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# Photodynamic antimicrobial therapy (A-PDT) using 1,9-Dimethyl-Methylene Blue zinc chloride double salt - DMMB and λ640 ± 5ηm LED light in patients undertaking orthodontic treatment

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ABSTRACT

Orthodontic treatment involves the use of apparatuses that impairs oral hygiene making patients susceptible to periodontal diseases and caries. To prevent increased antimicrobial resistance A-PDT has shown itself a feasible option. The aim of this investigation was to assess the efficiency of A-PDT employing 1,9-Dimethyl-Methylene Blue zinc chloride double salt - DMMB as a photosensitizing agent combined with red LED irradiation (λ640 ± 5 ηm) against oral biofilm of patients undertaking orthodontic treatment. Twenty-one patients agreed to participate. Four biofilm collections were carried out on brackets and gingiva around inferior central incisors; first was carried out before any treatment (Control); second followed five minutes of pre-irradiation, the third was immediately after the first AmPDT, and the last after a second AmPDT. Then, a microbiological routine for microorganism growth was carried out and, after 24-h, CFU counting was performed. There was significant difference between all groups. No significant difference was seen between Control and Photosensitizer and AmpDT1 and AmPDT2 groups. Significant differences were observed between Control and AmPDT1 and AmPDT2 groups, Photosensitizer and AmPDT1 and AmPDT2 groups. It was concluded that double AmPDT using DMBB in nano concentration and red LED was capable to meaningfully decrease the number of CFUs in orthodontic patients.

## 1. Introduction

Orthodontic treatment aims enhancement of both oral function and aesthetics and consequently a healthier life. The correction of occlusal unbalance often requires the use of fixed orthodontic apparatus, which facilitate biofilm growth, and, in most patients, this accumulation is associated to inadequate oral cleanliness. Oral biofilm is an ecosystem in constant change and its composition may vary in distinct locations in the oral cavity. It has been suggested that hundreds of microorganisms can colonize the mouth and a person can carry up to 200 different species [1]. Commonly, orthodontic patients present some level of gingival disease (gingivitis) during the treatment. Instructing and practice in oral hygiene, cautious scaling and specialized tooth cleansing are usually advised for maintenance of oral health [2].

It is known that some types of light may cause impairment of biological systems in certain conditions. One of those phenomena is the photodynamic effect, that occurs when light is absorbed by a photosensitizer (PS) located within a target cell or microorganism, leading to the creation of reactive oxygen species (ROS) and singlet oxygen. These can cause damage to cell or microorganism by a phototoxic reaction,

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frequently by oxidative damage [3,4].

A-PDT employs use of a specific photosensitizer agent that is applied then activated by light of the appropriate wavelength and intensity, which targets alesion. For PDT the target are usually cells and for AmPDT the target are different microorganisms. In both PDT and AmPDT, type I and/or type II photochemical reactions occurs. At present, photodynamic antimicrobial therapy (AmPDT) has been suggested as an alternative approach in treating oral infections including those initiated by biofilm accumulation. It is considered effective as it can kill various pathogens and disorganize the biofilm. Firstly, AmPDT was assessed in vitro [5–7], and later against oral biofilms [8–10].

The PSs to be used for AmPDT must take in account the microorganism as $Gram^+$ and $Gram^-$ bacteria are structurally different and therefore their interaction with the PS is different. It is established that efficacy of the PS depends on both their ionic charge (+ or -) and cellular structures. It is well known that $Gram^-$ bacteria walls possess an additional membrane covered in lipopolysaccharides, while $Gram^+$ walls contain only one membrane and have a thicker layer of peptidoglycan containing negatively charged teichoic acids. Gram-positive and Gram-negative bacteria both have a net negative surface charge, except in low pH. Phenothiazinic compounds can interact with both + and - molecules as they are either hydrophilic or hydrophobic being efficient against different types of microorganisms [11].

It was theorized that AmPDT may possibly improve microbiological control in patients undergoing orthodontic treatment. Hence, the purpose of this study was to evaluate the effectiveness of AmPDT employing 1,9-Dimethyl-Methylene Blue zinc chloride double salt - DMMB as a photosensitizing agent combined with red LED irradiation (λ640 ± 5 ηm, CW, 110 mW, 294 s, 30 J/cm$^2$) against oral biofilm of patients undertaking orthodontic treatment.

## 2. Methods

### 2.1. Ethical aspects and sampling

This cross-over clinical experimental study was approved by the Ethics Committee of the Faculty of Dentistry of the Federal University of Bahia (No. 2,857,839). The sample size was determined assuming a level of significance of 95%, 90% of power, with an estimated difference of 40% between the groups. The minimum sample size was defined in 19 individuals. An increase of 10% was assumed with the aim of homogenizing the groups. Thus, 21 individuals submitted to orthodontic treatment at the Center for Orthodontics and Facial Orthopedics Prof. José Édimo Soares Martins, School of Dentistry, UFBA entered the study. Patients that agreed to participate in the study signed an Informed Consent Form (ICF).

### 2.2. Reagents used on the present study

The following reagents were used to conduct the experiments: 1,9-Dimethyl-Methylene Blue zinc chloride double salt - DMMB, molecular weight 416.05, content 80% Sigma-Aldrich, St. Louis, MO, USA. Tryptic Soy Broth®, Merck Darmstadt, Hessen, Germany. Phosphate Buffered Saline®, Merck, Darmstadt, Germany, and Brain Heart Infusion®, Merck Darmstadt, Hessen, Germany.

### 2.3. Biofilm collection

The procedure was carried out as previously described by Soares et al. [10] with three biofilm collections in the gingival region and around the brackets of 41 and 42 teeth with sterile swabs. The first was taken prior any procedure to estimate the initial load of microorganisms in the patient's mouth. The second was carried out after the mouth was rinsed with the photosensitizer and the last two after irradiations with red LED.

### 2.4. Photosensitizer

The phenothiazine dye used was the DMMB that was diluted in a laminar flow chamber[1] using sterile distilled water and the compound was diluted to a concentration of 100 μg/mL, followed by 0.22 μm membrane filtration[2] to disinfect it. The concentrations used were in accordance with previously calculated $IC_{50}$ for bacteria and fungi [12, 13]. For all the subsequent protocols, a 5-min pre-irradiation time was adopted, which is necessary for the cell to absorb the compound.

### 2.5. AmPDT

For AmPDT1 and AmPDT2, 20 mL of the PS (700 ng/mL, DMMB, Sigma, Brazil) was given to each patient to do rinse for 5-min (pre-irradiation time) to permit the contact with the oral biofilm. After expelling the liquid, the irradiation protocol was conducted using a Red LED[3] (Table 1, Fig. 1). All procedures obeyed the standard regulation for infection control as well as photoprotection.

### 2.6. Microbiological analysis

The procedure was conducted as previously described by Soares et al. [10]. Briefly, the collected samples were placed in test tubes containing 4 ml of TSB broth and homogenized in a vortex device.[4] Sequential decimal dilutions ($10^{-2}$ to $10^{-5}$) were carried out in PBS. Lastly, 100 μL of the inoculum was streaked on Petri dishes with BHI Agar in triplicate for each dilution using a Drigalski loop. After 24-h incubation at 37°C,[5] colony forming units (CFU) were quantified by direct counting.

### 2.7. Statistical analysis

Data was tabulated and Log-transformed and descriptive analysis was carried out using Minitab®19 software (Minitab Ltd, UK). Central tendency and variability (mean and standard deviation) were calculated. ANOVA was used to verify possible differences between groups. In case of significant differences being observed an adjusted linear regression analysis was carried out.

## 3. Results

### 3.1. CFU count

Individual mean Logs of CFU counts are shown in Table 2. Data was Log-transformed and descriptive analysis was conducted. Central tendency and variability (mean and standard deviation) were found to validate the effectiveness of AmPDT in the decrease of CFU counts: Control group = 5.562 ± 0.542; Photosensitizer Group = 5.092 ± 0.478; AmPDT1 group = 4.448 ± 0.739; and AmPDT2 group = 3.742 ± 0.957 (Fig. 1).

**Table 1**
Parameters of the LED light source.

| Parameters | LED |
|---|---|
| Peak Wavelength (ηm) | λ640 ± 5 |
| Mode | CW |
| Irradiation time (s) | 294 |
| Power (mW) | 110 |
| Energy Density(J/cm$^2$) | 30 |

[1] Pachane, SP, Brazil
[2] KASVI, PR, Brazil
[3] MMOptics, São Carlos, São Paulo, Brazil
[4] Genie 2, Scientific Industries, New York, USA
[5] TE - 392 / I, Tecnal, Piracicaba, São Paulo, Brazil

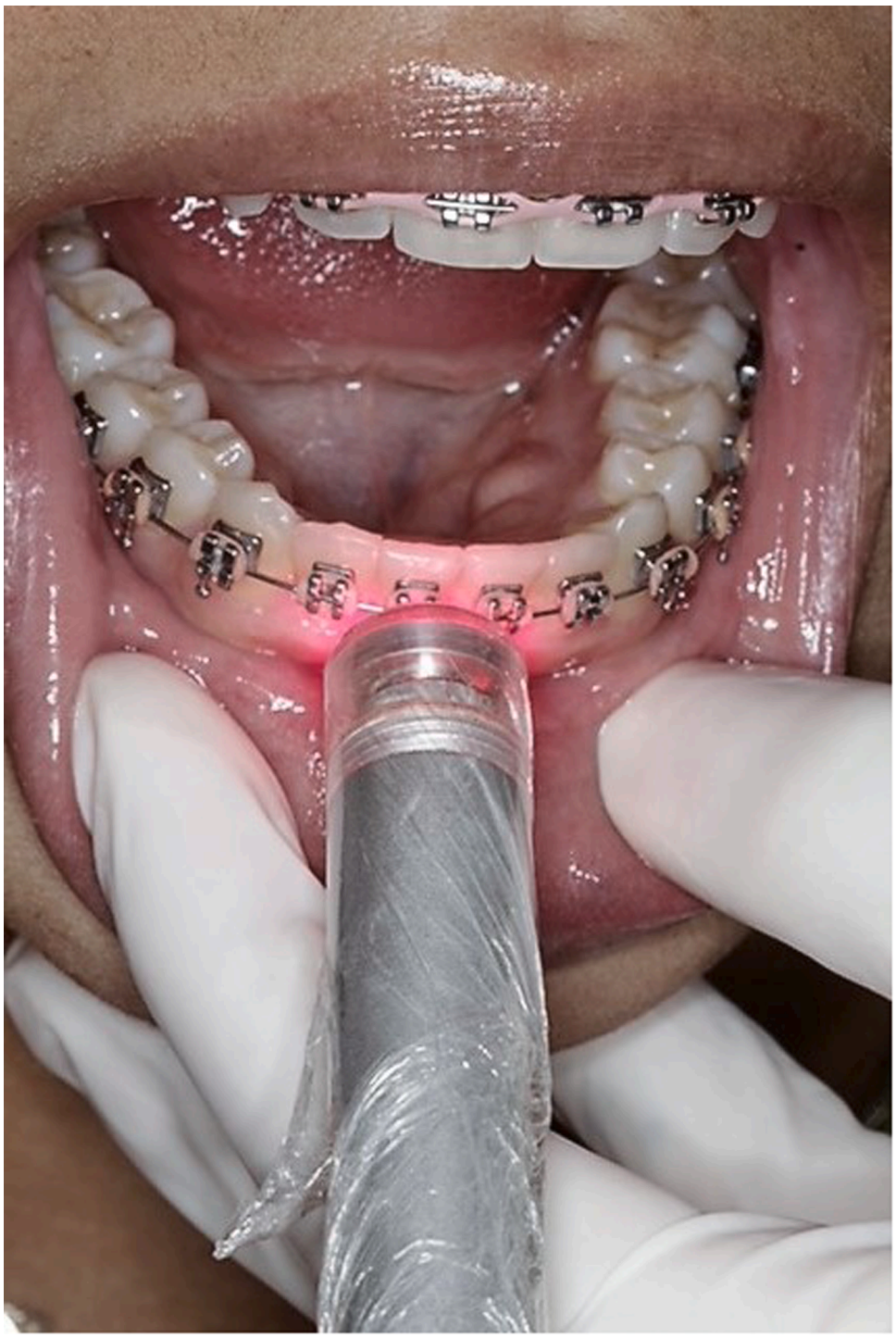

**Fig. 1.** Irradiation protocol used.

Data from the four groups (Control, Photosensitizer, AmPDT1 and AmPDT2) was analyzed. The difference between the groups was conducted by ANOVA. It was observed a significant difference between all groups ($p = 0.000$). After identifying the difference between the groups, an adjusted linear regression analysis was carried out. No significant difference between Control and Photosensitizer ($p = 0.052$) and AmPDT1 and AmPDT2 ($p = 0.10$) groups were observed. Significant differences were observed between Control and AmPDT1 and Control and AmPDT2 ($p<0.0001$) groups. Significant differences were also observed between Photosensitizer and AmPDT1 groups ($p = 0.0001$) and Photosensitizer and AmPDT2 ($p<0.0001$) groups. Fig 2

## 4. Discussion

The appearance of resistant multidrug strains prompted the search for more efficient protocols for the replacement of the routine use of antibiotics whose efficacy is diminishing due to the development of resistance [14,15]. Nevertheless, it is important to contemplate the complexity for the treatment of multispecies biofilm-forming consortia, in vivo, as conventional antimicrobial drugs usually have a single target and mode of action. It is known that several microbes can create ongoing resistance mechanisms when a unique attack point is present [1,3,16].

AmPDT has been proven to be both worthwhile and efficient in many

**Table 2**
Log CFU count for each patient in the biofilm collection.

| Patient | Control | Photosensitizer | AmPDT1 | AmPDT2 |
|---|---|---|---|---|
| 1 | 5.30103 | 5.403692 | 5.113943 | 4.213075 |
| 2 | 4.849215 | 4.01424 | 3.892095 | 3.156347 |
| 3 | 6.403692 | 6.09108 | 4.920819 | 4.823909 |
| 4 | 5.623249 | 5.293731 | 4.823909 | 4.477121 |
| 5 | 6.028029 | 4.778151 | 4.70757 | 3.425969 |
| 6 | 4.477121 | 4.750765 | 4.50515 | 3.477121 |
| 7 | 5.955848 | 5.544068 | 4.221849 | 2.753328 |
| 8 | 5.932812 | 4.906694 | 4.329059 | 3.778151 |
| 9 | 4.701856 | 4.735066 | 2.60206 | 1.124939 |
| 10 | 5.797037 | 5.48192 | 4.951014 | 4.591065 |
| 11 | 5.609239 | 5.293731 | 4.847161 | 4.564271 |
| 12 | 6.028029 | 4.778151 | 4.70757 | 3.425969 |
| 13 | 4.937852 | 4.425969 | 4.238882 | 3.954243 |
| 14 | 5.113943 | 4.884607 | 3.60206 | 2.367977 |
| 15 | 5.937852 | 5.636822 | 5.477121 | 4.920819 |
| 16 | 5 | 4.970037 | 3.221849 | 2.823909 |
| 17 | 5.965359 | 5.50515 | 5.041393 | 4.867271 |
| 18 | 5.31527 | 4.60206 | 3.985277 | 3.367977 |
| 19 | 5.801632 | 5.041393 | 3.69897 | 3.778151 |
| 20 | 6.286307 | 5.367977 | 5.146128 | 4.69897 |
| 21 | 5.726999 | 5.425969 | 5.367977 | 4 |

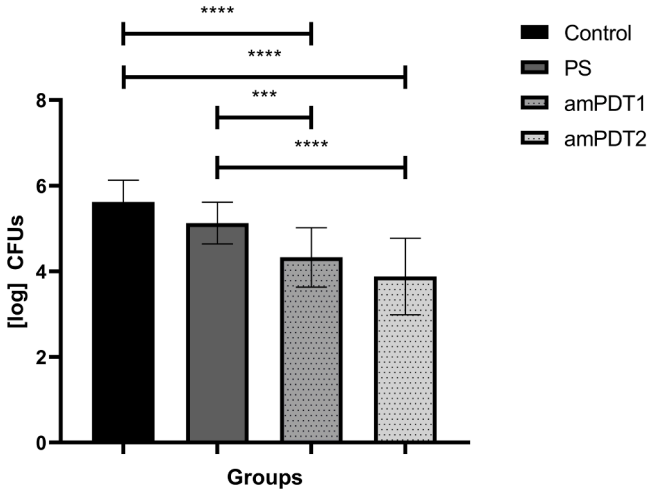


**Fig. 2.** Results of the ANOVA and Tukey analyzes between Control, Photosensitizer, AmPDT1, and AmPDT2 groups. Comparison of the exponential reduction of the microbial load after the addition of the photosensitizer and the treatment with AmPDT1 and AmPDT2. *** = $p = 0.001$ **** = $p < 0.0001$.

clinical conditions of problematical resolution [9,10,17]. It is well established that the photodynamic effect induces damage to biomolecules causing the loss of their biological functionality and consequent inactivation. Briefly, PSs act either by singlet oxygen or ROS production, both without specific targets, and capable of reacting quickly with different substratum [18,19].

Phenothiazines are hydrophilic photosensitive compounds that can form different ROSs and possess an efficacious germicide action against a varied spectrum of both $Gram^+$ and $Gram^-$ microbes [10]. Briefly, intracellular ROSs are produced by means of constant single-electron reduction and large concentrations of ROSs is known as oxidative stress, which antimicrobial effects are rapidly stimulated by their interaction with the thiol groups in proteins, DNA, and cell membranes through a dose-dependent effect [10]. ROS are categorized into those produced by Type I ($OH^•$) and those produced by the Type II photochemical ($^1O_2$) reactions which can kill microorganisms. Both reactions can occur at the same time, and the ratio between these processes varies according to the type of PS employed as well as on the microenvironment of the PS molecule [10,20]

The choice of the DMBB was due its phototoxicity, high lipophilicity and the ability to generate singlet oxygen. DMBB molecules intercalate with DNA and partition into membrane lipid causing photodamage. it could either affect the membrane and/or pass through it to damage intracellular structures. Under physiological conditions, DMMB tends to exist in a cationic form, and this positively charged nature would favor membrane targeting and binding whilst its structural amphiphilicity favors both membrane partitioning and accumulation [17].

A previous study suggested that Gram-positive bacteria are more sensitive to $^1O_2$ and Gram-negative are more susceptible to $HO^•$ [21] and, as we worked with a consortium in which both types were present, the use of the DMMB was justified as this PS has been shown effective to kill different strains of microorganisms [10,12,13,17]. As this is the first clinical report using the present protocol the employed concentration of the DMBB was based upon in vitro studies carried out by our team [12,13]. The originality of the present clinical essay is the use of the DMBB in microbial consortium in oral biofilm in vivo. As far as we are concerned there are no earlier reports on the literature using this protocol in vivo.

For the photodynamic effect to be efficacious, the selection of the PS stands of utmost significance. Consequently, the PS should be a kinetic and thermodynamically stable molecule of rapid synthesis, high yield, small price, good pharmacokinetics, and low toxicity in the dark, and do not kill cells with no activation. Satisfactory irradiation protocol and concentration of the PS in the target cells are important tasks for the effectiveness of the AmPDT as there is a need for adjusting them to accomplish results such as those observed in vitro [5,6,8,22,23].

A pre-irradiation period is needed to allow time for the PS interact with the biofilm allowing its dissemination and absorption by the microorganisms. Despite longer pre-irradiation time could cause a more effective interaction, so far, no ideal time has been set for either in vitro or in vivo protocols. Lengthier pre-irradiation period is not clinically practical because of the complexity of keeping the substance in the oral cavity [9,10,24,25]. Several previous reports using AmPDT in oral biofilms may be found in the literature [8–10,19,26,19,27]. Most clinical reports on orthodontic patients using Curcumin or MB alone as PSs failed to inform the pre-irradiation times [25,28,29]. However, a previous report from our team successfully used a pre-irradiation time of five minutes [10].

It is well accepted that, both the light source and wavelength, are important for the efficacy of the AmPDT. Usually, the wavelength used is in the visible and near-infrared spectrum (λ600–900 nm) [6,24,25,28,29]. Wavelengths above λ850 nm, because of their small energetic levels, decreases the effectiveness of the PS. The wavelength ought to be nearest to the absorption peak of the PS [4,30]. On the current investigation a red LED device emitting λ640 ± 5 nm was used [10]. Using inappropriate wavelengths will decrease or even do not photoactivate the PS hence making the procedure unsuccessful.

One may question the sample size of the present study. However, it was determined assuming a level of significance of 95%, 90% of power, with an estimated difference of 40% between the groups. The minimum sample size was defined in 19 individuals. An increase of 10% was assumed with the aim of homogenizing the groups.

The results of the present study evidenced significant difference between all groups. No significant difference between Control and Photosensitizer groups was observed. Significant differences were found between Control and AmTFD1 and AmTFD2 groups. Important to notice that comparable results were also observed when log transformation was not carried out (data not shown). These results are well aligned to our previous study by our group [10] in which the effectiveness of a similar protocol also carried out in orthodontic patients in which AmPDT was successful in significantly diminishing the number of microorganisms as seen on the CFU counts. CFU counting is advantageous in observing living cells as other techniques do not make a distinction between unviable and viable ones [10]. The ratio of reduction seen previously using just one AmPDT (Methylene Blue + Toluidine Blue) was ~90%. Important to observe that reductions on counting previously

observed in studies using phenothiazine byproducts were not superior to 70% [6–8,18,31]. The use of the DMBB in patients undertaking orthodontic treatment was never described.

The irradiation protocol used in this investigation was established according to a previous in vivo study using red LED combined with phenothiazine by-products and energy density of 30 J/cm$^2$ [10]. Irradiation protocol is an important constraint in clinical trials as large variations on these protocols are found studies using other PSs [24,25,28,29,32,]

Gram$^+$ and Gram$^-$ bacteria are different on regards to the sensitivity to AmPDT [8,9] the characterization of the bacteria observed in the samples was carried out by Gram staining (data not shown) being both types detected. The results of the present investigation demonstrated that, the use of the photosensitizer at low-concentration (700 ng/ml), was effective on killing most microorganisms after activation by LED light.

A significant decrease in microbial counting was found after AmPDT1 and AmPDT2 in comparison to the initial load of microorganisms detected. Comparing the present results with the ones described by Soares et al. [10] who found a reduction of ~90% after a single aPDT, using a bluish phenothiazinic solution, we found the same percentual of reduction when the DMBB was used, with the advantage of using a colorless solution that is unable to stain the teeth.

On the other hand, the sole use of the PS did not produce significant cell death [8–10]. These findings are in agreement with results observed in a previous study [32] which demonstrated that AmPDT causes morphological changes in the biofilm formed by both Gram$^+$ and Gram$^-$ bacteria causing larger decrease in the number of microorganisms.

Important to observe that the proposed protocol is incapable of causing microbial resistance, consequently, it can be repeated safely in the course of the clinical care, enhancing the usefulness of treatment [24,32]. The use of antibiotics may be associated with AmPDT, as AmPDT is capable to cause the disorganization of biofilms that impairs a few processes of resistance making the persisting microorganisms more sensitive to antimicrobials [5,7,27,32]

There are a few studies using AmPDT in orthodontics, as can be seen in a previously published systematic review [29], this reduced number of reports makes tricky to demonstrate the effectiveness of AmPDT in controlling the oral microbiota of these patients. Nevertheless, it is possible that the proposed procedure combined with preservation of oral hygiene and routine dental checkups, is a trustworthy procedure to diminish the possibility of developing gingival illnesses in these patients [10].

It is known that inadequate oral hygiene is a major reason for biofilm formation and many patients, including the orthodontics ones, as they may have difficulty in doing oral cleaning in an effective way and may demand professional cleaning. However, besides being effective, it will increase the cost of the treatment. Other aspect to be considered is that most bacteria have a quick reproduction time and so far, there is no effective method clinically usable that change this. The results of the present protocol demonstrated a reduction on microbial count, that is clinically important. The practical benefit from this work consists in using a technique that is easy to be carried out, require small time to be performed, it is low-cost, and presents no reported side-effect.

Lastly, it is important the constant pursuit for more efficient AmPDT protocols to get even higher ratios of microbial killing in oral biofilms, preventing microbial resistance and the manifestation of infections in patients undertaking dental treatment.

## Conclusion

It was concluded that double AmPDT using DMBB in nano concentration and red LED was capable to meaningfully decrease the number of CFUs in orthodontic patients.

## Declarations

*Code availability*

Not applicable

*Compliance with ethical standards*

The authors of the present investigation state that they have no competing conflict of interest to declare. Author (ALBP) received Productivity Fellowship from the Brazilian National Council for Scientific and Technological Development (CNPq) (No.304279/2018-8). The author (LGPS) received a research grant from the Brazilian National Council for Scientific and Technological Development (CNPq) (No.427767/2018-0). The author (PFA) received a research grant from Brazilian National Council for Scientific and Technological Development (CNPq) (No.302753/2020-6). The author (PJLG) received a PhD grant from the Bahia State Research Support Foundation) FAPESB (BOL0777/2016), and authors (IPFN and APLTS) received PhD grants from the Bahia State Research Support Foundation – FAPESB (BOL0220/2020 and BOL0667/2021). Author (JAA) received an undergraduate researcher grant from the Brazilian National Council for Scientific and Technological Development (CNPq) (No.118923/2022-6)

## Ethics approval

This cross-over clinical experimental study was approved by the Ethics Committee of the Faculty of Dentistry of the Federal University of Bahia (No. 2857839).

## Consent to participate

According to Brazilian regulations all participant who agreed to entry the study signed a written informed consent.

## Consent for publication

Not Applicable

## CRediT authorship contribution statement

**Luiz G.P. Soares:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Cristiane Galdino de Almeida:** Investigation. **Pedro J.L. Crugeira:** Investigation, Conceptualization. **Iago P.F. Nunes:** Investigation. **Anna Paula L.T. da Silva:** Investigation. **Jeovana A. Almeida:** Investigation. **Maria C.T. Cangussú:** Writing – review & editing, Writing – original draft, Formal analysis. **Paulo F. de Almeida:** Methodology, Formal analysis, Conceptualization. **Fernando A.L. Habib:** Writing – original draft, Methodology, Investigation, Conceptualization. **Antônio L.B. Pinheiro:** .

## Declaration of Competing Interest

None

## Availability of data and material

Not Applicable

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