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Randomized *in vivo* evaluation of photodynamic antimicrobial chemotherapy on deciduous carious dentin

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Abstract. The aim of this randomized *in vivo* study was to compare antimicrobial chemotherapies in primary carious dentin. Thirty-two participants ages 5 to 7 years underwent partial caries removal from deep carious dentin lesions in primary molars and were subsequently divided into three groups: control [chlorhexidine and resin-modified glass ionomer cement (RMGIC)], LEDTB [photodynamic antimicrobial chemotherapy (PACT) with light-emitting diode associated with toluidine blue solution and RMGIC], and LMB [PACT with laser associated with methylene blue solution and RMGIC]. The participants were submitted to initial clinical and radiographic examinations. Demographic features and biofilm, gingival, and DMFT/DMFS indexes were evaluated, in addition to clinical and radiographic followups at 6 and 12 months after treatments. Carious dentin was collected before and after each treatment, and the number of *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus casei*, *Fusobacterium nucleatum*, *Atopobium rimae*, and total bacteria was established by quantitative polymerase chain reaction. No signs of pain or restoration failure were observed. All therapies were effective in reducing the number of microorganisms, except for *S. sobrinus*. No statistical differences were observed among the protocols used. All therapies may be considered as effective modern approaches to minimal intervention for the management of deep primary caries treatment. © 2015 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.20.10.108003]

Keywords: photochemotherapy; dental caries; dentin; primary teeth; real-time polymerase chain reaction.

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1 Introduction

The widespread use of fluoride has led to the decline of dental caries worldwide.¹ However, it is still a very common disease in preschool children, with high risk of developing caries in both primary and permanent teeth.² This high-risk caries group therefore requires special dental care and innovative treatment techniques that prioritize minimal intervention.

In the dynamic process of dental caries, after enamel demineralization, the lesion may progress slowly toward the dentin, initially characterized by an area of widely demineralized dentin located under a partially demineralized zone and infected with bacteria.³ The inner layer, known as the affected dentin, is usually contaminated with fewer bacteria, and although these microorganisms are capable of dissolving this mineralized tissue, the ultrastructure of the crossed bands from remaining collagen matrix makes it capable of being remineralized. The microbial composition of deep caries in dentin is diverse and the most prevalent species are *Fusobacterium nucleatum*, *Atopobium genomospecies*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Bifidobacterium* species, and the *Streptococcus* species, mainly *Streptococcus mutans*.⁴

Traditionally, total removal of carious tissue can cause damage to the pulp, pain, and even weakening of the tooth structure.

Nowadays, more conservative approaches, known as minimal intervention techniques for the management of caries, have proven to reduce adverse consequences of restorative treatment such as postoperative pain and pulp exposure⁵. Minimal intervention can be described as “removal of the superficial necrotic and demineralized dentin with complete excavation of the peripheral demineralized dentin, avoiding excavation close to the pulp”⁶. Therefore, this indirect pulp treatment is recommended for primary teeth with deep caries or reversible pulp inflammation. A proper diagnosis and treatment selection are based on history, clinical, and radiograph examinations, in addition to a good marginal seal of the restoration, in order to create an environment that would be inhospitable to the remaining bacteria.⁷ The use of hand or rotary instruments does not guarantee the cleanliness of the infected dentin and elimination of residual bacteria usually present in the cavity before restorative procedures.⁷ Thus, photodynamic antimicrobial chemotherapy (PACT) may be a promising alternative for elimination of the microorganisms still present in the remaining affected dentin and increasing the chances of successful treatment.

PACT associates a photosensitive agent, activated by irradiation, with a light source at a specific wavelength to generate cytotoxic species, including singlet oxygen and free radicals.⁸ These products are capable of damaging the essential

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components of cells, or modifying the metabolic activity irreversibly, and may result in bacterial death.⁹ This mechanism of action avoids the development of bacterial resistance, since this is a limitation of conventional antimicrobial therapies.¹⁰

Studies have demonstrated that cariogenic bacteria are susceptible to death as a result of the photosensitization of microbial components, with the use of photosensitive agents such as *ortho*-toluidine blue^{11–13} and methylene blue¹⁴ in association with light sources. Furthermore, *S. mutans*, the most cariogenic microorganism, may be killed under conditions similar to those found in a caries lesion.¹¹ This has also been observed in different studies using dentin collagen matrix, *in vitro* bovine carious dentin, *in vitro* human carious dentin, *ex vivo* carious dentin, and *in vivo* carious dentin.^{13–20}

If the microorganisms associated with dental caries could be reduced, *in vivo*, by PACT, the results could be very positive for conservative dentistry, since it does not imply removal of the affected dentin, thereby reducing the risk of pulp exposure, and can thus be considered less traumatic treatment for children.¹⁰ Therefore, the aim of this study was to test, *in vivo*, two PACT protocols in the treatment of deep caries lesions in primary teeth by means of microbiological and clinical evaluations.

2 Material and Methods

2.1 Ethical Considerations

This parallel-designed clinical trial was conducted in accordance with the Helsinki declaration and was approved by the ethics committee of the School of Dentistry, University of São Paulo (#150/10). Each child's parent signed a written term of free and informed consent allowing the children to participate in the study, and the children themselves also granted permission for the treatment. This study was conducted in accordance with the standards of reporting trials—Consort Statement²¹ and was registered with ClinicalTrials.gov (NCT# 02479958).

2.2 Sample Collection

Participants, recruited from the School of Dentistry of the University of São Paulo between May and October 2011, were children in the age range from 5 to 7 years. The sample size was estimated based on the means and standard deviations of a previous study using BioEstat 5.3 software (Mamirauá, Tefé, AM, Brazil).¹⁴ Calculation of statistical significance was estimated using the paired *t*-test for the paired samples ($\alpha = 0.05\%$; $\beta = 0.20\%$).

The following inclusion criteria were applied: children with good general health, without syndromes or chronic systemic diseases. They should also have at least one primary molar with deep carious lesion, which should extend two-thirds of the inner half of dentin (Fig. 1) without pain symptoms and be compatible with reversible pulpitis. Children whose parents refused to sign the informed consent document, who did not cooperate with the clinical exams, whose teeth had the treatment choice changed (pain or evolution to irreversible pulpitis), who did not keep the appointments for the scheduled treatments, or who needed antibiotic treatment for other medical reasons were excluded from the study without prejudice. For all of these reasons, out of the 58 children enrolled, 32 (18 girls and 14 boys) were included in the final sample, according to the CONSORT flow diagram (Fig. 2).²¹ Digital photographs and

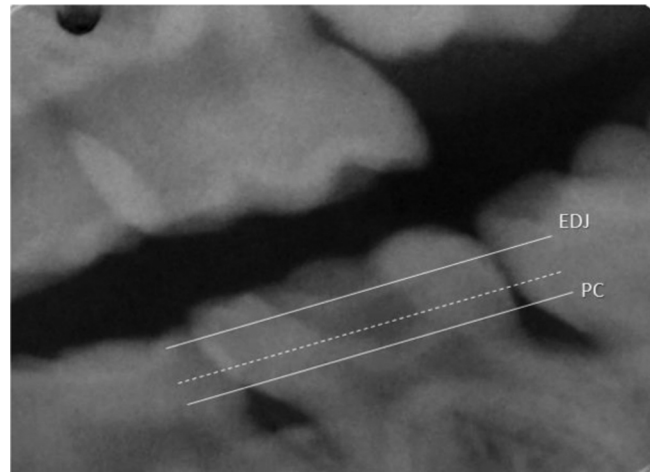


Fig. 1 Radiograph showing the extent of the carious lesions included in the study. EDJ indicates the enamel–dentin junction and PC, the pulp chamber limit. Lesions should extend two-thirds of the inner half of dentin (interrupted line).

initial radiographs were taken of the selected teeth; demographic features of the children were evaluated by DMFT (decayed, missing, and filled teeth) and DMFS (decayed, missing, and filled surfaces) indexes of primary dentition, as well as the bio-film and gingival indexes.^{22,23}

The participants were randomly assigned to their groups by a simple randomization method, generated by a software spreadsheet (Excel 2007; Microsoft, Redmond, Washington). Clinical procedures were all performed by one experienced operator, and both the operator and patient were able to see which treatment was being carried out (although they were children), because the light source could not be disguised. However, dentin samples collected from each patient were coded, so the researcher was blind to the laboratory phase of the microbiological analysis. Encoding was performed by applying previously created labels to the microcentrifuge tubes into which the carious dentin was introduced. Labels were only decoded at the end of the experiment, after the microbiological analysis.

2.3 Treatment Groups

All treatments were conducted after isolation with rubber dam (Table 1). The carious tissue was partially removed from the lateral walls, and only the superficial demineralized necrotic tissue was removed from the pulp wall with the help of a conventional dentin curette. The dentin was initially collected from the pulp wall with the active part of a sterile 1-mm diameter micropunch (Ritcher, São Paulo, Brazil) with a penetration depth of 0.5 mm.¹⁴ After this, the teeth were treated according to the designated groups as follows:

1. Control (C): The remaining dentin was washed with 2% chlorhexidine, a new dentin collection was performed, at another site of the cavity, and restoration was performed with resin-modified glass ionomer cement (RMGIC) Vitremer (3M/ESPE St. Paul, Minnesota).
2. Photodynamic antimicrobial therapy with light-emitting diode (LED) (group LEDTB): Treatment of the remaining dentin was performed with 200 μ L of *ortho*-toluidine blue dye (0.1 mg/mL) for 60 s. After

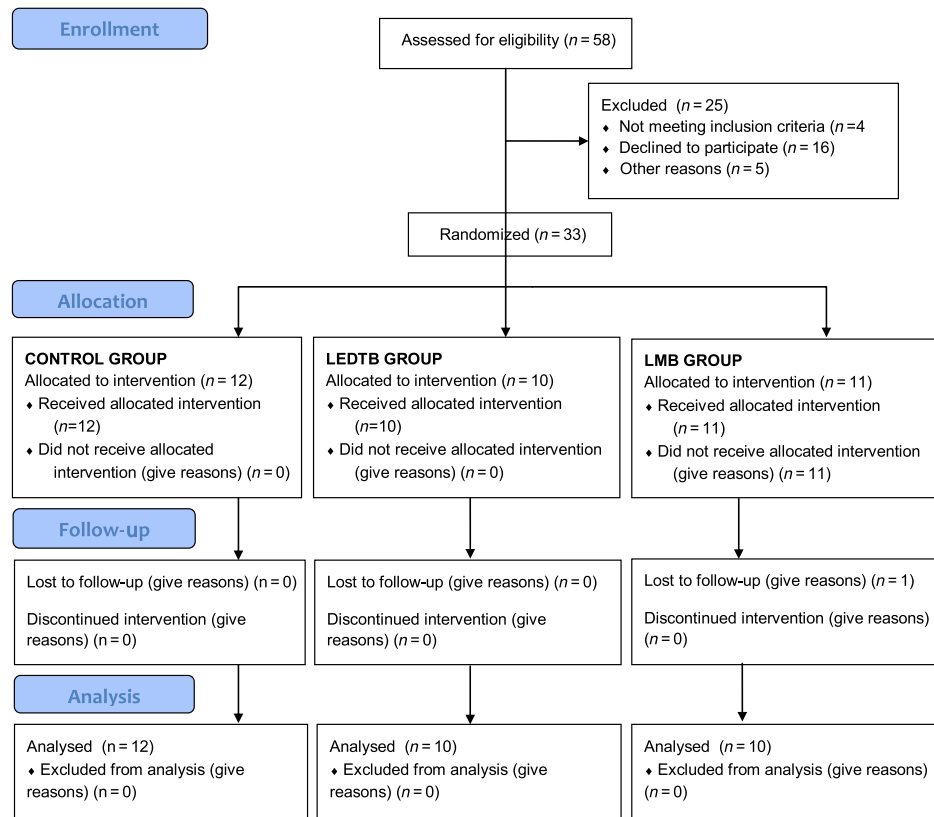


Fig. 2 CONSORT flow diagram.

this period, a red LED light source from the Special Laboratory of Lasers in Dentistry—LELO/FOUSP (MM Optics Ltda, São Carlos, São Paulo, Brazil) with a wavelength of 630 nm was used, at 100 mW power; 9.0 J of energy, and energy density of 30.0 J/cm², for 60 s. The photosensitizer was washed out with water and dried with a sterile cotton pellet, dentin was collected again, at another site of the cavity, and restoration was performed with RMGIC Vitremer (3M/ESPE St. Paul, Minnesota).

- Photodynamic antimicrobial therapy with laser (group LMB): Treatment of the remaining dentin was performed with 200 μ L of methylene blue dye 0.01% (Chimiolux[®]—Hyrofarma, BeloHorizonte, Minas Gerais, Brazil) for 5 min, and after this, a red low-power LASER light source from the Special Laboratory of Lasers in Dentistry—LELO/FOUSP (Photon Lase III—DMC, São Carlos, São Paulo, Brazil) with a wavelength of 660 nm was used, at 100 mW power;

9.0 J of energy, and energy density of 320.0 J/cm², for 90 s.¹⁴ The photosensitizer was washed out with water and dried with a sterile cotton pellet, the dentin was collected again, at another site of the cavity, and restoration was performed with RMGIC Vitremer (3M/ESPE St. Paul, Minnesota). A power meter was used to ensure the correct emission of light by the laser equipment.

Table 2 shows the demographic features of the participants, such as age and gender distribution, and clinical variables such as DMFT and DMFS values, % of visible biofilm, gingival and biofilm index values.

All patients were clinically and radiographically followed up 6 and 12 months after the end of the treatments. They were also submitted to full dental care, when a complete treatment was performed. They were instructed to make contact if they experienced any signs of pain in any of their teeth.

2.4 Microbiological Analysis

The carious dentin collected before and after each treatment was placed in separate microtubes containing 100 μ L of TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 7.6) and stored at -20°C until use. Microbiological analyses of the samples were performed by quantitative analysis of total bacteria and five specific species, by real-time polymerase chain reaction (PCR).

2.4.1 Bacterial strains and growth conditions

S. mutans (Sm, ATCC 25175), *Streptococcus sobrinus* (Ss, ATCC 44378), *L. casei* (Lc, L324m), *F. nucleatum* (Fn,

Table 1 Characteristics of the experimental groups.

Group	LED	Low-power laser	Photosensitizer	Restoration
C	-	-	-	Vitremer
LEDTB	+	-	<i>ortho</i> -Toluidine blue	Vitremer
LMB	-	+	Methylene blue	Vitremer

Table 2 Means (\pm SD) of clinical variables such as age and gender distribution, DMFT and DMFS values, % of visible biofilm, gingival, and biofilm index values evaluated.

	<i>n</i> = 32
Age (years)	6.5 \pm 1.7
Gender (M/F)	14/18
DMFT	5.3
DMFS:	8.3
% Visible biofilm	32
Biofilm index	0.80
Gingival bleeding index	0.16

ATCC 25586), and *Atopobium rimae* (Ar, CCUG 31168) were used as a source of chromosomal deoxyribose nucleic acid (DNA) for amplification and later as standard in PCR quantitative curves. Sm strains were cultured in mitis salivarius agar (Difco, Sparks, Maryland) with 0.2 units/ml bacitracin at 37°C under microaerophilic conditions (10% CO₂ in air; Shel Lab, Cornelius, Oregon) for 48 h; Ss strains were cultured in mitis salivarius agar (Difco, Sparks, Maryland) at 37°C under microaerophilic conditions (10% CO₂ in air; Shel Lab, Cornelius, Oregon) for 48 h; Lc strains were cultured in Rogosa agar (Difco, Sparks, Maryland) at 37°C under microaerophilic conditions (10% CO₂ in air; Shel Lab, Cornelius, Oregon) for 48 h; Fn strains were grown at 37°C in BHI agar under anaerobic conditions (85% N₂, 5% H₂, and 10% CO₂) generated in an anaerobic chamber (Plaslabs, Lansing, Michigan) for 96 h; Ar strains

were grown at 37°C in BHI agar under anaerobic conditions (85% N₂, 5% H₂, and 10% CO₂) generated in an anaerobic chamber (Plaslabs, Lansing, Michigan) for 96 h.

2.4.2 Deoxyribose nucleic acid extraction

The carious dentin collected was thoroughly vortexed, and total DNA from bacterial standard strains and carious dentin collected was extracted using a MasterPure™ Complete DNA and RNA EPICENTRE kit (Epicentre Biotechnologies, Madison, Wisconsin), according to the manufacturer's instructions. DNA was eluted in TE buffer; the quantity and quality were estimated by spectrometry (Nanodrop ND1000, Thermo Fisher Scientific Inc., Wilmington, Delaware), and then were stored at -20°C until use. In order to check the specificity and to produce amplicons to PCR standard curves of each standard DNA, conventional PCR was performed using the primers described in Table 3.

2.4.3 Quantitative polymerase chain reaction

Quantitative analysis of the different bacteria was performed by real-time PCR with the thermal cycler Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, California), and the product was detected by fluorescence using SYBR® Green system (Applied Biosystems, Foster City, California) in accordance with the manufacturer's recommended protocol. PCR reactions were set up in 96-well plates in a total volume of 20 μ l containing 10- μ l Sybr Green mix, 100 ng of sample DNA, and 200 nM of each primer (Table 3). Internal region of the *16SrRNA* gene specific to identify Universal, Lc, Fn, and Ar, while an excerpt inside *gtfB* and *gtfU* was used to identify Sm and Ss, respectively. Temperature cycling profiles included a preincubation at 94°C for 5 min followed by 40 cycles of 94°C for 15 s, annealing temperature according to

Table 3 Type of primers, sequence (5'-3'), amplicon size (bp), and annealing temperatures (°C) used for each group/species.

Group/species	Primers	Sequence (5'-3')	Amplicon size (bp)	Annealing temperature (°C)	Reference
<i>S. mutans</i>	Smut3368	GCCTACAGCTCAGAGATGCTATTCT	114	60	24
	Smut3481	GCCATACACCACTCATGAATTGA			
<i>S. sobrinus</i>	Ssob287	TTCAAAGCCAAGACCAAGCTAGT	88	56	24
	Ssob374	CCAGCCTGAGATTCAGCTTGT			
<i>L. casei</i>	sg-Lcas	ACCGCATGGTTCTTGGC	296	60	25
	sg-Lcas	CCGACAACAGTTACTCTGCC			
<i>F. nucleatum</i>	Fnuc 134	TGCGATAAGCCTAGATAAGTTGCA	330	58	26
	Fnuc 203	CTTAATAGATTGCTCCATTCCGAAA			
<i>A. rimae</i>	c-Atopo	GGGTTGAGAGACCGACC	190	56	25
	c-Atopo	CGGRGCTTCTTCTGCAGG			
Universal	Uni-F (D88)	GAGAGTTTGATYMTGGCTCAG	500	56	27
	Uni-R (E49)	GAAGGAGGTGWTCCARCCGCA			

Table 3, for 30 s, 72°C for 1 min, and final extension at 72°C for 10 min. As negative control for each reaction, MilliQ sterile water was added instead of a DNA template. After the amplification reactions, the melt curve analysis was performed using the following temperatures: 95°C for 15 s, 65°C for 1 min, and 0.5°C to 95°C for 15 s.²⁸ Fluorescence was detected after each cycle and represented on a graph using the Step One Plus Real-Time PCR System software (Applied Biosystems, Foster City, California).

Quantification of Universal, Sm, Ss, Lc, Fn, and Ar gene copies of purified amplification DNA products was obtained by comparison with standard curves (10^8 to 10 copies of each specific microorganism gene). Curve standards and clinical samples were run in duplicate, and mean values were used to calculate the bacterial load. Data were analyzed using iQ5 Optical System Bio-Rad software (Hercules, California).

2.5 Statistical Analysis

The number of DNA gene copies of each species/bacterial group was evaluated by two-way analysis of variance, treatment, and time, as the repeated measures factor. The homogeneity of variances was checked and the data were transformed to \log_{10} . The Spearman correlation test was used to check if there was any correlation between the bacterial gene copies and the clinical variables. The level of significance for all statistical tests was $p < 0.05$.

3 Results

After the follow-up periods of both 6 and 12 months, no signs of pain or restoration failure were observed. The radiographs also showed no abnormal images. Figure 3 shows the number of gene copies of each microorganism (\log_{10}) before and after each treatment group. Table 4 shows the statistical results with p values. Independent of the treatment, a significant reduction in microorganisms was found for universal bacteria, Sm, Lc, Fn, and Ar before and after treatments when values were compared. No differences were found between treatments. No strong correlations were found among any of the clinical variables tested.

Table 4 Average and standard error of quantification of microorganisms (\log_{10}) before and after each treatment.

		C (n = 12)	LEDTB (n = 10)	LMB (n = 10)
Universal Bacteria ^a	Before	5.68 ± 0.67	4.45 ± 0.80	4.73 ± 0.41
	After	5.34 ± 0.53	4.02 ± 0.67	3.53 ± 0.33
<i>S. mutans</i> ^b	Before	2.37 ± 0.43	1.97 ± 0.49	2.04 ± 0.42
	After	1.80 ± 0.39	1.59 ± 0.44	1.59 ± 0.44
<i>S. sobrinus</i> ^c	Before	0.72 ± 0.32	0.32 ± 0.18	0.53 ± 0.26
	After	0.77 ± 0.33	0.83 ± 0.36	0.53 ± 0.27
<i>L. casei</i> ^d	Before	3.85 ± 0.73	2.99 ± 0.70	3.89 ± 0.60
	After	3.59 ± 0.68	2.58 ± 0.70	3.11 ± 0.50
<i>F. nucleatum</i> ^e	Before	1.81 ± 0.34	1.03 ± 0.38	1.62 ± 0.42
	After	1.52 ± 0.34	0.75 ± 0.35	1.17 ± 0.31
<i>A. rimaee</i> ^f	Before	3.18 ± 0.38	2.99 ± 0.58	2.93 ± 0.53
	After	2.69 ± 0.37	2.16 ± 0.52	2.31 ± 0.36

^aANOVA: Treatment × Time, $p = 0.366$; Treatment, $p = 0.146$; Time, $p = 0.019$.

^bANOVA: Treatment × Time, $p = 0.818$; Treatment, $p = 0.851$; Time, $p = 0.002$.

^cANOVA: Treatment × Time, $p = 0.207$; Treatment, $p = 0.840$; Time, $p = 0.135$.

^dANOVA: Treatment × Time, $p = 0.192$; Treatment, $p = 0.586$; Time, $p < 0.001$.

^eANOVA: Treatment × Time, $p = 0.855$; Treatment, $p = 0.270$; Time, $p = 0.020$.

^fANOVA: Treatment × Time, $p = 0.665$; Treatment, $p = 0.808$; Time, $p < 0.001$.

4 Discussion

The aim of the present study was to evaluate the *in vivo* effects of photodynamic antimicrobial therapy on the carious dentin of primary teeth. In both *in vitro* and *in situ* studies, the literature has shown this therapy to be effective in reducing the number of microorganisms in planktonic cultures and in carious

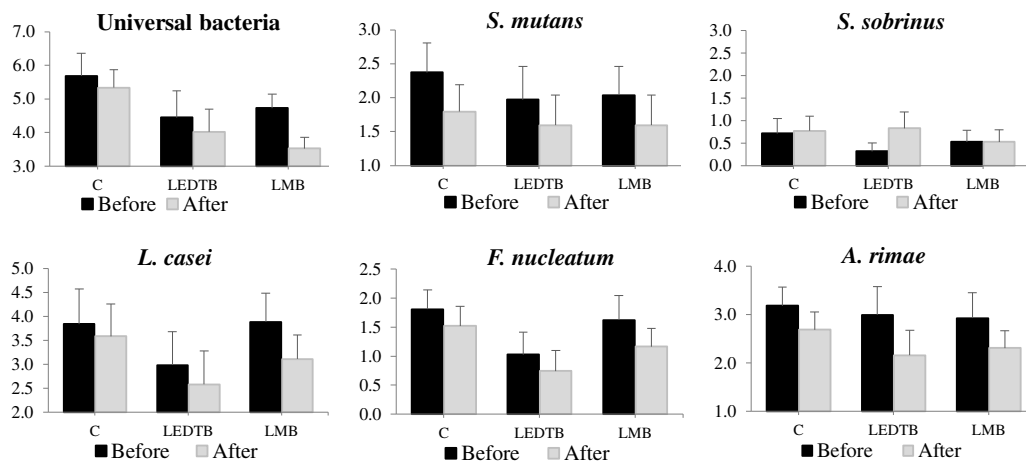


Fig. 3 Quantification of microorganisms (\log_{10}) and standard deviation (SD) before and after each treatment.

dentin.^{11–20} The results of the present study demonstrated that PACT reduced the number of all the microorganisms tested after each treatment, except for *S. sobrinus*; however, for the LED and low-power laser parameters used, no differences could be found among the groups.

The classic treatment for deep caries lesions, as was performed in our control group, is to remove the infected dentin, clean the remaining affected tissue, and seal the cavity with a restorative material. These procedures alone guarantee a reduction in bacteria,⁷ as shown in our results. However, the main goal of the present study was to perform a conservative technique for treating deep caries lesions, to reduce the number of microorganisms in the remaining affected dentin even further, by means of PACT, and in many cases, to avoid the need for endodontic treatment. A tendency for this to be achieved in the PACT groups was observed; however, the reduction did not differ statistically from that of the control group. This is in agreement with studies conducted by Teixeira et al.,²⁹ who were unable to demonstrate the antimicrobial effect of PACT in bacteria present in oral biofilm, and by Tonon et al.,²⁰ who found only a discrete bacterial reduction in a clinical isolate strain of *S. mutans*. Bacteria have different behaviors when treated in planktonic cultures, in biofilms, and in carious dentin.^{15,17,20} PACT is less prone to work in biofilms or in the carious dentin matrix due to the limited penetration of the photosensitizing agent into this surface and the deficient light propagation through the demineralized tissues.^{18,19,30} A relevant *in vivo* study conducted with 26 young adult patients with deep caries in permanent molars, by using methylene blue as the photosensitive agent and low-power red laser (InGaAlP $\lambda = 660$ nm, 100 mW, 320 J/cm², 90 s, 9 J), was able to show a modest reduction in microorganisms (1 log₁₀),¹⁴ however, differing from the high values achieved in *in vitro* or *in situ* studies. In the LMB group in the present study, we used the same PACT parameters as those used in the above-mentioned study, and we were unable to find this statistical microbial reduction *in vivo*, although there was a numerical trend toward this (Fig. 3).

Several photosensitive agents have been proposed for PACT in carious dentin, and they have shown good results *in vitro* and *in situ*. Among them, toluidine blue,^{11–13,15–18} methylene blue,^{14,17} and, more recently, curcumin.^{19,20} The authors did not expect to find differences between the two PACT techniques proposed, because provided the PS agent selected is activated by a proper light source wavelength, the reactive products, such as singlet oxygen, would be generated and make direct or indirect contact with bacteria and damage their vital structures.³¹ Although there was a lack of difference between the PACT groups and the control group, the trend toward bacteria reductions highlights the need for future clinical studies testing new PACT protocols for carious dentin, since there have been new findings on the choice of light source and type of photosensitive agents. A recent study evaluated *ortho*-toluidine blue dye penetration into carious dentin and showed it could be detected up to ~50- μ m deep *in situ*, as measured by confocal Raman microspectroscopy. This reveals the capacity of the photosensitizer to really penetrate into a carious lesion, resulting in the death of microorganisms deep within dentin.¹⁸

A limitation of this clinical trial was the final number of patients enrolled to participate. It is especially critical to maintain children within the inclusion criteria, because of their higher rates of sickness and parents' cooperation. During the follow-up recalls at 6 and 12 months, none of the treated teeth showed any

pain symptoms or radiographic abnormalities. The demographic features of the children confirmed the high caries risk group, as shown by the DMFT of 5.3 and DMFS of 8.3. The acceptable value proposed by the WHO for the year of 2000 was of 50% of caries-free children at 5 years of age and DMFT lower than 3.0 at 12 years.³² The values of the present investigation are way above these predictions and emphasize the urgent need for primary dental care for this population.

This study was unable to show that PACT protocols were better than the control protocol used, nevertheless, all therapies reduced the number of microorganisms in dentin, except for *S. sobrinus*. Therefore, all therapies can be considered effective modern approaches to minimal intervention for the management of deep primary caries. However, it is important to emphasize that these results could be a starting point for new clinical searches for parameters for the use of low-power lasers and LED in PACT for microbial reduction.

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Biographies of the authors are not available.