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In Vitro Comparative Evaluation of the Efficacy of Antimicrobial Photodynamic Therapy, Chlorhexidine, Sodium Fluoride, and Hydrogen Peroxide for Acrylic Resin Disinfection

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Abstract

Introduction: Plaque accumulation on the surface of removable orthodontic appliances could lead to dental caries, periodontitis, and fungal infections. This study evaluated the effectiveness of antimicrobial photodynamic therapy (aPDT), chlorhexidine (CHX), sodium fluoride (NaF), and hydrogen peroxide (H_2O_2) for the disinfection of acrylic resin.

Methods: In this in vitro experimental study, 100 acrylic resin specimens were randomly divided into five groups (n = 20 each): *Enterococcus faecalis*, *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus sanguinis*, and *Lactobacillus acidophilus*. Each group was immersed separately in 5 mL of microbial suspension. They were then incubated until biofilm formation on their surface. Of each microorganism, one biofilm sample in phosphate-buffered saline was considered as negative control, and other biofilm samples (n = 80) were subjected to aPDT with curcumin, 0.12% CHX (positive control), 1% H_2O_2, and 0.2% NaF. Finally, the number of colonies was counted. Data were analyzed by the Kruskal-Wallis and Mann-Whitney tests, two-way ANOVA, and Bonferroni adjustment at a significance level of 0.05.

Results: The interaction effect of the treatment modality and type of microorganism was significant on the microbial count (effect size: 0.91, $P < 0.05$). Maximum bacterial proliferation was noted in the following combinations: NaF/*E. faecalis*, H_2O_2/*E. faecalis*, and H_2O_2/*S. salivarius*. Microorganisms had no or insignificant growth and proliferation in the aPDT and CHX groups.

Conclusion: The results supported the optimal antimicrobial efficacy of PDT which was comparable to that of CHX. aPDT showed superior antimicrobial efficacy to NaF and H_2O_2 for the disinfection of acrylic resin.

Keywords: Antimicrobial photodynamic therapy; *Enterococcus faecalis; Lactobacillus acidophilus; Streptococcus mutans; Streptococcus salivarius; Streptococcus sanguinis.*

Introduction

Removable orthodontic appliances are extensively used in clinical orthodontics aiming to correct malocclusions or maintain the treatment results.[1] However, the presence of a removable orthodontic appliance changes the equilibrium of the microbial ecosystem of the oral cavity.[2] Shortly after using an orthodontic appliance, oral microorganisms colonize the surface of the appliance and adhere to its different components, especially its acrylic plate, since it has tiny porosities on the internal and external surfaces that provide a suitable environment for the accumulation of microorganisms.[3] Oral diseases and infections caused by these bacteria can affect the teeth, oral mucosa, and periodontal tissues including the cementum, periodontal ligament, and supporting bone.[4] Therefore, the disinfection of removable appliances is highly important to decrease plaque accumulation on their surface and minimize the risk of dental caries, periodontitis, and fungal infections as such.[1]

Correct toothbrushing with fluoridated toothpaste is an effective method for controlling biofilms formed on the surface of removable orthodontic appliances. However,

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poor hand skills and low quality of homecare are among the factors that compromise the patients' ability to effectively control mechanical biofilms.[3] Moreover, toothbrushing cannot efficiently clean the tiny pores on the surface of acrylic plates.[5] Chemical agents are also extensively used for the disinfection of acrylic surfaces. However, they have limitations such as their limited penetration into the acrylic surface and the formed biofilm. Also, some bacterial species are genetically resistant to chemical cleaning agents.[6]

Antimicrobial photodynamic therapy (aPDT) refers to treatments based on light dynamics.[7] It is defined as the inactivation of cells, microorganisms, or molecules mediated by light.[8] It is also known as aPDT and photodynamic disinfection.[9] The eradication of pathogenic microorganisms by this method is referred to as photodynamic inactivation[10] or lethal photosensitization.[11] In dentistry, it is also referred to as photo-activated disinfection and light-activated disinfection.[12] The history of aPDT dates back to ancient Greece, Egypt, and India.[13] Despite its introduction in 1900,[9] the application of aPDT was shadowed by the advent of antibiotics.[14] PDT was first introduced as an alternative treatment for cancer. PDT is an alternative to conventional cancer treatments including surgery, radiotherapy, and chemotherapy because it has no serious side effects and can be used repeatedly. It can be utilized to destroy viable tissue with abnormal development because it destroys cells through necrosis or apoptosis.[13,15] Thus, bacterial, fungal, and viral infections can be treated by aPDT since they share one common characteristic, that is, uncontrolled cell proliferation and the presence of pathogenic microorganisms.[13]

PDT requires three main components of photosensitizer (PS), light, and oxygen.[16] The presence of a non-toxic PS activated by the proper wavelength of oxygen-containing visible light is the basis for the mechanism of action of aPDT.[13,17] The type of PS, its concentration, and the type of microorganism (gram-positive or gram-negative bacteria, fungi, or viruses) all affect how effective aPDT is against certain pathogens.[13] aPDT is more successful in the deactivation of Gram-positive bacteria because the external part of their cell wall is significantly more porous and allows the PS to reach the cytoplasmic membrane.[18] The morphology of gram-negative bacteria is significantly more complex. The negatively charged lipopolysaccharides, lipoproteins, and purine proteins in their cell wall serve as a physical barrier and prevent the penetration of the PS.[19] It is noteworthy that the photodynamic activity of the PS decreases for microorganisms in the form of biofilm.[20] aPDT has demonstrated beneficial results on periodontal pathogens such as *Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Prevotella intermedia*, *Capnocytophaga gingivalis, Porphyromonas gingivalis,* and *Streptococcus sanguinis*. In addition, aPDT has demonstrated optimal results on *Actinomyces israelii* and *Prevotella intermedia* in endodontics.[21,22] aPDT has other advantages such as optimal safety, non-invasiveness, and not generating resistant species. It can be repeated several times with no cumulative toxic effects; thus, it is also suitable for use in the elderly and medically compromised patients.[13,23]

Several studies have reported the utilization of aPDT for inhibition and microbial control in treatment and decontamination in different surfaces such as the oral cavity.[24-29] Considering the few numbers of studies on the efficacy of aPDT in comparison with different mouthwashes for the disinfection of orthodontic appliances, this study evaluated the efficacy of aPDT, chlorhexidine (CHX), sodium fluoride (NaF), and hydrogen peroxide (H_2O_2) for the disinfection of acrylic resin.

Materials and Methods

In this in vitro experimental study, 100 acrylic resin specimens were used (n = 20 per each of the five types of microorganisms).

Preparation of Acrylic Specimens

A total of 100 acrylic specimens were fabricated by mixing the polymer and monomer as instructed by the manufacturer (Marlic, Tehran, Iran). The specimens had a 10 mm diameter and a 2 mm height. After finishing, they were cleaned with water and soap and stored in distilled water for 3 days to ensure no residual monomer was on their surface. The discs were then autoclave-sterilized at 121°C for 60 minutes.[16,30]

Microbial Culture

Streptococcus mutans (PTCC 1683), *Streptococcus salivarius* (PTCC 1448), *Streptococcus sanguinis* (PTCC 1449), *Lactobacillus acidophilus* (PTCC 1643), and *Enterococcus faecalis* (PTCC 1858) were obtained in lyophilized vials. Under sterile conditions, lyophilized bacterial vials were diluted with 1 to 2 mL of brain heart infusion broth and then cultured. To prepare a subculture of *S. mutans*, *S. salivarius*, *S. sanguinis*, and *E. faecalis*, a swab dipped in microbial suspension was used for bacterial culture on a Petri dish containing sheep blood agar. *L. acidophilus* was cultured on Mueller Hinton agar and incubated under vacuum (anaerobic condition). Other Petri dishes were incubated at 37 °C in the presence of 10% CO_2 for 48 hours. The colonies were collected by a sterile inoculating loop and dissolved in brain heart infusion broth in microtubes. Next, they were incubated at 35-37 °C for 1-2 hours. To determine the concentration of each microorganism in sterile tubes, their optical density was read at a 600-nm wavelength to prepare 0.5 McFarland standard concentration containing 1.5×10^8 colony forming units per milliliter (CFUs/mL).[7,23]

Biofilm Formation on Acrylic Surfaces

Acrylic specimens were randomly assigned to five groups and were separately immersed in 5 mL of each microbial suspension. They were then incubated at 37 °C for 24 hours in a shaker incubator operating at 80 rpm until biofilm formation on their surface.[23]

Antimicrobial Photodynamic Therapy

Curcumin (Merck, Darmstadt, Germany) was used as the PS. For this purpose, curcumin salt was diluted with 10% dimethyl sulfoxide to prepare 800 µM/L solution. Right before use, this solution was mixed with saline (0.85% NaCl) to reach a final concentration of 40 µM/L. The specimens were then placed in the dark in 2 mL of curcumin solution for 5 minutes, and they were then light-cured with an LED curing unit (Woodpecker, Guangxi, China) at a 420-480 nm wavelength with a light intensity of 1000 ± 100 mW/cm^2 for one minute.[30]

Study Groups

Phosphate buffered saline (PBS) was used as the negative control of the study, and 0.12% CHX was used as the positive control of the study. Other experimental groups included 0.2% sodium fluoride (NaF) mouthwash, 1% hydrogen peroxide (H_2O_2), and aPDT.[31]

The abovementioned solutions were added to Petri dishes containing acrylic specimens in such a way that both surfaces of the acrylic specimens were immersed in the solution. The acrylic specimens remained in the solutions for 10 minutes in all groups.[32]

Colony Counting

All solutions were removed and acrylic specimens were rinsed with PBS to detach the loosely attached bacteria. Next, acrylic specimens were separately placed in Falcon tubes containing 1 mL of PBS and underwent sonication for 10 minutes to detach the adhered biofilm. Biofilm-containing suspensions were homogenized, and 20 µL of each sample was diluted with 80 µL of PBS to obtain 10^{-2} dilution. Next, 20 µL of the diluted solution was cultured on brain heart infusion broth agar, followed by incubation in a candle jar for 24 hours at 37 °C in the presence of 10% CO_2. It should be noted that the *L. acidophilus* samples were cultured on Mueller Hinton agar and were then incubated at 37 °C under vacuum for 24 hours.[30,31,33] Finally, the colonies were visually counted. The following equation was used to compute the colony count (CFUs/mL), and the result was reported as log10.

$$\frac{\text{colony count} \times \text{dilution factor}}{\text{volume plated}}$$

Statistical Analysis

The number of microorganisms was reported as median (first - third quartiles). The Kruskal-Wallis test was used to compare the microbial count among different groups, followed by pairwise comparisons with the Mann-Whitney test and Bonferroni adjustment. Two-way ANOVA was applied to assess the effects of biofilm type and disinfection method on the microbial count. All statistical analyses were conducted using SPSS version 16 at a significance level of 0.05.

Results

In the assessment of the overall count of microorganisms based on the grown biofilms, the median (first-third quartile) of the microbial count in biofilms treated with PDT, CHX, NaF, and H_2O_2 was found to be 0 (0-0), 0 (0-0), 173 (85-375), and 200 (0-645), respectively.

Table 1 presents the measures of central dispersion for the mean microbial count in biofilms in different treatment groups. The results showed a significant difference in the microbial count between the treatment groups ($P<0.001$). Thus, pairwise comparisons were carried out regarding the microbial count, which revealed that the microbial count in the aPDT group was significantly lower than that in the NaF ($P<0.001$) and H_2O_2 ($P=0.004$) groups, and the mean bacterial count in the CHX group was significantly lower than that in the NaF ($P<0.001$) and H_2O_2 ($P=0.004$) groups. No other significant differences were noted ($P>0.05$).

With respect to the type of microorganisms, the median (first-third quartile) of the microbial count for *E. faecalis*, *L. acidophilus*, *S. mutans*, *S. salivarius*, and *S. sanguinis* was 620 (33-1950), 0 (0-0), 0 (0-112.5), 40 (0-412.5), and 50 (0-275), respectively. The results showed a significant difference in the microbial count of different microorganisms ($P<0.001$). Pairwise comparisons regarding the microbial count showed a significantly higher count of *E. faecalis* than *S. mutans* ($P<0.001$), *S. salivarius* ($P=0.015$), *S. sanguinis* ($P=0.015$), and *L. acidophilus* ($P<0.001$). Also, the microbial count of *L. acidophilus* was significantly lower than that of *S. salivarius* ($P=0.015$) and *S. sanguinis* ($P=0.015$). No other significant differences were noted ($P>0.05$).

Table 2 summarizes the median and the first and third quartiles of the microbial count in different combinations of treatment groups/microorganisms. Two-way ANOVA showed a significant effect of the type of treatment on the microbial count, irrespective of the type of microorganism (Figure 1, $P<0.001$). The effect size was considerable. The

Table 1. Measures of Central Dispersion for the Mean Microbial Count in Biofilm in Different Treatment Groups

Treatment	Microbial Count			
	Minimum	Maximum	Mean	Std. Deviation
PDT	0	40	7.35	15.13
CHX	0	33	5.9	12.19
NaF	0	2500	526.5	806.12
H_2O_2	0	2000	480.5	642.38

Table 2. Median (First-Third Quartiles) of Microbial Count in Different Combinations of Treatment Groups/Microorganisms

Treatment	Biofilm					Effect Size (*P* Value)		
	S. mutans	*E. faecalis*	*S. salivarius*	*S. sanguinis*	*L. acidophilus*	Treatment	Biofilm	Treatment and Biofilm
PDT	0 (0-0)	37 (33.5-39.75)	0 (0-0)	0 (0-0)	0 (0-0)	0.838 (<0.001)	0.909 (<0.001)	0.910 (<0.001)
CHX	0 (0-0)	32 (25.75-32.75)	0 (0-0)	0 (0-0)	0 (0-0)			
NaF	173 (154-190.5)	2100 (1625-2425)	105 (85-140)	300 (225-375)	0 (0-0)			
H_2O_2	0 (0-0)	1650 (1275-1950)	575 (512.5-645)	200 (125-275)	0 (0-0)			

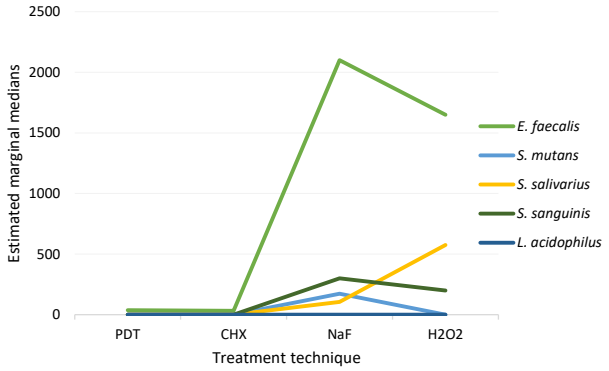

Figure 1. Effect of Different Treatments on the Microbial Count of Different Microorganisms

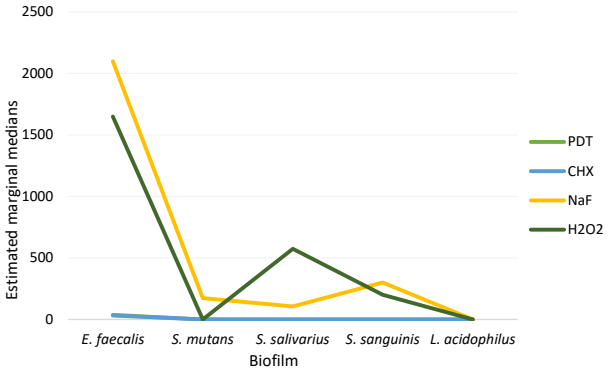

Figure 2. Effect of Microorganism Type on the Microbial Count in Different Treatment Groups

lowest microbial count was recorded in biofilms subjected to PDT and CHX, while the highest count was noted in biofilms treated with NaF and H_2O_2.

Furthermore, the results showed that the type of microorganism had a significant effect on the microbial count, irrespective of the treatment technique (Figure 2, $P<0.001$). The effect size was considerable. The lowest microbial count was observed for *L. acidophilus* and *S. mutans,* and the highest count was noted in *E. faecalis*, *S. sanguinis,* and *S. salivarius*.

The interaction effect of the treatment technique and the type of microorganism on the microbial count was also significant (Figure 3, $P<0.001$). The effect size was considerable (0.91). Maximum bacterial proliferation was noted in the following combinations: NaF/*E. faecalis*, H_2O_2/*E. faecalis*, and H_2O_2/*S. salivarius*. Microorganisms had no or insignificant growth and proliferation in the aPDT and CHX groups.

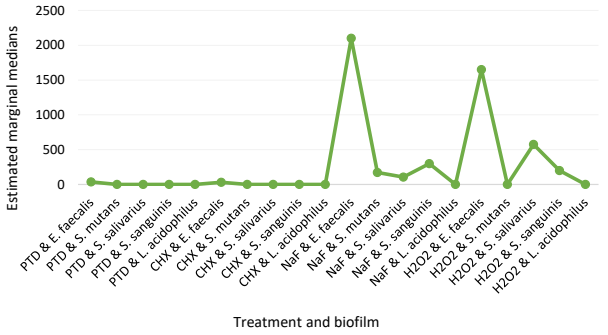

Figure 3. Interaction Effect of Microorganism Type and Treatment Type on the Microbial Count

Discussion

This study assessed the efficacy of antimicrobial aPDT, CHX, NaF, and H_2O_2 for the disinfection of removable orthodontic appliances. The results showed the lowest microbial count (irrespective of the type of microorganism) in the PDT and CHX groups, followed by NaF and H_2O_2. On the other hand, the lowest microbial count, irrespective of the type of treatment, was noted in *S. mutans* and *L. acidophilus,* followed by *S. salivarius*, *S. sanguis*, and *E. faecalis*. The results showed no significant difference in the microbial count between the aPDT and CHX groups. Lamarque et al[34] compared the effectiveness of aPDT with CHX and curcumin PS against *S. mutans* and *L. acidophilus* immediately and after 24 hours. They confirmed the optimal efficacy of CHX and the immediate efficacy of aPDT against these microorganisms. de Sousa Farias et al[31] showed that both 0.12% CHX and aPDT decreased the microbial load of *S. mutans*. The results regarding the optimal efficacy of PDT in the current study agreed with the abovementioned findings. Malhotra et al[35] compared the antimicrobial effects of 0.12% CHX, 0.2% NaF, and 10% propolis tincture mouth rinse in water (5:1 ratio) on *S. mutans*, *L. acidophilus*, and *Candida albicans*. They reported that NaF had the smallest effect on *S. mutans*, but its efficacy was superior to that of the propolis mouth rinse against *L. acidophilus* and *C. albicans*.

In the current study, the median *S. mutans* count in the NaF group was 173; this value was 0 for *L. acidophilus*. These outcomes and those of Malhotra et al were in agreement.[35] Silhacek and Taake[36] evaluated the effects

of H_2O_2 on *S. mutans* and showed that H_2O_2 inhibited *S. mutans*. In the present study, H_2O_2 had the smallest effect on *E. faecalis,* followed by *S. salivarius and S. sanguis*, and the greatest effect on *S. mutans* and *L. acidophilus*. The present findings were in line with those of Silhacek and Taake.[36]

In the current study, the significant effect of the type of treatment, type of microorganism, and their interaction on the microbial count was confirmed. The present results showed the comparable antimicrobial efficacy of aPDT and CHX against all the tested microorganisms including *E. faecalis*. *E. faecalis* is a major culprit in primary and refractory endodontic infections.[37,38] It is a Gram-positive facultative anaerobic microorganism and a part of oral microflora. It is responsible for many cases of primary endodontic infections.[37,39] Rocha et al[40] evaluated the effect of aPDT with blue diode light along with curcumin on *E. faecalis* biofilm and showed a significant reduction of this microorganism as such. Shahmoradi et al[41] assessed the effect of selenium nanoparticles and aPDT with methylene blue (MB) on *E. faecalis* biofilm. They showed that this combination effectively eliminated *E. faecalis* from the root canal system. Yamamoto et al[42] evaluated the efficacy of aPDT with MB, curcumin, and indocyanine green with a laser for the reduction of *E. faecalis*. They found that aPDT was the most effective method. Mustafa et al[43] assessed the efficacy of aPDT as an adjunct to mechanical instrumentation for the elimination of *E. faecalis* from C-shaped canals and showed its highly favorable efficacy for this purpose.

The main culprit in dental caries development is *S. mutans*. It has high adhesion to the tooth structure and causes demineralization and a pH drop on the tooth surface by acid production [44]. The obtained results regarding the effects of aPDT on *S. mutans* in the current investigation were consistent with the findings of Quishida et al[30] and Pereira et al[45] who showed the significant efficacy of PDT for the reduction of *S. mutans* load. In contrast to the present findings, de Freitas-Pontes et al[23] showed that aPDT with MB and a 630-nm LED with 150 mW power only caused a slight decrease in *S. mutans* count. Terra-Garcia et al[46] assessed the efficacy of aPDT with Fotoenticine PS against several *S. mutans* clinical strains and showed that it was capable of eliminating microorganisms adhering to the enamel. They also showed its optimal disinfecting efficacy. Hwang et al[47] evaluated the effectiveness of aPDT with *Chlorella* and *Curcuma* extracts against *S. mutans*. They showed a reduction in the viable cell count after 24 hours and concluded that PDT with a 405 nm wavelength of light and Chlorella and curcuma PSs can significantly decrease *S. mutans* count. Mocuta Bojoga et al[48] evaluated the efficacy of MB and a mixture of chlorophyll-phycocyanin with and without aPDT in comparison with 2% CHX against *S. mutans*. They found that aPDT with MB and a mixture of chlorophyll-phycocyanin significantly decreased the bacterial colony count. Benine-Warlet et al[49] evaluated the effect of the addition of potassium iodide to aPDT with a red laser (660 nm) and MB on *S. mutans* biofilm. They found that the addition of potassium iodide to aPDT with MB and a red laser had a synergistic effect on the elimination of *S. mutans*. Pourhajibagher et al[50] assessed the antimicrobial, antiviral and mechanical characteristics of the orthodontic acrylic resin containing various concentrations of *Ulva lactuca* following aPDT against *S. mutans*. They found that the addition of 1% *Ulva lactuca* to orthodontic resin and the simultaneous use of aPDT increased antibacterial and anti-biofilm properties and led to the elimination of this microorganism. However, de Freitas-Pontes et al[23] found that aPDT with MB and an LED at a 63-nm wavelength and 150 mW power slightly decreased *S. mutans* count.

L. acidophilus is among the microorganisms involved in dental caries. It can be abundantly found in active carious lesions.[44,51] Azizi et al[51] compared the efficacy of aPDT with indocyanine green and MB against *L. acidophilus* and reported that aPDT with MB alone or along with 660-nm laser irradiation significantly inhibited the growth and proliferation of *L. acidophilus*. Also, Araújo et al[52] evaluated the sensitivity of *S. mutans* and *L. acidophilus* in the biofilm phase versus dentin caries to aPDT with curcumin as a PS and 450-nm blue light. They concluded that aPDT with curcumin and blue light had significant photo-toxic effects on both *S. mutans* and *L. acidophilus* biofilms. However, bacteria in carious dentin were more resistant to aPDT, and the phototoxic effects on the bacteria in carious dentin depended on the concentration of curcumin.

S. sanguinis is an initiator of microbial plaque formation. It adheres to the surface of the teeth, enhancing the attachment of other bacteria and the formation of biofilms.[53] de Paula et al[54] evaluated the effect of aPDT with MB and beta-cyclodextrin particles and red laser light on a number of microorganisms in oral biofilm, including *S. sanguinis,* and showed that it effectively decreased the count of this microorganism in the primary oral biofilm.

Also, *S. salivarius* is among the first bacteria that colonize the oral cavity.[55] Khatibi et al[56] tested the antimicrobial properties of aPDT with indocyanine green against *S. salivarius*. They reported that aPDT with a laser and indocyanine green had comparable antimicrobial efficacy to an 810-nm diode laser and CHX. They concluded that aPDT can decrease the microbial count. However, in the present study, aPDT completely inhibited bacterial growth and yielded a median count of zero.

This study had some limitations. The biofilm created in vitro has a different composition than the biofilm formed in the oral environment, which limits the generalizability of the results to the clinical circumstances. Additional clinical research is necessary to obtain more

dependable results.

Conclusion

The results of this study showed the lowest microbial count in the PDT and CHX groups, followed by NaF and H_2O_2, irrespective of the type of microorganism. On the other hand, the lowest microbial count, irrespective of the type of treatment, was noted in *S. mutans* and *L. acidophilus*, followed by *S. salivarius*, *S. sanguis*, and *E. faecalis*. The results supported the optimal antimicrobial efficacy of aPDT which was comparable to that of CHX. aPDT showed significantly superior antimicrobial efficacy to NaF and H_2O_2 for the disinfection of removable orthodontic appliances.

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Writing–review & editing: Farhad Sobouti, Sepideh Dadgar, Mohadeseh Heidari.

Competing Interests

The authors declare no conflict of interest.

Ethical Approval

All investigational procedures used in this study were reviewed and approved by the Research Ethics Committee at Mazandaran University of Medical Sciences (ethical approval code: IR.MAZUMS.REC.1400.11804).

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