ORIGINAL ARTICLE

The Efficacy of Photodynamic Therapy against Streptococcus mutans Biofilm on Orthodontic Brackets: An In-vitro Study

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ABSTRACT

Background and Objective. Orthodontic brackets predispose dental biofilm accumulation causing caries and gingivitis. Chlorhexidine is an adjunct to mechanical plaque removal, but has side-effects (tooth staining, bacterial resistance) due to long term use. This study tested the efficacy of Photodynamic Therapy, which produces reactive oxygen species, to reduce *Streptococcus mutans* in dental biofilm on orthodontic brackets.

Methods. A 5-day *S. mutans* biofilm was grown on forty enamel-bracket specimens. Thirty-nine specimens were randomized to three treatment groups: A. Distilled Water; B. 0.12% Chlorhexidine (CHX); C. Photodynamic Therapy (PDT) using Toluidine Blue O (TBO) as a photosensitizer, activated by red LED (630nm). After treatment, one random specimen from each group was viewed under Environmental Scanning Electron Microscopy (ESEM); the other 12 specimens, biofilms were collected, weighed, and cultured onto BHI agar plates to determine the number of CFU/mg. For baseline evaluation, one clean and one untreated specimens were preserved for ESEM.

Results. Based on Tukey HSD test, group A had the most *S. mutans* (37.0573 CFU/mg) and was significantly different ($p < 0.05$) from groups B (0.1712 CFU/mg) and C (1.1193 CFU/mg), where both showed less bacteria than group A. The statistical difference between groups B and C was insignificant. ESEM images showed specimen A covered with more abundant and denser *S. mutans* biofilm than specimens B and C, with almost similar morphology showing sparse, less dense, and disintegrated biofilm with unclear cellular walls and presence of amorphous masses.

Conclusion. Both Photodynamic Therapy and 0.12% Chlorhexidine showed a significant reduction of *S. mutans* in dental biofilm on orthodontic brackets. However, there is no significant difference between them in reducing *S. mutans* CFU/mg. Photodynamic therapy could be an alternative adjunctive tool to mechanical removal of plaque adhered to orthodontic brackets.

Keywords: photodynamic therapy, scanning electron microscopy, biofilm, orthodontic brackets, chlorhexidine

* *Dr. Barrameda and Dr. Karganilla shared first authorship for this manuscript.*

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INTRODUCTION

Orthodontic brackets on teeth provide artificial niches for dental plaque, a biofilm, which is an essential precursor to caries and periodontal disease.[1,2] *Streptococcus mutans (S. mutans),* a Gram-positive facultative anaerobe, is considered the main etiologic agent.[3,4]

Brackets surface roughness protects from shear forces, but will rapidly progress to supragingival plaque build-up and the risk for enamel decalcification.[5-10] *S. mutans* levels were seen higher in orthodontic children with White Spot Lesions (WSL), an early and reversible stage of caries, and with gingivitis than in those without.[11] WSL at the bracket peripheries are frequently permanent and can progress to cavitation if left untreated.[9,11] Plaque also accumulates more on the excess composite around the bracket and the distinct 10μm gap at the enamel-composite interface, an effect of the inherent resin polymerization shrinkage and the difference in coefficient of thermal expansion between composite and tooth.[12]

Primarily, dental plaque is removed mechanically by tooth brushing, flossing, the use of interdental brushes, and professional scaling and polishing. With increased caries rate, chemotherapeutics can augment mechanical removal and control biofilm adherence.[1,13,14] Chlorhexidine (CHX) is bacteriostatic at low concentrations and bactericidal at high concentrations. It inhibits the glycosyltransferase enzyme, which promotes biofilm accumulation on teeth.[15] A 30 second-rinse of CHX twice a day, morning and evening after brushing, is sufficient for an 18mg optimum dose of CHX (15 ml) in a 0.12% solution to be effective.[16,17] However, it has disadvantages: taste alteration, staining of teeth and restorations, burning sensation, and bacterial resistance with indiscriminate use.[14,18,19] CHX varnish showed no influence on the caries increment of high-risk orthodontic patients, with a high rate of *S. mutan*s recolonization showing short-term bactericidal effect.[1,20,21]

Photodynamic therapy (PDT) is a promising method for the inactivation of bacteria and efficient intra-oral disinfection.[18] A photosensitizer, such as Toluidine Blue Ortho (TBO), is used to penetrate the bacteria's cytoplasmic membrane, which is then activated by light to produce reactive oxygen species (ROS), which are bactericidal but not toxic to host cells.[13] It is rapid, non-invasive and low cost with high therapeutic success.[18] TBO most effectively reduce *S. mutans* by 99.9%.[13] It can diffuse even in the hydrophobic region due to its low molecular weight (305.83 g/mol).[22] Its peak of maximum absorption is 640 nm, efficient in absorbing red light with predominant 636 nm wavelength, resulting in phototoxic effects against *S. mutans* in-vitro.[13] Photosensitization of in-vitro *Streptococcus mutans, Streptococcus sobrinus,* and *Streptococcus Sanguinis* biofilms using TBO and red Light Emitting Diode (LED) showed significant bacterial reduction by more than tenfold.[22,23] Confocal laser scanning microscopy revealed that majority of the photosensitization occurred in the outermost layers of the biofilms.[23] There was bacterial reduction within a layer of 10 μm in an artificial *S. mutans* biofilm model after PDT.[24]

Many of the oral bacteria do not express proteins, like catalase and superoxide dismutase that neutralize ROS.[13] PDT reduces the bacteria's glucose consumption, delaying its growth and causing death, thus reducing plaque deposition on teeth.[25] It efficiently inactivates antibiotic-sensitive/resistant strains, with low mutagenic potential of photoresistant microbial cells.[26,27] For patients' use at home, a toothpaste containing photosensitizer and a light-emitting toothbrush could be a promising tool in the future.[28]

This pilot *in-vitro* study aimed to determine the anti-microbial efficacy of PDT with TBO and red LED against *S. mutans* biofilm on conventional metal orthodontic brackets bonded to bovine enamel slabs. Specifically, it aimed: 1) To determine the number of CFU/mg of *S. mutans* after exposure of the enamel-bracket specimens to distilled water, 0.12% Chlorhexidine, and PDT, 2) To compare the number of CFU/mg of *S. mutans* after exposure of the enamel-bracket specimens to 0.12% Chlorhexidine (positive control), PDT, and distilled water (negative control), and 3) To describe the *S. mutans* biofilm morphology on the bracket surface and enamel-bracket interface, particularly the abundance of layers, cluster binding, pattern of formation and alterations after treatment with distilled water, 0.12% Chlorhexidine, and PDT, by examining under the Environmental Scanning Electron Microscope (ESEM) (Quanta 450 model, FEI).

As part of the limitation of the study, the performance of light-activated disinfection (LAD) depended on the technical specifications of FotoSan® 630 kit with LAD device used. Unlike Chlorhexidine (CHX), PDT today is professionally used and not commercially available for the patients. The study did not aim to provide a recommendation for home-application of PDT. It aimed at comparing the two concepts: chemical vs light-activated disinfection to know which has better antimicrobial efficacy.

Since it did not involve human participants, the study was exempted from ethical review by the Research Ethics Board in accordance with the guidelines set by the University of the Philippines Manila. There are no other ethical issues involved in the study.

MATERIALS AND METHODS

The sample size in the study has a power of 96.34.[29] Forty-one (41) recently extracted sound bovine mandibular incisors, with intact labial surface, no cavities, fractures, or enamel lesions were derived from Malvar slaughterhouse in Pasay City, NCR, certified by the City Veterinarian Office.[30,31]

The teeth were washed with distilled water and immersed for seven days in 0.1% thymol-water solution at room temperature, for asepsis and dehydration prevention.[31] After this period, the remaining soft tissues, calculus, and bone fragments were removed using ultrasonic scaler and

scalpel blade#15. Teeth were polished with rubber cups and pumice slurry then stored in a sealed jar containing 0.1% (w/v) thymol solution at 4˚C in a refrigerator and used within one month after extraction.[30,31]

From the labial surface, enamel slabs with the dimensions 5 mm high x 5 mm wide x 2 mm thick were prepared using a water-cooled diamond fissure bur in a high-speed handpiece and measured with Vernier caliper. The slabs were serially finished and polished with aluminum oxide-coated abrasive discs (course, medium, fine, superfine: 3MSof Lex) and secured in a refrigerator.[30]

Enamel-bracket Specimens

Forty-one lower first premolar conventional metal orthodontic brackets, without hook (0.018 slot, Roth, 3MUnitek™ Miniature Twin) were used.

The labial surface of enamel slabs was cleaned with pumice slurry and prophylaxis brush in a low-speed handpiece for 10 seconds, then water-rinsed for 10sec and dried with an oil/humidity-free air spray. The surface was then etched with 37% phosphoric acid (3MUnitek™) for 15 seconds then rinsed with water for 10 seconds to ensure total removal of etchant. It was dried with air spray which resulted in the enamel acquiring a chalk-white color.

A thin uniform coat of Adhesive primer (Transbond™XT) was applied on the labial surface and light-cured for 10 seconds.[31]

Bracket cement (3M Transbond™ XT) was applied to the bracket base and centered on the enamel slab using a bracket holder. To standardise the bonding pressure and cement thickness, a 453.6-g Gilmore needle (Soiltest, CT5) was held vertically on the bracket center while a clinical probe was used to remove excess material around the bracket base. The mesial and distal sides were light-cured for 20 seconds with LED (Ti-Lite GT1500, Monitex) with 1,500 mW/cm^2 at a fixed distance and angle to the surface.[30]

The enamel-bracket specimens were secured in a sterile plastic pouch (Defend®) and autoclaved at 121˚C at 15 psi for 15 mins.[32]

Out of the 41 specimens, 40 specimens were subjected to *S. mutans* growth, 39 of which were treated and one left untreated, while one randomly chosen specimen was left clean and without bacterial growth and assigned for ESEM evaluation.[12]

S. mutans Mono-species Biofilm on Enamel-bracket Specimens

S. mutans ATCC 25175 (TNC Everlight) were rehydrated according to manufacturer's instructions. It was grown overnight in brain heart infusion (BHI) (Scharlau) medium in an anaerobic jar at 37˚C for 24 to 48hrs.

Checking of purity of culture was done using Gram staining, then plating samples onto a new fresh BHI agar medium and incubated at 37˚C in an anaerobic jar for 24 hours.[32]

Confirmation of purity and genus was done by observing growth of one type of characteristic colonial morphology, Gram-positive cocci in chains, and catalase-negative colonies.

Standardization of Inoculum: BHI broth with 5% sucrose and *S. mutans* were compared to 0.5 Mcfarland. Streaking of solution was done on BHI agar then confirmation of counts followed (~1-2 x 10^8 CFU/ml).

After sterilization, the bracket specimens were distributed in four quadrant plates. Ten specimens were placed in each plate, arranged at the corners of each quadrant, separate from each other. 10 ml of sterile BHI broth with 5% (w/v) sucrose was added in each quadrant to immerse the specimens, a total of 40 ml broth in each plate.[22]

All BHI-containing quadrant plates were inoculated with 1 ml of standardized inoculum, performed only once on the first day in an anaerobic jar. After every 24 hours, the specimens were transferred into a new quadrant plate with fresh BHI with 5% sucrose. This was performed during the next five days for biofilm to grow and adhere to specimens.

Inspection for contamination was done every day at each transfer time, by Gram staining and plating a loopful of samples onto a new fresh BHI agar, incubated at 37˚C in an anaerobic jar for 24 hours. Catalase and morphologic characteristics in Gram stain were utilized for genus confirmation.[22,30,32]

After five days of biofilm growth, one untreated specimen was randomly assigned (fishbowl technique) and preserved in 1% Hank's Balanced Salt Solution,1 ml (HBSS) contained in 2 ml microcentrifuge tube, in a refrigerator (4˚C) for less than six hours and was evaluated under ESEM for baseline record of biofilm growth.[33,34]

Enamel-bracket specimens were picked up with sterile forceps from the quadrant plates and immersed twice, one second each, in 3 ml sterile saline solution contained in each well of a culture plate to remove loosely bound material.[22]

Treatment Groups

There were three groups with 13 samples per group, with a power of 96.34.[29] Twelve samples per group were used for quantitative evaluation while one sample per group was used for qualitative (ESEM) evaluation. After washing with saline, the specimens were randomly grouped (fishbowl technique) into three different 65-mm Petri dishes. In each group, 13 specimens were arranged separately from each other in three rows to prevent cross-contamination wherein one specimen per group was randomly chosen for ESEM evaluation.

Group A: (Distilled water)

The specimen for ESEM was immersed in a 15 ml covered test tube containing 10 ml distilled water for 30 seconds, while the test tube was being agitated in a test tube lateral shaker (2 shakes/sec), to simulate rinsing inside the oral cavity. The same procedure was done for the 12 specimens.

Group B: (0.12% CHX)

The ESEM specimen was immersed in a 20 ml covered test tube containing 15 ml 0.12% Chlorhexidine (Orahex®) for 30 seconds, while in a test tube lateral shaker (2 shakes/sec), then set aside in a covered Petri dish for 30 minutes to ensure the efficacy of the solution, as per manufacturer's instruction. The same procedure was done for the 12 specimens.

Group C: (PDT)

FotoSan630® device (CMS DentalAps) was used with red LED, 620-640 nm wavelength, peak 630 nm and 2-4 W/cm^2 power density. Its "green" mode indicates time: small button (10 seconds), large button (30 seconds). The study used 60 seconds (2 x 30 sec).[35] The low-viscosity photosensitizer (FotoSan® agent) is Toluidine Blue O (0.1 mg/ml). A blunt tip (8 mm diameter) was attached to the LED.[35]

The ESEM specimen was submerged in a microcentrifuge cap containing (0.2 ml) TBO for five minutes (pre-irradiation while inside a Petri dish). It was exposed to red LED for 60 seconds, using a blunt tip (8 mm diameter) directly in contact with the TBO and specimen.[22,23,35]

Same procedure was done for the 12 specimens. The blunt tip was replaced with a new one for each specimen to avoid cross-contamination. The researcher used protective eyewear during irradiation.

Two FotoSan® LED units were used alternately, recharging one while waiting to be used. This allowed consistent light intensity when checked on the Visible Curing Light Meter (Cure Rite, Caulk) before each radiation.[32,36] The LED should display an output level of ≥ 300 before each use, otherwise it is returned to the docking station.

Quantitative Evaluation

After the treatments, biofilms were collected from 12 specimens. To avoid cross- contamination, each specimen was assigned one scalpel blade and one sterile forceps.

One researcher scraped off the biofilms using scalpel blade#11 from all bracket surfaces and labial surface of enamel slab.[32] Forceps was used to stabilize the specimen contained in a Petri dish layered with tissue. Collected biofilms were placed into individual pre-weighed 2 ml-micro-centrifuge tubes containing 0.9% (w/v) NaCl (1.5 ml) and analytically weighed.

Biofilms in microcentrifuge tubes were sonicated on ice using a water bath sonicator (Rocker™) at 60 W for 15 seconds to obtain suspensions containing single cells.[22] Caps were opened during sonication to prevent explosion of tubes during vibration. To further disperse the biofilm, microcentrifuge tubes were subjected to a high speed vortex mixer (speed 8) for five seconds upright and five seconds longitudinally.

Undiluted and 1:100 dilution of suspensions were prepared using normal saline solution as the diluent. Prepared suspensions were further subjected to a vortex mixer for five seconds before plated onto BHI agar then incubated at 37°C in an anaerobic jar for 48 hours. The number of surviving microorganisms was determined by colony counting from the undiluted plates only, which had consistent countable colonies.

The values were expressed as CFU per milligram of biofilm.[22]

Computation for the number of *S. mutans* CFU/mg from each enamel-bracket specimen:

Raw count from plate x Dilution factor (1) x
(biofilm wt. in mg / 1500 ul) x 10 ul = CFU/mg

Qualitative Evaluation

Five specimens were evaluated for ESEM: one clean bracket, one untreated bracket with biofilm and three specimens with biofilm from the treatment groups were preserved separately in 1% (1 ml) HBSS contained in microcentrifuge tubes, in a refrigerator (4°C) for less than six hours.[33,34]

The underside of fresh, hydrated specimens were dried with tissue and mounted on the aluminum stub using double-adhesive tape made of carbon-impregnated disc.

The specimens were individually and consecutively placed inside the ESEM chamber and viewed under "wet mode" with 2°C, gas pressure of 3-5 Torr and settings: 10-15 Kv, spot 4.[33,34,37,38] Biofilm morphology was assessed from the bracket slot area and tie wings, and enamel bracket interface lateral to the bracket base.[10,12]

RESULTS

Quantitative Evaluation

In each group, one microcentrifuge tube containing 0.9% (w/v) NaCl was accidentally not pre-weighed, reducing the observations to 11 for groups A and C. In addition, for group B, three microcentrifuge tubes had repeatedly lower weight after biofilm collection than its pre-weight, which yielded negative results and excluded from the data together with one outlier, resulting in seven observations for group B.

Group A (37.0573) had the highest number of CFU/mg from all specimens, followed by Group C (1.1193) then Group B (0.1712) (Figure 1). The p-value corresponding to the F-statistic of one-way ANOVA is lower than 0.05, suggesting one or more treatments are significantly different (Table 1).

The number of *S. mutans* CFU from plate culturing met the 12 sample size per group, with a statistical power of 96.34%.[29] *S. mutans* colonies after treatment with distilled water (1619) were more than that of CHX (229) and PDT (39) treatments (Figure 2). The p-value corresponding to the F-statistic of one-way ANOVA is lower than 0.05 (Table 2).

Based on the statistical tests done for quantitative evaluation (CFU and CFU/mg of *S. mutans*), one-way ANOVA revealed that there were differences among all groups. Post-hoc Tukey HSD tests (Tables 3 and 4) revealed

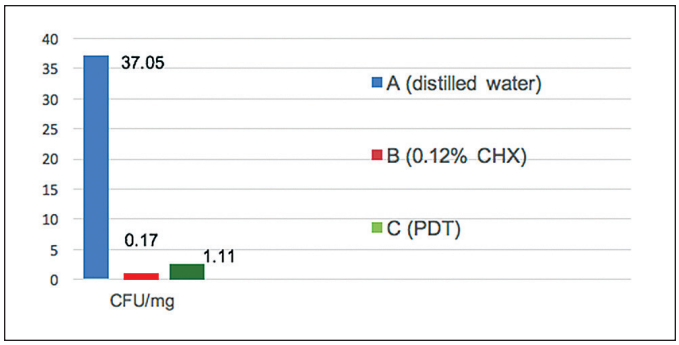


Figure 1. Number of *S. mutans* biofilm on enamel-bracket specimens after treatment with **(A)** distilled water, **(B)** 0.12% Chlorhexidine, and **(C)** Photodynamic therapy (CFU/mg).

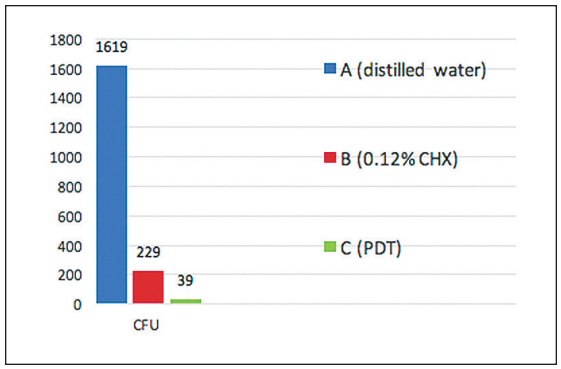


Figure 2. The number of CFU of *S. mutans* on enamel-bracket specimens after treatment with **(A)** distilled water, **(B)** 0.12% Chlorhexidine, and **(C)** Photodynamic therapy.

Table 1. One-way ANOVA of the Number of CFU/mg of *S. mutans* of Three Independent Treatments

Source	Sum of squares SS	degrees of freedom vv	mean square MS	F statistic	*p*-value
Between-treatments	74.2496	2	37.1248	6.15265	0.006491
Within-treatments	156.8829	26	6.034		
Total	231.1326	28			

Table 2. One-way ANOVA of the Number of CFU of *S. mutans* of Three Independent Treatments

Source	Sum of squares SS	degrees of freedom vv	mean square MS	F statistic	*p*-value
Treatment	124,016.6667	2	62,008.3333	6.0894	0.0056
Error	336,038.0833	33	10,182,9722		
Total	460,054.7500	35			

that the treatment effect in group A (distilled water) was significantly different from groups B (0.12% chlorhexidine) and C (photodynamic therapy), while the difference between treatment groups B and C was not significant. Statistical tests had 87% power.

Given the lack of significant difference between treatment groups B and C, it can be inferred that the effects of CHX and PDT treatments against *S. mutans* biofilm were comparable.

Qualitative Evaluation

ESEM images in Figures 3.1A, 3.2, 3.3, and 3.4 show clean bracket surface: slot area, tie wings, and enamel bracket interface. Figure 3.1B shows an untreated specimen.

Three enamel-bracket specimens treated separately with (a) distilled water, (b) 0.12% chlorhexidine, and (c) photodynamic therapy were analyzed under ESEM to evaluate the enamel-bracket interface and *S. mutans* biofilm. (Figures 4.1 and 4.2).

The enamel-bracket interface in the specimen (A) remained covered with abundant dense biofilm after distilled water treatment. Specimens (B) and (C) displayed gaps (arrows) between the enamel and bracket base, indicating sparse biofilm not completely covering the junction, after

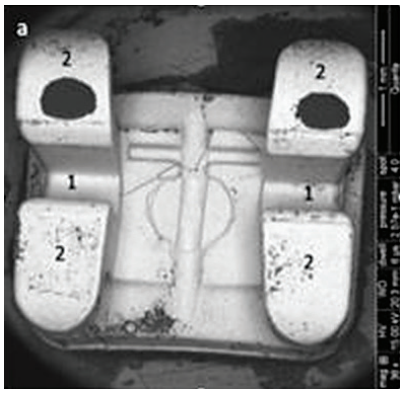
Table 3. Tukey HSD Result of the Number of CFU/mg of *S. mutans* of Three Independent Treatments

Treatment pairs	Tukey HSD Q statistic	Tukey HSD *p*-value	Tukey HSD inference
A vs B	3.9824	0.0240065	*p<0.05 (significant)
A vs C	4.4112	0.0118184	*p<0.05 (significant)
B vs C	0.0920	0.8999947	insignificant

Table 4. Tukey HSD Result of the Number of CFU of *S. mutans* of Three Independent Treatments

Treatment pairs	Tukey HSD Q statistic	Tukey HSD *p*-value	Tukey HSD inference
A vs B	3.9764	0.0218314	*p<0.05 (significant)
A vs C	4.5199	0.0083813	**p<0.01 (significant)
B vs C	0.5435	0.8999947	insignificant

treatment with 0.12% CHX and PDT, respectively. Notably, there were slightly fewer areas of biofilm in the specimen treated with PDT (C) compared to the specimen treated with 0.12% CHX (B). Clean specimen (D) displayed a clear enamel-bracket interface with excess adhesive cement (Figure 4.1A-D).



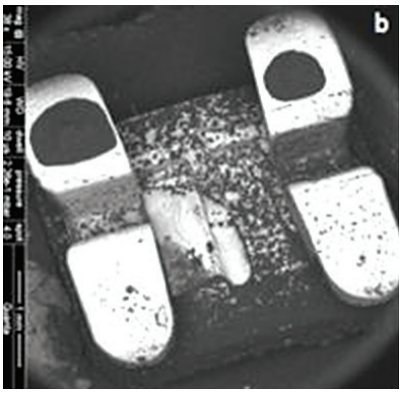


Figure 3.1. ESEM image (36x magnification) of a clean enamel-bracket specimen: **(a) (1)** slot area, **(2)** tie wings, and **(b)** untreated specimen with *S. mutans* biofilm.

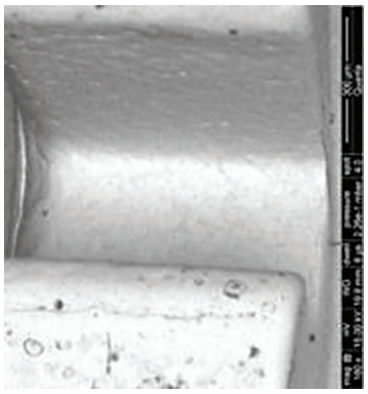


Figure 3.2. ESEM image (160x magnification), a closer view of the slot area of a clean bracket surface.

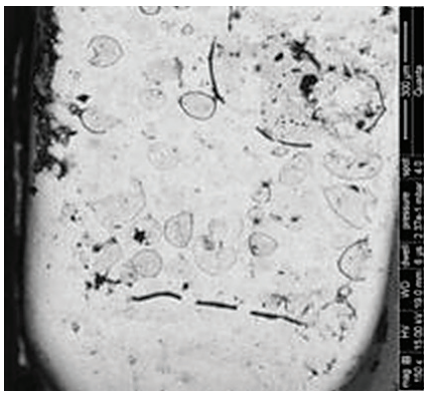


Figure 3.3. ESEM image (150x magnification), a closer view of the tie wing of a bracket, showing liquid droplets from the storage solution, 1% Hank's Balanced Salt Solution (HBSS).

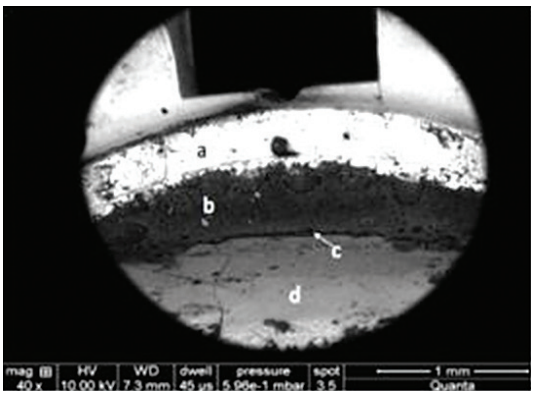


Figure 3.4. ESEM image (40x magnification) of a clean enamel-bracket interface showing the: **(a)** bracket base, **(b)** excess adhesive resin, **(c)** polymerization gap between the resin and enamel, and **(d)** enamel.

Abundant *S. mutans* in long chains of diplococci with clear cellular walls in dense thick confluent biofilm were seen in the specimen treated with distilled water (A). While in the specimen treated with 0.12% CHX (B), *S. mutans* were less dense with unclear cellular walls showing biofilm disintegration and amorphous masses. At a higher magnification (10,000x), the PDT-treated specimen (C) showed more *S. mutans* with indistinct cellular walls, biofilm disintegration, and an increase of amorphous masses. The undisrupted *S. mutans* biofilm in the untreated specimen (D) seemed to have a layer covering the aggregate of long chains making it denser, with morphology similar to the specimen treated with distilled water (A) (Figures 4.2A-D).

From a dense (A) to least dense biofilm (C), there was a decrease in *S. mutans* chains with an increase in amorphous masses. All specimens exhibited unclear cellular walls in the bracket slot area (Figure 5).

In bracket tie wings, there was a decrease in *S. mutans* clustering from specimens (A) to (C). Distilled water-treated specimen (A) showed more aggregated cocci than the PDT-treated specimen (C), which showed sparsely distributed cocci (Figure 6).

DISCUSSION

Photodynamic Therapy

Bovine enamel slabs, a suitable medium for *S. mutans* biofilm growth, were used. The results align with previous studies of Williams et al. and that of Burns et al. employing PDT with TBO, which effectively eradicated *S. mutans* in collagen matrices, carious human teeth, and demineralized dentin.[39,40] Melo et a.l recommended PDT as an adjunct in disinfecting residual carious dentin as seen in the *S. mutans* susceptibility in *in-vitro* dentin caries treated with LED plus TBO.[32] Thus, oral decontamination of orthodontic patients is possible using PDT, as supported by the study of Panhoca et al., which used blue-light and curcumin as photosensitizer, optimized with sodium dodecyl sulfate as surfactant.[41]

The use of a narrow band of LED radiation does not necessarily result in adverse side effects.[42] A low-level PDT

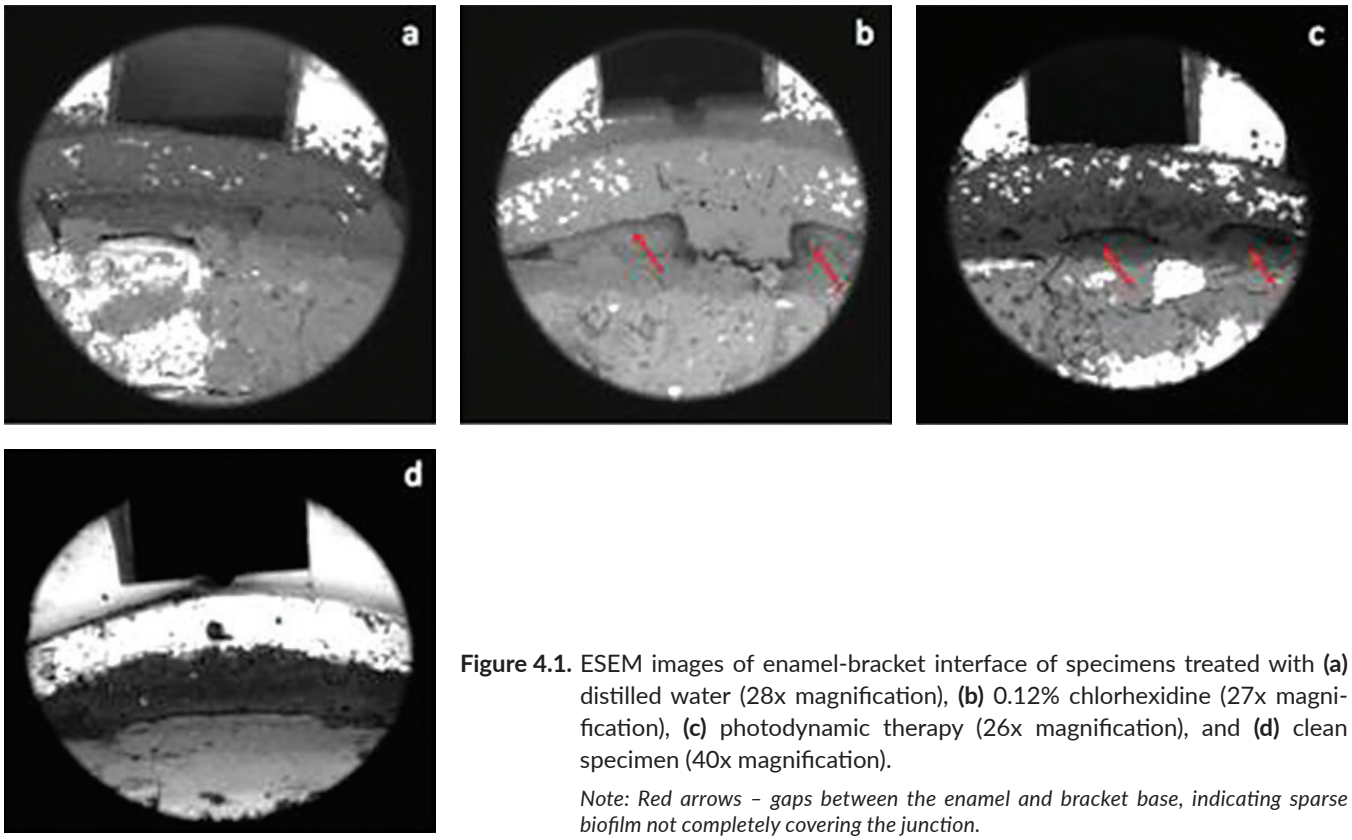


Figure 4.1. ESEM images of enamel-bracket interface of specimens treated with **(a)** distilled water (28x magnification), **(b)** 0.12% chlorhexidine (27x magnification), **(c)** photodynamic therapy (26x magnification), and **(d)** clean specimen (40x magnification).

Note: Red arrows – gaps between the enamel and bracket base, indicating sparse biofilm not completely covering the junction.

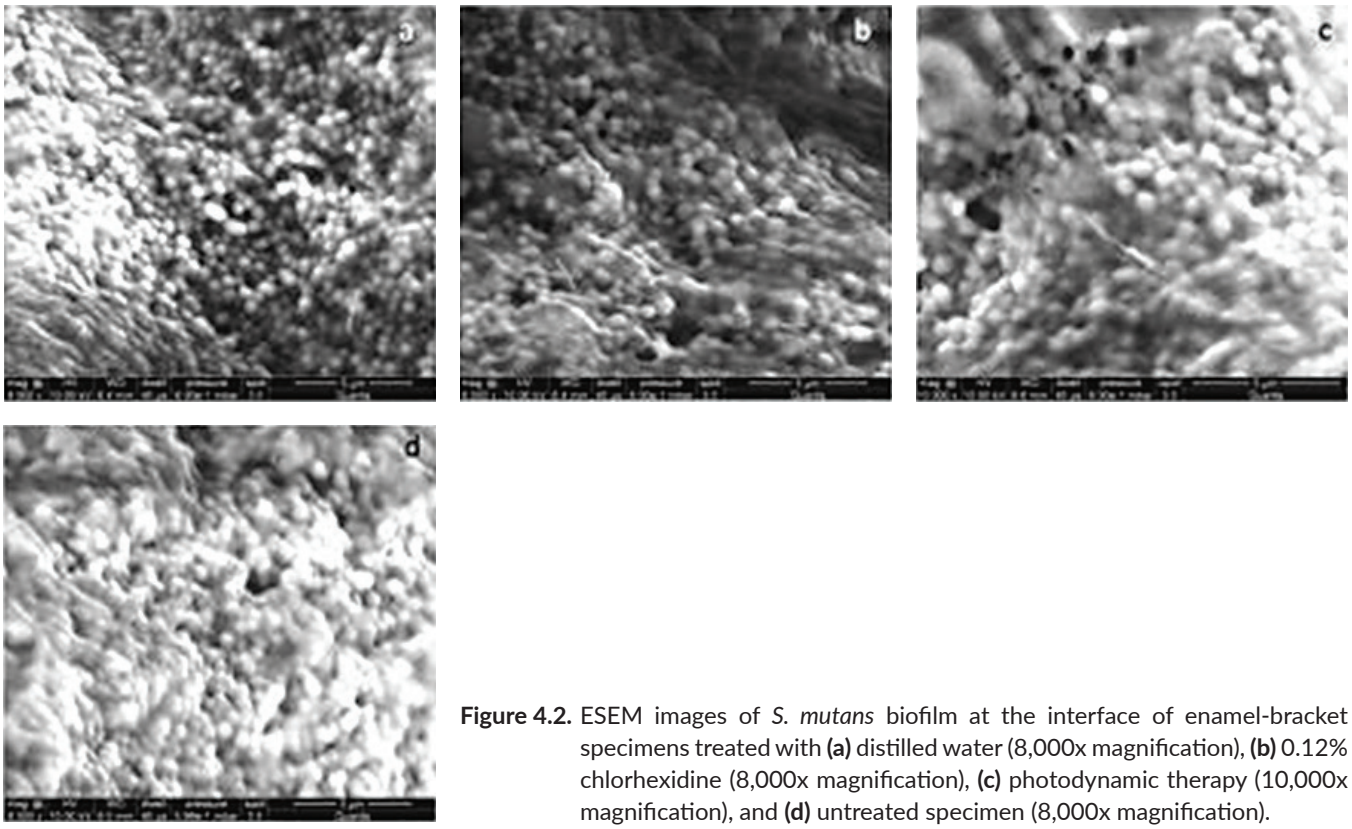


Figure 4.2. ESEM images of *S. mutans* biofilm at the interface of enamel-bracket specimens treated with **(a)** distilled water (8,000x magnification), **(b)** 0.12% chlorhexidine (8,000x magnification), **(c)** photodynamic therapy (10,000x magnification), and **(d)** untreated specimen (8,000x magnification).

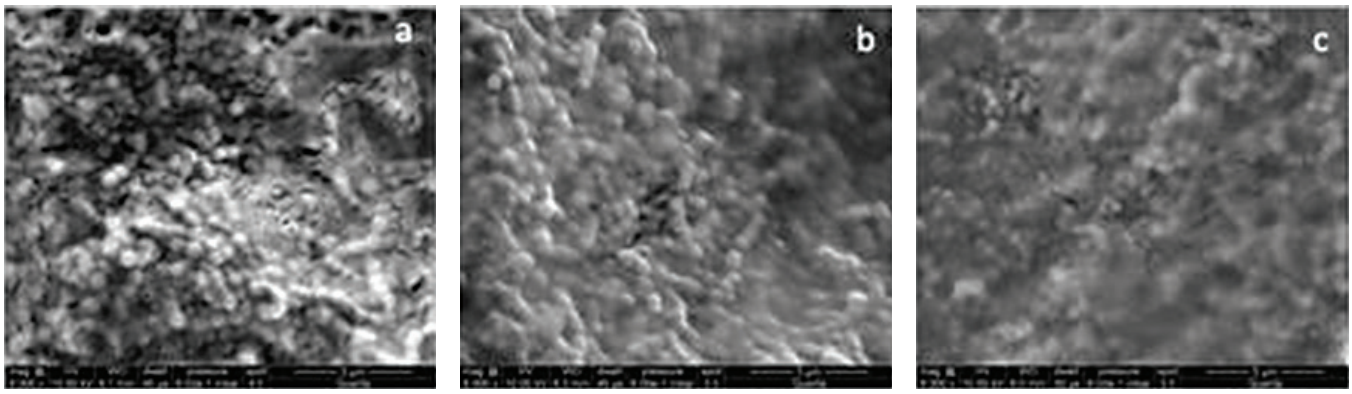


Figure 5. ESEM images (8,000x magnification) *S. mutans* biofilm in the bracket slot area of specimens treated with **(a)** distilled water, **(b)** 0.12% chlorhexidine, and **(c)** photodynamic therapy.

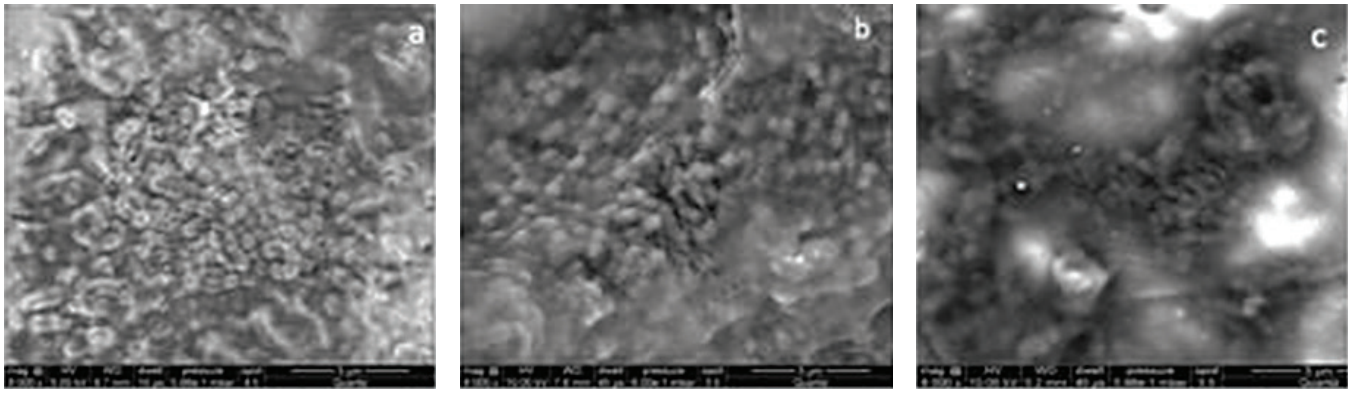


Figure 6. ESEM images (8,000x magnification) *S. mutans* biofilm in the bracket tie wing area of specimens treated with **(a)** distilled water, **(b)** 0.12% chlorhexidine, and **(c)** photodynamic therapy.

with a short irradiation time does not result in thermal changes within the gingival tissues and root surfaces.[43] In a study conducted by Qiao et al., a 60-second irradiation, the same time as the present study, was performed using a non-thermal diode laser with 675 nm wavelength and 0.01% Methylene blue photosensitizer with 10 seconds pre-irradiation time. PDT exhibited no cytotoxicity to human periodontal ligament cells and human gingival fibroblasts, but promoted their proliferation, attachment, and collagen synthesis.[44]

In orthodontics, a Fotosan® device using TBO may disinfect areas with persistent plaque accumulation and promote remineralization of WSL.

Chlorhexidine

Chlorhexidine, the positive control in this study, has been proven to effectively disrupt biofilm formation. A study by Ogaard et al. showed *S. mutans* suppression in patients with fixed orthodontic appliances, 20 weeks after low concentration CHX-thymol varnish (Cervitec) application, performed before the band and bracket placement.[45] Emilson et al. have demonstrated a higher CHX efficacy with slow recolonization of bacteria for initially low colonized teeth. It works as preventive and adjunctive tool to mechanical plaque removal.[46]

Conversely, a study by Jenatschke et al. found that CHX varnish treatment did not reduce the caries rate in high-risk orthodontic patients. The duration of *S. mutans* suppression depends on the extent of varnish coating on the plaque-retentive niches, such as orthodontic appliances, which also impede CHX application. Prolonged CHX varnish therapy may also lead to bacterial resistance.[20]

PDT vs CHX Treatment

The present study showed that PDT and CHX were both effective antimicrobials, with an insignificant difference in their efficacy in reducing *S. mutans*. This was in line with the study by Panhoca et al., that used PDT with blue light irradiation and curcumin, optimized with surfactant sodium dodecyl sulfate showing an insignificant difference in the oral bacterial reduction compared to 0.12% CHX swished by patients for 30 seconds.[41]

ESEM Assessment

ESEM imaging without specimen fixation preserved the *S. mutans* biofilm architecture. The residual tooth-colored

bonding adhesive around the bracket base added to the surface roughness (Figure 3.4B).[12] The polymerization gap of the adhesive made the enamel-bracket interface retentive to biofilm (Figure 3.4C). Based on the amount, thickness, and coverage of *S. mutans* biofilm that remained, CHX and PDT treatment effects were similar, displaying more biofilm and cellular damage with increased amorphous masses than distilled water (Figures 4,5, and 6). A higher magnification 10,000x in Figure 4.2C, needed for the PDT specimen may imply more bacterial killing and greater reduction of biofilm thickness after treatment.

S. mutans biofilm treated with distilled water (Figure 4.2A) was almost similar to the untreated specimen (Figure 4.2D), with dense, undisrupted thick and confluent morphology that seemed to have a protective layer covering the aggregate of long chains. With ESEM imaging of hydrated specimens, glycocalyx can be visualized, which comprise 85-90% of volume to serve as a protective layer against biocides.[37,47]

In the bracket slot areas (Figure 5) and tie wings (Figure 6), PDT treatment resulted in greater bacterial kill compared to 0.12% CHX and distilled water. The antimicrobial effects of PDT and 0.12% CHX were apparent in the brackets, which act as niches for biofilm and barriers to external agents. This was reflected in the number of CFU and CFU/mg after treatments.

In this study, mature biofilms were subjected mainly to antimicrobials without prior mechanical debridement, which resulted in indistinct cellular differences. This was similar to a previous study by Koban et al., where oral biofilms that received no mechanical debridement prior to treatment with 0.1% CHX showed almost intact architecture, similar with 0.9% NaCl control group based on SEM images.[48] While in a study by Melo et al., scraping off from carious dentin slabs was performed prior to PDT, which resulted in unclear cellular walls, lost wall bands with amorphous masses and mini cells due to division at the inappropriate junction of *S. mutans* cells.[32]

In the present study, biofilm was preserved carefully prior to ESEM, which showed changes in *S. mutans* morphology rather than its viability after treatments and no treatment done. To determine viability, bacterial culturing was performed.

CONCLUSION

With no significant difference between Photodynamic therapy and 0.12% Chlorhexidine, both treatments significantly reduced *S. mutans* based on CFU/mg counts and ESEM images.

Photodynamic therapy (red LED and TBO) demonstrated notable reduction of *S. mutans* biofilm on conventional metal orthodontic brackets bonded to bovine enamel slabs. PDT could serve as an alternative adjunctive tool for the mechanical removal of plaque adhered to orthodontic brackets.

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Author Disclosure

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