36.48 µm
50 µm
Z (nm)
Y (µm)
X (µm)
F
52.13 µm
50 µm
Z (nm)
Y (µm)
X (µm)

Figure 5

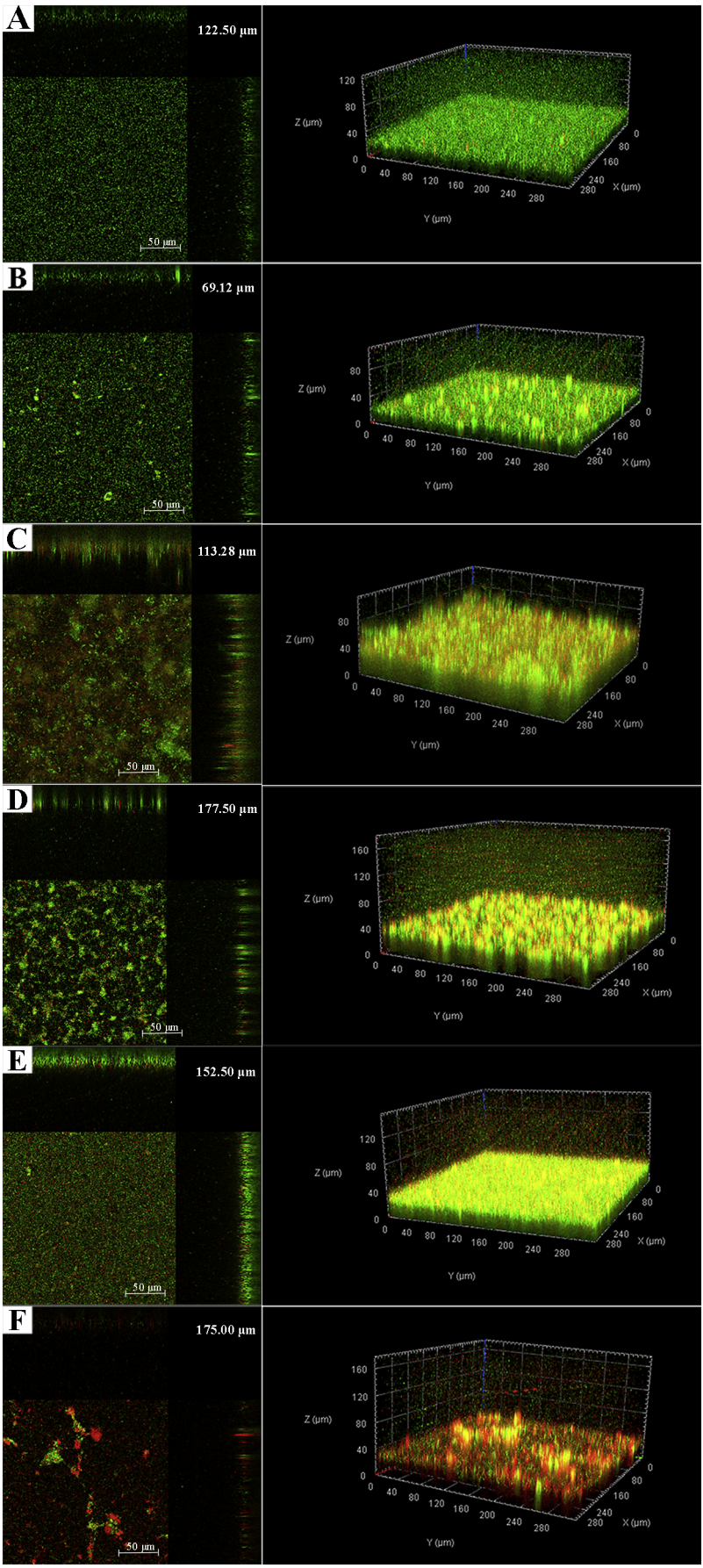
A
122.50 µm
50 µm
Z (µm)
Y (µm)
X (µm)
B
69.12 µm
50 µm
C
113.28 µm
50 µm
D
177.50 µm
50 µm
E
152.50 µm
50 µm
F
175.00 µm
50 µm

Figure 6