



UNESP – Universidade Estadual Paulista
Faculdade de Odontologia de Araraquara



MARCO AURELIO BENINI PASCHOAL

EFEITO DA TERAPIA FOTODINAMICA ANTIMICROBIANA MEDIADA POR
CURCUMINA SOBRE *STREPTOCOCCUS MUTANS*

Araraquara
2013



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EFEITO DA TERAPIA FOTODINAMICA ANTIMICROBIANA MEDIADA
POR CURCUMINA SOBRE *STREPTOCOCCUS MUTANS*

Tese apresentada ao Programa de Pós-Graduação
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EFEITO DA TERAPIA FOTODINÂMICA ANTIMICROBIANA MEDIADA
POR CURCUMINA SOBRE *STREPTOCOCCUS MUTANS*

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Dedicatória

*A*creditar e proporcionar sonhos,

Conselheiro, amigo, ouvinte e guia,

Pai, avô e bisavô,

Ao meu querido avô-pai, Carlos Benini (*in memoriam*) dedico
este trabalho.

“O correr da vida embrulha tudo.

A vida é assim: esquentada e esfria,

aperta, daí afrouxa,

sossega e depois desinquieta.

O que ela quer da gente é coragem”

João Guimarães Rosa

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Resumo

Paschoal MAB. Efeito da terapia fotodinâmica antimicrobiana mediada por curcumina sobre *Streptococcus mutans* [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2013.

Resumo

Proposição: O trabalho investigou o potencial antimicrobiano fotodinâmico (PACT) mediado por um fotossensibilizador (FS) natural, curcumina (C), exposto a uma fonte de luz LED azul (L) aplicado sobre biofilme maduro de *Streptococcus mutans* (SM) formado sobre discos de hidroxiapatita e comparou esse potencial com biofilmes submetidos a imersão única com solução de clorexidina a 0.12% (CHX). Adicionalmente, verificou-se a eficácia de uma fonte de luz de amplo espectro (luz branca) de alta intensidade de potência na presença de C e azul de toluidina (T) a baixas concentrações expostos a um tempo extra curto de iluminação. **Material e Método:** Biofilmes de SM foram submetidos a tratamento com soluções de C e iluminadas com um LED azul (L) a 48 J/cm² e 72 J/cm² e a imersão em CHX. Adicionalmente, uma fonte de luz branca (42 J/cm²; 3410 mW/cm²) foi utilizada na presença de baixas concentrações de C e T por um período extra curto de iluminação sobre suspensões de SM. A eficácia das tratamentos foi realizada por meio da contagem de unidades formadoras de colônia (UFC/mg de biofilme seco e UFC/ml de suspensão) e análise morfológica dos biofilmes foram realizadas por meio de microscopia confocal a laser e microscopia eletrônica de varredura ambiental. **Resultados:** PACT (C a 2.5 mM e L a 48 J/cm²) apresentou a maior redução bacteriana substancial quando comparada a CHX. A luz branca apresentou fotossensibilização letal nos parâmetros utilizados. **Conclusão:** A utilização da curcumina, um corante natural e a luz branca, capaz de ativar FS em curtos períodos de tempo, são avanços nesse campo antimicrobiano alternativo.

Palavras-chave: Curcumina, Fotoquimioterapia, *Streptococcus mutans*.

Abstract

Paschoal MAB. Effect of photodynamic antimicrobial chemotherapy mediated by curcumin on *Streptococcus mutans* [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2013.

Abstract

Purpose: This work investigated the antimicrobial photodynamic potential (PACT) mediated by a natural photosensitizer (PS), curcumin (C), exposure to an source of blue LED (L) applied over a *Streptococcus mutans* (SM) mature biofilm formed on hidroxiapatite discs and compared this potential with biofilms submitted to single immersion of chlorhexidine at 0.12% (CHX). Additionally, it was verified the efficacy of a broad visible spectrum light source (white light) of high potency intensity in the presence of C and toluidine blue (T) at low concentrations and exposure to an extra short illumination time. **Materials:** SM biofilms were submitted to a treatment with C solutions and exposure to a 48 J/cm² e 72 J/cm² of L and immersion in CHX. Furthermore, a white light source (42 J/cm²; 3410 mW/cm²) was used in the presence of low concentrations of C and T for an extra short period over SM suspensions. The efficacy of the treatments were performed by colony forming units counting (CFU/mg of dry biofilm and CFU/ml of suspension) and the morphological analysis of biofilms were performed using confocal laser scanning microscopy images and environmental scanning electron microscopy. **Results:** PACT (C at 2.5 mM and L at 48 J/cm²) presented a bacterial substantial reduction when compared with CHX treatment. The white light showed lethal photossensibilization in the utilized parameters. **Conclusion:** The use of curcumin, a natural compound and the white light, able to activate FS at shorts periods, are advance on this antimicrobial alternative field.

Keywords: Curcumin, Photochemotherapy, *Streptococcus mutans*.

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1 Introdução

1 INTRODUÇÃO

A cárie dentária está entre as doenças crônicas contagiosas mais significantes na população humana mundial, afetando, sobretudo, populações de condições socioeconômicas precárias, muito embora os estudos epidemiológicos demonstrem uma redução de sua incidência. (Marsh ²⁵, 2003; Ribeiro, Longo ³⁰, 2013).

Essa doença, de caráter heterogêneo é caracterizada, quimicamente, pela perda de tecido duro em função de frequentes quedas de pH ocasionadas pela produção de ácidos por bactérias presentes no biofilme dentário (Buzalaf et al.⁶, 2008). Este biofilme, fortemente aderido as superfícies dos dentes, representa o fator biológico primordial e principal fator de virulência controlável na modulação da patogênese (Ribeiro, Longo ³⁰, 2013).

Biofilmes são comunidades complexas de micro-organismos organizados em uma estrutura tridimensional envolvida em uma matriz extracelular. Biofilme oral ou dentário é o termo utilizado para descrever o acúmulo de microrganismos na superfície dos dentes e, em geral, são formados sobre outras superfícies, além da superfície dentária. Ele é constituído por microcolônias de células bacterianas distribuídos em uma matriz de glicocalice. Estão presentes no biofilme oral: polissacarídeos, células epiteliais descamadas, leucócitos, enzimas, sais minerais, glicoproteínas salivares, proteínas, pigmentos e restos alimentares (Costa et al. ⁹, 2013).

O biofilme proporciona, como vantagens para os microrganismos, proteção diante de fatores ambientais como os mecanismos de defesa do hospedeiro e proteção em relação a substâncias potencialmente tóxicas (p. ex. antibióticos e antissépticos). O crescimento na forma de biofilme também pode facilitar a

obtenção de nutrientes, a utilização de nutrientes produzidos por outras bactérias, a remoção de produtos metabólicos tóxicos, assim como o desenvolvimento de um meio ambiente físico e quimicamente apropriados (Costa et al.⁹, 2013).

A relação entre a presença de carboidratos fermentáveis, o aumento da proporção de bactérias ácido-tolerantes, formação de um biofilme cariogênico e desmineralização dentária encontra amplo suporte científico (Bowen, Koo,⁴ 2011; Duarte et al.¹³, 2008; Loeshe²⁴, 1986; Marsh²⁶, 2006; Paes Leme et al.²⁸, 2006; Rolla³¹, 1989; Koo et al.²², 2009).

Dessa maneira, medidas preventivas para a cárie dentária devem incluir desorganização periódica da estrutura do biofilme, por ações mecânicas, realizadas por meio de escovação e fio dental. Entretanto, este hábito resulta em apenas 40% da remoção do biofilme, o que acarreta uma rápida reorganização e recolonização, não sendo adequado para se atingir um ótimo nível de remoção do substrato (Slot et al.³², 2007). Além disso, a efetividade da escovação está atrelada diretamente a fatores como destreza, adesão ao hábito e maturidade do indivíduo (Frandsen¹⁷, 1986; Teitelbaum et al.³³, 2009; Wilson³⁵, 1987). Sendo assim, práticas adjuntas ao controle mecânico da placa são necessários.

Numerosos agentes químicos tem sido avaliados para a suplementação do controle mecânico da placa do paciente e, conseqüentemente, reduzir a atividade ou prevenir doenças bucais, como a cárie dentária (Slot et al.³², 2007). Os compostos a base de bisbiguanida, os quais incluem o gluconato de clorexidina e alexidina, são os agentes mais efetivos utilizados atualmente (Slot et al.³², 2007). Clorexidina é uma biguanida catiônica com excelentes propriedades bacteriostáticas (Slot et al.³², 2007). A superioridade desse agente como oposto por outros agentes químicos usados no controle da placa deriva da persistência desse agente (substatividade) que prolonga sua ação anti-bacteriana (Kornman

²³, 1986). Resultados otimizados quando do uso adjunto da clorexidina e a remoção de placa rotineira tem sido amplamente bem documentado em pesquisas clínicas a curto e longo prazo (Corbet et al.⁸, 1997; Charles et al.⁷, 2004).

Entretanto, investigações tem apontado que seu uso por períodos prolongados sem supervisão ou utilizada de forma aleatória, pode levar a formação de cálculo dentário, manchamento do tipo extrínseco amarronzado das superfícies dentárias e descamação da mucosa (Flotra et al.¹⁶, 1971; Overholser et al.²⁷, 1990). Twetman ³⁴ (2013) reportou, após análise minuciosa de estudos clínicos randomizados atuais e metanálise relativa a essa substância, não existir consenso quanto a segurança na prescrição da clorexidina ao que concerne a prevenção e controle da cárie e gengivite na prática da higiene bucal diária.

O desenvolvimento e a inserção de novas estratégias terapêuticas na prevenção da cárie dentária podem contribuir para o controle da população microbiana cariogênica sobretudo em pessoas com alto risco a cárie, agregando valores as manobras terapêuticas existentes. Sendo assim, alternativas para inativação eficiente de microrganismos presentes no biofilme bucal, como a terapia fotodinâmica antimicrobiana (PACT, do inglês, *Photodynamic Antimicrobial Chemotherapy*) tem sido proposta (Ribeiro, Longo ³⁰, 2013).

A ação fotodinâmica requer a sensibilização das células/tecidos alvos por um agente fotossensibilizador (FS), em geral exógeno, que, ao ser ativado por uma fonte de luz na presença do substrato oxigênio, resulta na formação de espécies de oxigênio reativas capazes de promover a morte celular (Ribeiro, Longo ³⁰, 2013). Trata-se de uma técnica não invasiva e localizada, cujos benefícios estimulam a sua inserção na rotina clínica devido a seletiva e a rápida inativação da viabilidade microbiana após fotoativação (Wood et al. ³⁶, 2006;

Paschoal et al.²⁹, 2013). Isso demonstra mínima capacidade mutagênica sem favorecer o desenvolvimento de resistência microbiana decorrente da grande toxicidade das espécies reativas de oxigênio (ROS, do inglês, *Reactive Oxygen Species*) e em função de atingir diferentes alvos, como membrana plasmática, mitocôndria e núcleo (Dougherty ¹¹, 2002).

Dificuldades relacionadas ao custoso processo de purificação de FS sintéticos, potencial de corar a estrutura dentária, restaurações a base de resina e cavidades recém-preparadas fazem com que a PACT ainda seja limitada na prática clínica (Giusti et al.¹⁸, 2008).

Partindo-se dessa problemática, a busca de corantes ou FS naturais que apresentem propriedades antimicrobianas e que venham a sobrepor as dificuldades atualmente encontradas é necessária.

Sendo assim, a curcumina, um pigmento amarelo extraído do rizoma da planta *Curcuma Longa L* tem apresentado destaque no campo fotodinâmico. Historicamente, esse corante tem sido usado pela medicina oriental, principalmente a Indiana, devido a suas propriedades farmacológicas, dentre elas, anti-inflamatórias, anticarcinogênicas e antifúngicas (Aggarwal et al.¹, 2007; Epstein et al.¹⁵, 2010; Goel et al.¹⁹, 2008; Hatcher et al.²¹, 2008). Estudos atuais atestam que suas propriedades antimicrobianas poderiam ser exacerbadas quando de sua iluminação com comprimentos de onda adequados (Bruzell et al.⁵, 2005; Dujic et al.¹⁴, 2009; Dovigo et al.¹², 2011; Araújo et al.³, 2012). Além disso, seu baixo custo, eficiência na geração de ROS e fácil manipulação fazem desse corante um potencial FS para a inativação de espécies presentes em biofilmes (Araújo et al.², 2012; Araújo et al.³, 2012; Dovigo et al.¹², 2011).

O tempo dispendido para a fotoativação dos FS também é um desafio na PACT. Trabalhos reportam tempos que variam de 15 a 60 minutos para

sensibilizar espécies de *S. mutans* (Wood et al.³⁶, 2006), *Escherichia coli*, *Enterococcus faecalis* (Denis et al.¹⁰, 2011), *Metarhizium anisopliae* and *Aspergillus nidulans* - espécies fúngicas (Gonzales et al.²⁰, 2011) na presença de diferentes FS e luz de amplo espectro, o que, clinicamente, são propostas inviáveis.

Nesse contexto, investigar o potencial antimicrobiano da curcumina, um corante natural e a eficácia de uma nova fonte de luz de amplo espectro a uma alta intensidade de potência com vistas a diminuir o tempo de iluminação são propostas viáveis relativas a essa temática.

2P_{proposição}

2 PROPOSIÇÃO

Objetivo geral

Investigar o potencial fotodinâmico antimicrobiano da curcumina quando exposta a comprimento de onda no espectro azul por um diodo emissor de luz sobre biofilme maduro formado por *Streptococcus mutans* comparando essa eficácia com o “padrão-ouro”; além de avaliar o efeito de uma nova fonte de luz de amplo espectro e alta densidade de potência sobre suspensões planctônicas de *Streptococcus mutans*.

Objetivos específicos

Estudo 1

Avaliar, *in vitro*, o potencial fotodinâmico antimicrobiano da curcumina exposta a uma fonte de luz LED no comprimento de onda azul sobre biofilme maduro de *Streptococcus mutans*.

Estudo 2

Avaliar, *in vitro*, o potencial fotodinâmico antimicrobiano da curcumina exposta a uma fonte de luz LED no comprimento de onda azul sobre biofilme maduro de *Streptococcus mutans* e comparar a clorexidina a 0.12% em solução.

Estudo 3

Investigar o efeito fotodinâmico de uma nova fonte de luz de amplo espectro e alta densidade de potência na presença de azul de toluidina e curcumina sobre suspensões planctônicas de *Streptococcus mutans*.

3 Capítulos

3 . 1 Capítulo 1*

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EFFECTS OF PHOTODYNAMIC ANTIMICROBIAL CHEMOTHERAPY MEDIATED BY CURCUMIN ON *STREPTOCOCCUS MUTANS* MATURE BIOFILM

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ABSTRACT

Background: Photodynamic antimicrobial chemotherapy (PACT) studies have shown promising results for inactivation of cariogenic microorganisms organized in planktonic suspensions, but minor attention is given to biofilm models. The present investigation verified the efficacy of curcumin, a natural compound used as photosensitizer excited by a blue LED light on a mature *Streptococcus mutans* biofilm. **Methods:** The treatments were performed using 2.5 mM or 5 mM of curcumin (C) excited by LED light (L) in the blue wavelength (240.1 mW/cm²; 450 nm $\pm$ 30) operated at 48 and 72 J/cm² corresponding to 200 and 300 s of light exposure, respectively. *S. mutans* UA159 biofilms were formed on saliva-coated hydroxyapatite discs in batch culture at 37°C, 5% CO₂. Tryptone-yeast extract broth containing 1% sucrose was changed daily. After the 5th day, the mature biofilm was treated with C and L (C⁺L⁺; PACT group); without C and L (C⁻L⁻; control); with C and without L (C⁺L⁻); without C and with L (C⁻L⁺). Biofilms were assessed by microbial viability (colony forming units - CFU/mg of biofilm dry weight) and morphological analyses by variable pressure scanning electron microscopy (VPSEM) and confocal laser scanning microscopy (CLSM). **Results:** The application of PACT demonstrated a substantial photokilling reduction rate in comparison to control groups. The morphology of biofilms did not differ among the experimental conditions and confocal images showed that PACT treatment resulted in a high proportion of dead cells. **Conclusion:** Curcumin is a natural, non-toxic photosensitizer that presented a potential photodynamic antimicrobial effect against a mature *S. mutans* biofilm.

Key-words: bacteria; biofilm; dental caries.

INTRODUCTION

Dental caries is still the most significant human chronic contagious disease and results from interactions over time between specific groups of bacteria associated with a rich diet constituted by fermentable carbohydrates and dental biofilms known as dental plaque [1-3]. The persistence of these structures over the hard tissues allows the irreversible destruction of mineralized structures of teeth, compromising the dental vitality and the fixation of the element in the maxillomandibular complex [4].

Streptococcus mutans, gram-positive aerotolerant anaerobic bacteria, is considered as one of the most cariogenic microorganism present in the dental biofilm [5,6] due to extracellular polysaccharides (EPS) production, mostly glucans, from sucrose using glucosyltransferase (GTFs) [7,8]. This property, associated with acidogenic and acid-tolerant characteristics, are critical virulence factors that maintain the low pH of the oral environment, and, consequently, enhancing demineralization of the dental tissue [5,9,10]. Furthermore, the presence of glucans promote the accumulation of microorganisms on tooth surface, and contribute to the establishment of EPS matrix, which provides bulk, structural integrity for dental biofilms, and acts as a protection factor against antimicrobial substances [2,11,12].

Mechanical tooth cleaning by means of a toothbrush is considered as the most common way of controlling the plaque accumulation. However, the effectiveness is dependent on factors such as dexterity and compliance of the individuals, sometimes, not achieving an optimal level of plaque removal [13]. On this way, the adjunctive use of mouthwashes containing antimicrobial agents such as chlorhexidine, triclosan or fluoride [14-16] in combination with mechanical debridement has been shown to be effective in the prevention of caries [17].

Biofilms exhibit several antibiotic-resistance mechanisms to antimicrobial agents [18-20]. In addition, the use of chlorhexidine have demonstrated some adverse effects such as a moderate to severe staining of teeth, excess formation of supragingival calculus, allergic responses and taste alteration [21,22]. Furthermore, disruption of the oral microbiota and the difficulty of maintaining therapeutic concentrations of the active agent in the oral cavity are also problems associated with the use of that substances [23]. Thus, there is a need on the development of alternative antimicrobial techniques [24].

Photodynamic antimicrobial chemotherapy (PACT) has been suggested as an alternative approach to antimicrobial agents aiming inactivation of microorganisms involved in the etiology and development of oral biofilms [25-28]. PACT is a therapeutic modality which employs the combination of visible light, a drug (called photosensitizer – PS or dyes) and molecular oxygen. The combination of these components produces reactive oxygen species (ROS) which are capable of reacting with intra and extracellular structures causing irreversible damage and cell death [29,30].

The PS most used are the merocyanine derivates, phtalocyanines, hematoporphyrin, xanthene dyes and phenotiazinium [31]. The choice of an ideal agent is one of the challenges in the PACT due to possibility of teeth staining and high costs of purification which limits the clinical applicability of this novel approach [32,33].

Curcumin, a compound isolated from *Curcuma longa L.*, is the most biologically active phytochemical component of a popular Indian spice and has been used for centuries as a medicine, and as dietary pigment as well. The drug displays a variety of biological properties such as antiproliferative activity against cancer cells and antioxidant activity [34,35]. This dye exhibits significant

antimicrobial activity *in vitro* against a number of Gram-negative and Gram-positive bacteria including *Bacillus subtilis*, *Escherichia coli*, *Helicobacter pylori*, and some *Staphylococcus aureus* strains [36,37]. Recent investigations showed that the antimicrobial effects of curcumin may be enhanced by combination with light, especially in the blue spectral region [38,39]. Great effectiveness, low cost and simple manipulation represent some advantages when using this PS [40].

Several studies have shown that oral bacteria in planktonic cultures and in dental plaque scrapings are susceptible to PACT [41,42]. However, the current literature presents an absence of investigations regarding PACT applicability on biofilm models and its effectivity. Thus, more evidence concerning the impact of PACT on microorganisms present on cariogenic biofilm is necessary aiming to guide future clinical photodynamic applications.

Based on these findings, the aim of this present study was investigate the effects of PACT mediated by curcumin on a mature biofilm model formed by *S. mutans*.

MATERIAL AND METHODS

Photosensitizer and light source

The photossensitizer used was curcumin (C) (Sigma Aldrich, St Louis, MI, USA) at final concentrations of 2.5 and 5 mM (diluted in DMSO, 10% final concentration). A light emitting-diode (LED) (L) in the blue wavelength was used to curcumin activation. The device (Prototype, Project Finep/ Gnatus LED Edixeon, Edison Opto Corporation, New Taipei City, Taiwan) provided an emission with a central wavelength of 450 nm $\pm$ 30 and the intensity (power density) of this light was 240.1 mW/cm². The irradiances (energy fluency) tested in the study were 48

and 72J/cm². The work distance utilized was 5 mm and the light exposure times were 200 and 300 s, respectively.

In vitro biofilm formation

Hydroxyapatite discs (Clarkson Chromatography Inc, South Williamsport, PA) were coated with filter-sterilized whole saliva (sHA). Cells of *S. mutans* UA 159 (ATCC 700610) were grown in buffered tryptone yeast extract (TSBYE) containing 1% (wt/vol) glucose. *S. mutans* biofilms were formed on sHA discs placed horizontally in a 24-well plate containing 1% (wt/vol) sucrose in batch culture at 37°C in 5% CO₂. The culture medium was replaced daily for 5 days [43,44]. At the end of the experimental period (120h-old biofilms), the biofilms were submitted to the treatments.

PACT on in vitro biofilms

After 5 days of biofilm formation, sHA discs containing the biofilms were transferred to other 24-well plates containing curcumin at 2.5 mM or 5 mM (groups C+L+ and C+L-) or 10% of DMSO (groups C-L- and C-L+) during the pre-irradiation time (PIT) of 1 min in the dark. The PIT was selected based on preliminary data (data not shown). Following this time, the biofilms were exposed for 200 or 300 s to LED irradiation (groups C+L+ and C-L+) or kept at room temperature during the same period (groups C-L- and C+L-). The biofilms were scraped with a sterile spatula and transferred to tubes containing 5 ml of PBS and subjected to sonication using three 15 s pulses with an interval of 15 s at an output of 6 W (Branson Sonifier 150; Branson Ultrasonics, Danbury, CT) [44]. The volume of 1 ml of the homogenized suspension was transferred to a preweighed microtube and was dried in a Speed Vac concentrator and used for the determination of dry weight. A sample of 100 µl was used to perform a ten-fold serial dilution and

aliquots of the diluted samples were plated onto blood agar and then incubated at 37°C, 5% CO₂ for 48 h to investigate the number of viable microorganisms. The results were expressed as colony-forming units (CFU) per milligram of biofilm dry weight and transformed into log₁₀ constituting the quantitative analysis.

Biofilm characterization

Confocal laser scanning microscopy (CLSM)

Two groups (C+L+ and C-L-) were selected for confocal laser scanning microscopy (CLSM). The viability of bacteria within the biofilms was determined by staining the biofilms with LIVE/DEAD *BacLight* Bacterial Viability kit (Molecular Probes, Inc., Eugene, OR, USA) which includes two fluorescent nucleic acids stains: green-SYTO 9 and red-propidium iodide. The biofilms were treated according to the manufacturer's instructions, being stained immediately after the treatments, kept in the dark and protected from light until the analysis. Stained biofilms were examined with a confocal scanning fluorescence microscope (Leica TCS SP 5 II Confocal microscope, Leica Microsystems, Wetzlar, Germany) using specific filters, 488/507 nm for detection of SYTO 9 and 503/615 nm for the detection of propidium iodide.

Variable pressure scanning electron microscopy (VPSEM)

To verify if there was any changes on the topography/morphology of the biofilms after the application of PACT, samples were analyzed by variable pressure scanning electron microscopy (VPSEM) with an EVO® 50 Series microscopy (Carl Zeiss, AG, Germany). This microscope is able to obtain images without the need of samples pre-treatment, keeping the natural aspect and characteristics of fresh biofilms right after the application of the therapies. The acquisition of these images

was performed by the combination of vacuum and pressure at different field widths.

Statistical analysis

The assays of PACT effect were performed in duplicate for each group and the procedure was repeated three times on different days ($n = 6$). The mean and the standard deviation (SD) of the numbers of CFU/mg of dry weight biofilm for each treatment were calculated. CFUs were transformed into $\log_{10}$ in order to reduce variance heterogeneity. A two way ANOVA test followed by Dunnet-t test were used to verify the differences among all the studied groups. The cut off level of significance was set at 2.5%.

RESULTS

The antimicrobial effect of PACT using two different curcumin concentrations under light dosimetries at 48 and 72 J/cm² on the viability of *S. mutans* biofilms after 5 days is showed in Figures 1a and 1b, respectively. There was a significant reduction in CFU/mg of *S. mutans* biofilm when exposed to light irradiation in the presence of PS (PACT group; C+L+) in comparison to control group ($p < 0.025$). On the other hand, there was no antimicrobial effect when sensitizer (C+L-) and light (C-L+) were tested alone ($p > 0.025$).

INSERT FIGURE 1

The effect of PACT on *S. mutans* biofilm was analyzed by images performed by CSLM. In the Figure 2a it can be visualized that the biofilms treated with PACT (C+L+) presented more red fluorescence than the control biofilms

(C-L-). Red cells represent dead cells (Figure 2b). The control biofilm (C-L-) presented more green fluorescence, indicating a high level of viable cells.

INSERT FIGURE 2

The analysis of VPSEM images demonstrated no morphological changes when the biofilms were treated with PACT (C+L+) or all the controls (C+L-, C-L+, or C-L-). Since the experimental conditions presented high morphological similarity, just C-L- and C+L+ groups are shown (Figures 3 and 4, respectively).

INSERT FIGURES 3 AND 4

DISCUSSION

The present study investigated the photodynamic effect of PACT mediated by curcumin exposure to a blue LED light on *in vitro* *S. mutans* mature biofilm. The biofilms illuminated by the LED light source in the presence of the studied dye presented a significant decrease on viable microorganisms, showed by CFU/mg of biofilm and CLSM. The bacterial photokilling rate achieved log reductions of 3.09 and 1.74 when treated with 2.5 mM and 2.51 and 1.78 when treated with 5 mM of PS, both at 48 and 72J/cm², respectively. On the other hand, no statistical difference was observed in the control groups blue LED (C-L+) or curcumin alone (C+L-) ($p > 0.025$). Similar results were obtained by several other authors, in which neither the irradiation in the absence of PS nor alone incubation had a significant effect on the viability of *in vitro* microorganisms in both planktonic and biofilm models [27,28,45-48]. The most acceptable hypothesis for the photodynamic

action of curcumin is related to the lipid membrane and protein binding photodamage [42].

In accordance with our results, increasing the parameters did not enhance the antimicrobial effects of PACT. This same outcome pattern was confirmed by previous studies using different PS and light dosages [27,49,50]. This fact is directly related with ROS production and its interaction with bacteria [51]. Dovigo et al. [27] suggested that longer irradiation times produced lower quantities of ROS due to high photobleaching rate. They observed that the light absorption and fluorescence of curcumin decreased as a function of illumination time (light fluence). The spectral changes monitored were a measurement of the induced photobleaching of the curcumin molecules and was used as an indirect measurement of the potential photodynamic response. Furthermore, the excess of dye in the solution at high concentrations could result in optical quenching by preventing the light from reaching the bacteria [49].

This investigation used a light-emitting diode in a blue wavelength to excite curcumin, promoting optimization of PACT process. The advantages in the use of LED are simple manipulation, affordable price and the light is already present in the dental offices. In order to evaluate the performance of both light sources, Zanin et al. [45] demonstrated that the use of a He-Ne laser or a red LED in combination with TBO presented the same antimicrobial effect on *S. mutans* biofilm viability. To corroborate with this finding, Giusti et al. [32] verified a preponderant bactericidal effect using a LED light at 24 J/cm² in combination with 2 mg/ml of Photogem® on *S. mutans* and *Lactobacillus acidophilus* presented in artificial carious bovine dentin. Similar results were obtained when *S. mutans* mature biofilms were irradiated by an energy density at 55 J/cm² of a red LED in the presence of TBO

[47]. Hence, the usage of light-emitting diodes at different wavelengths that cover a broad visible spectrum of light is a trend in PACT approach.

Recently, the antimicrobial role of curcumin enhanced by specific light application has been investigated [38,52]. This PS presents absorption ranging from 300 to 500 nm with a central peak at 450 nm that corresponds with the emission of the tested LED. Curcumin exhibits a wide range of pharmacological effects, including anti-inflammatory, anti-carcinogenic, and anti-infection activities [53]. As a potent anti-oxidant, this dye also has shown anti-proliferative and anti-carcinogenic properties in a wide variety of cell lines and animals. [54,55]. One of the disadvantages is that curcumin has a low solubility in water. Thus, in the present study, curcumin was dissolved at 10% DMSO (based on previous study) [27] that demonstrated no toxic effects on the microorganism *S. mutans* viability. Further studies need to be performed to improve curcumin solubility. Investigations in planktonic cultures, [49,56] clinical isolates [57] and biofilms [27] showed that curcumin was an effective PS in PACT. Dovigo et al. [27] inactivated *C. albicans* species in a planktonic model using a blue LED (440 – 460 nm) at 5.28 J/cm² in the presence of 20 µM of curcumin. Yet, this same study applied this same light protocol using curcumin 4 times more concentrated in a biofilm model achieving a statistical significant difference in fungi reduction, however with a lower degree of photokilling. Still concerning fungi species, a combination of curcumin at 40 µM and an 18 J/cm² of a blue LED light source was effective in the inactivation of clinical isolates of *C. albicans*, *C. tropicalis*, and *C. glabrata* [57]. Considering cariogenic microorganisms, Araujo et al. [40,56] reported that photodynamic therapy mediated by a curcumin salt and exposed to a light source at 450 nm was

found to be effective in the reduction of both salivary microorganisms in an *in vivo* approach and *S. mutans* and *L. acidophilus* on planktonic cultures as well.

In the present study we evaluated the effect of PACT mediated by curcumin on mature biofilms. Bacteria in biofilms have been shown to be less affected by a photodynamic procedure than bacteria in the planktonic phase [58]. The presence of biofilm matrix allows stability and structural integrity, limits the diffusion of substances and, at same time, provides protection to the bacteria from inimical influences of antimicrobials and other environmental assaults [9,59]. Furthermore, that the more complex the composition of biofilms, the more resistant it seems to PACT process [60]. One of the explanations is that the interactions between the different matrix polymers, produced by different microorganism, might result in a more viscous matrix [60].

The average bacterial reduction of this investigation was $1.74 - 3.08 \log_{10}$ in PACT groups. Biofilms formed by *S. aureus* treated with MB (0.1 mg/ml) and an indium-galium-alumini-phosphide (InGaAlP) laser for 98 s presented more sensitivity to photodynamic therapy achieving a reduction of $3.29 \log_{10}$ when compared to an average reduction of $2.81 \log_{10}$ of *S. mutans* biofilms [60]. Sharma et al. [61] found $4.5 \log_{10}$ of CFU reduction methicillin-resistant *S. aureus* biofilms, using Toluidine-Blue O (TBO), followed by irradiation with 640-nm laser diode. Similar results were found using MB (0.008 mg/ml) irradiated with a 400 W source of light [42] and TBO (0.1 mg/ml) treated with a He-Ne laser, [45] presenting $1.5-2.6 \log_{10}$ and $2.10-3.11 \log_{10}$ CFU reduction, respectively.

The morphological analysis of biofilms after the procedures was performed by CLSM and VPSEM images. CLSM investigated the proportion of live and dead cells after PACT application and is considered a method that confirms therapy

effectiveness [62]. This study used the Live/dead BacLight™ bacterial viability kit (Invitrogen) that distinguishes between bacteria with damaged and undamaged cell membranes, which provides a two-color fluorescence assay of bacterial viability. The SYTO 9 dye penetrates both viable and nonviable bacteria, while the propidium iodine penetrates bacteria with damaged membranes and quenches SYTO 9 fluorescence. Dead cells that take up propidium iodine fluoresce red while viable cells fluoresce green [61]. Overall, the Figure 2b illustrates the effectiveness of PACT by mostly red fluorescence (dead cells) whereas no treated biofilms showed viable cells by green fluorescence in the Figure 2a.

The VPSEM images complemented the confocal images in this study. VPSEM abolishes the need for fixation of the sample, and is able to reveal, right after the PACT application, the modifications in the biofilm without desiccation or structural damages, usually verified in scanning electron microscopy or transmission electron microscopy [63]. As observed, there were no morphological changes between biofilms treated with PACT and control [60].

CONCLUSION

Further studies need to be performed to verify curcumin penetration within the biofilm, toxicity in mammalian cells, and also improvement on its solubility, but the results reported in this work demonstrated that a single application with the natural compound curcumin-mediated PACT activated with a blue LED light is a promising antimicrobial approach for the treatment of mature *S. mutans* biofilm.

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FIGURES LEGENDS

Figure 1. (a) L at 48J/cm² and C at 2.5 and 5 mM. Treatment effects with C and L (PACT group: C+L+), with C and without L (PS group: C+L-), without C and with L (Light group: C-L+) without C and without L (control group: C-L-) on the viability of *S. mutans* biofilms. Data represent mean values (n = 6). **(b)** L at 72J/cm² and C at 2.5 and 5 mM. Treatment effects with C and L (PACT group: C+L+), with C and without L (PS group: C+L-), without C and with L (Light group: C-L+), and without C and without L (control group: C-L-). Data represent mean values (n = 6). Error bars represent standard deviation and data followed by different letters differ statistically (p < 0.025).

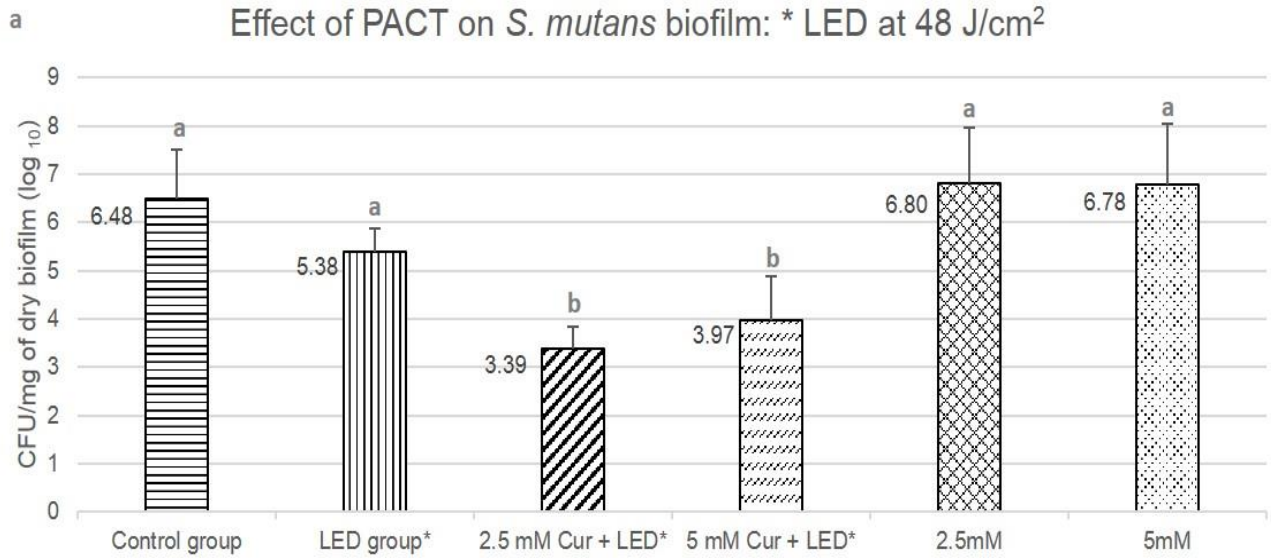
Figure 2. CLSM images of biofilms at 120 h incubation time in 1% sucrose. Viable and affected bacteria are represented by green and red fluorescence, respectively. **(a)** Control group submitted to 10% DMSO for 1 min. **(b)** Biofilm submitted to PACT at 5 mM of curcumin exposure at 72J/cm² of blue LED light.

Figure 3. VPSEM image of control group (C-L-). **(a)** Magnification at 3000 µm. **(b)** Magnification at 500 µm.

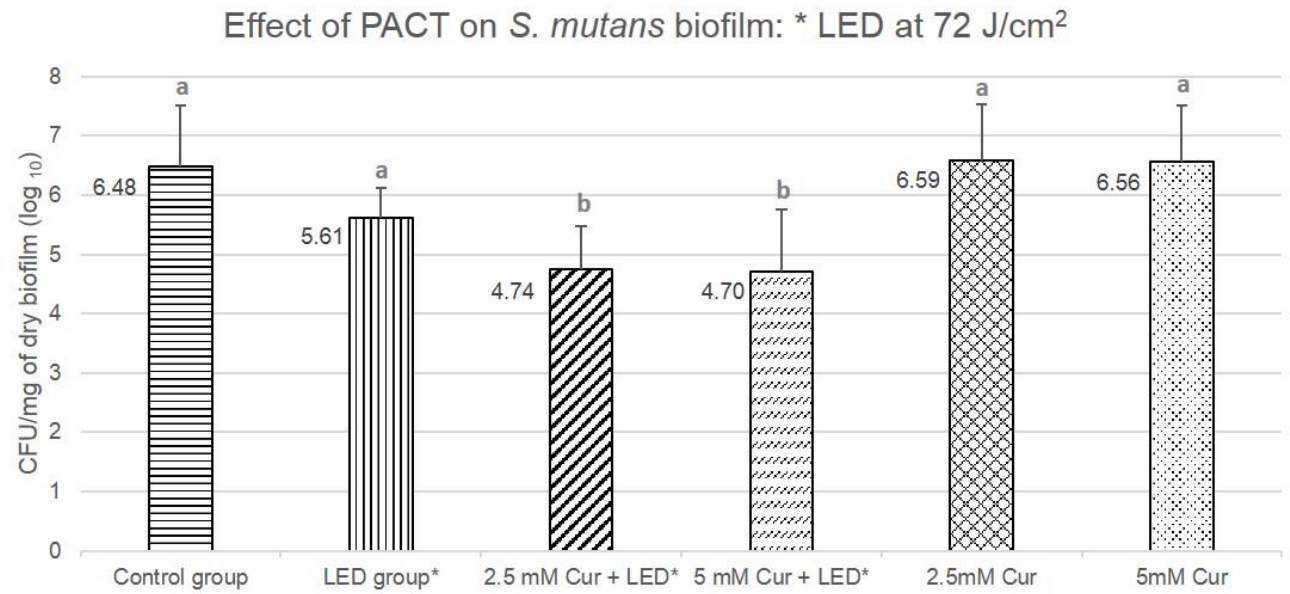
Figure 4. VPSEM image of PACT group submitted to 5mM of curcumin exposure at 72J/cm² of blue LED light. **(a)** Magnification at 3000 µm. **(b)** Magnification at 500 µm.

FIGURES

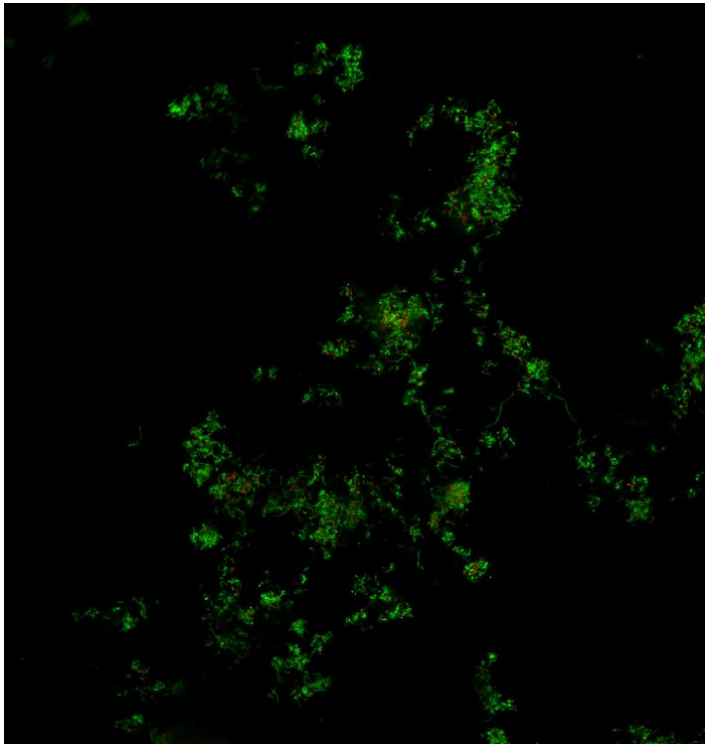
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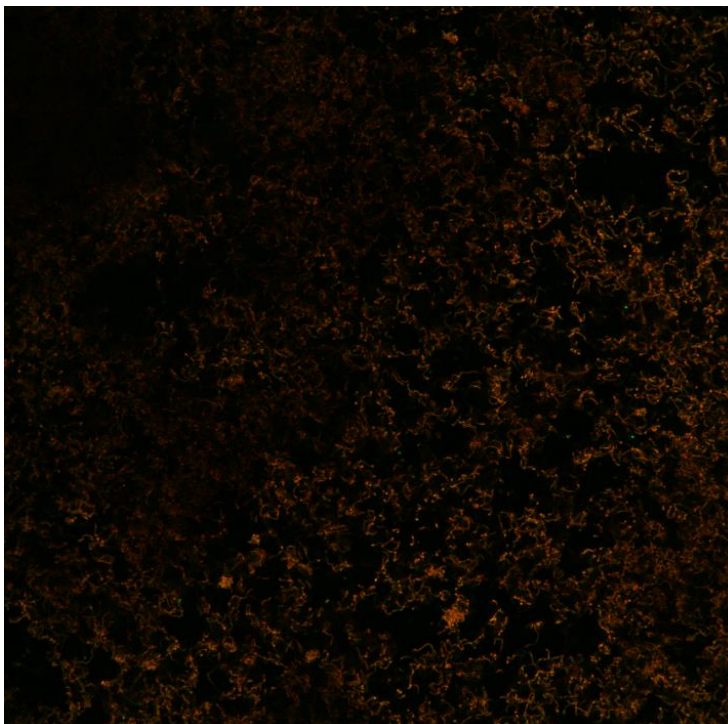
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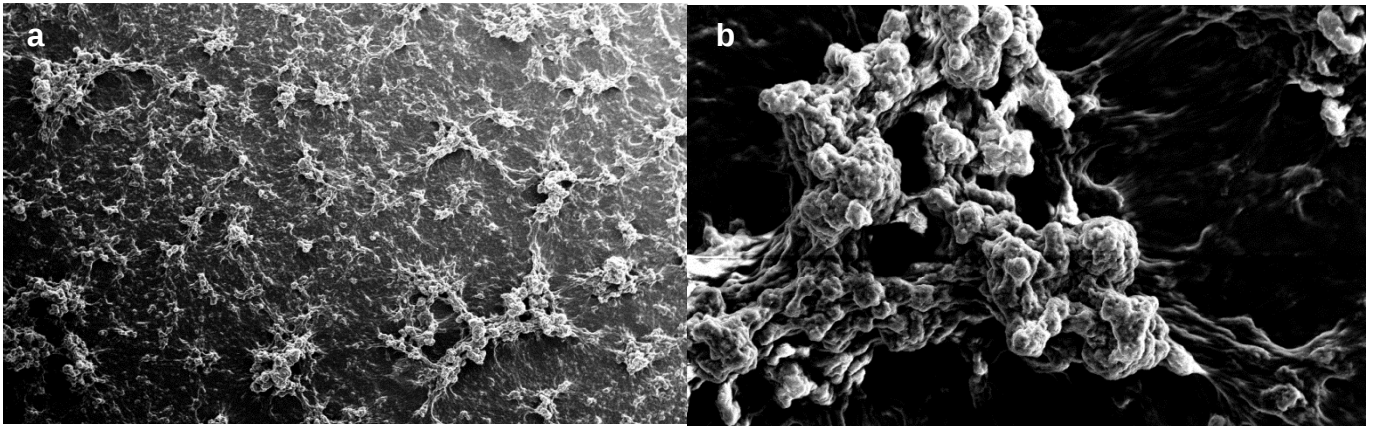
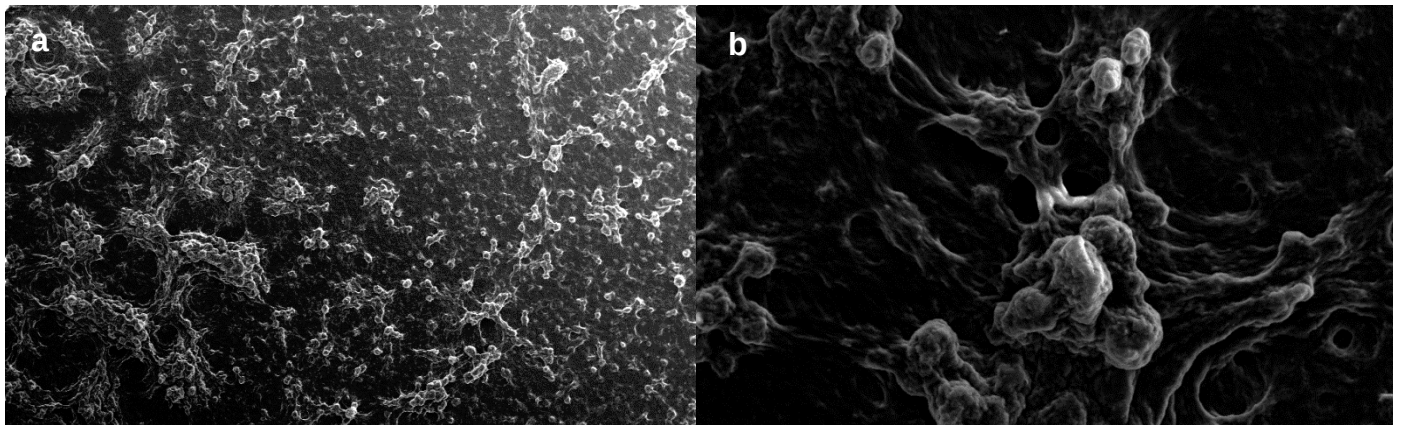


2.a.



2.b.



3.a and 3.b.**4.a. and 4.b.**

3 . 2Capítulo2*

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EFFECTS OF CURCUMIN PHOTODYNAMIC ANTIMICROBIAL CHEMOTHERAPY AND CHLORHEXIDINE ON *STREPTOCOCCUS MUTANS* MATURE BIOFILM

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ABSTRACT

Maintaining an adequate low level of plaque through brushing is often not feasible. Effective chemotherapeutic agents as an adjuvant to mechanical plaque control would therefore be valuable. Chlorhexidine mouthwash has been proved to be an effective inhibitor of plaque accumulation. However, undesirable side effects has arising to uncontrolled use of this substance. Photodynamic antimicrobial chemotherapy (PACT) is a promising antimicrobial alternative that presents a broad spectrum of action, including the efficient inactivation of microorganisms in dental plaque capable of prevent oral diseases organized in biofilms, such as dental caries. **Objectives:** This investigation aimed to compare the efficacy of PACT mediated by curcumin, a natural compound used as photosensitizer, excited by a blue LED light and a chlorhexidine solution over a mature *Streptococcus mutans* biofilm. **Methods:** The treatments were performed using 2.5mM of curcumin (C) submitted to a LED light activation in the blue wavelength (BL) ($240.1\text{mW}/\text{cm}^2$; $450\text{ nm} \pm 30$) operated at $48\text{ J}/\text{cm}^2$ and a 0.12% of chlorhexidine solution (CHX). *S. mutans* UA159 biofilms were formed on saliva-coated hydroxyapatite discs in batch culture at 37°C , 5% CO_2 . Tryptone-yeast extract broth containing 1% sucrose was changed daily. After the 5th day, the mature biofilm was treated without C and BL (C⁻BL⁻, control group); with C and without BL (C⁺BL⁻, curcumin group); without C and with BL (C⁻BL⁺, LED group) and with C and L (C⁺BL⁺, PACT group) and 0.12% chlorhexidine rinsing (CHX group). Biofilms were assessed by microbial viability (CFU/mg of biofilm dry weight) and morphological analyses by environmental scanning electron microscopy (ESEM) and confocal laser scanning microscopy (CLSM). **Results:** The biofilms submitted to PACT application demonstrated a significant decrease in the CFU counts with no antimicrobial effect to CHX group when compared to control group. Confocal images supported the quantitative analysis; high proportion of viable cells emitting green fluorescence was found in CHX group whereas PACT biofilms demonstrated dead cells by red fluorescence. Contrarily, ESEM images provide no changes on the morphology of the tested biofilms to different treatments. **Conclusion:** *S. mutans* mature biofilms were photosensitized by curcumin, a natural photosensitizer, demonstrating that PACT can be considered an adjunctive antimicrobial tool aiming to decrease the levels of dental plaque accumulation expanding the preventive options related to dental caries.

INTRODUCTION

The oral cavity is colonized by a diverse community of microorganisms living in equilibrium (“normal oral microflora”).¹ One peculiarity of this environment is that most of bacteria is found as complex aggregates known as biofilms and are present on the surface of the teeth.²

The accumulation of these bacterial biofilms in combination with a high intake of dietary fermentable carbohydrates decreases the pH allowing the development of pathogenic bacteria as *Streptococcus mutans*, main organism involved with the etiology of dental caries and towards the breakdown of microbial homeostasis to dental demineralization.³

Current treatment regimens for biofilm-related diseases involve the mechanical removal of the causative organisms. However, the efficiency of conventional therapy may not be completely satisfactory in certain cases; thus, the adjuvant use of antimicrobial agents are desired to achieve a high reduction of bacterial levels.⁴

Numerous authors have pointed that chlorhexidine has a greater *in vivo* immediate antibacterial effect and a greater substantivity than other antiseptics used in the oral cavity.⁵⁻⁷ Although, it is discussed the difficulty in obtaining a long and significant decrease in the *Streptococcus mutans* resident cells after chlorhexidine regimen.⁸ Furthermore, the indiscriminate use of this substance can generate some side effects as alteration in taste, teeth and restorations staining, burning sensation.^{9,10} Therefore, alternatives for an efficient adjunctive removal of cariogenic bacteria are needed. Such a novel possibility is the application of photodynamic antimicrobial chemotherapy (PACT).¹¹ Briefly, upon illumination, a photosensitizer (PS) is exposed to a specific wavelength from a light source. During this process, free radicals of

singlet oxygen and reactive oxygen species (ROS) are formed, which then produce an toxic effect to the membrane, mitochondria or the nuclei of the target cells previously PS-binded.¹² Light and PS are non-toxic by themselves; hence only cells containing dyes and receiving light are affected by this intervention.¹³

PACT is bacterial-resistance free due to different ROS action sites of damaged cells; allied to this main characteristic, rapidness, non-invasiveness, easy repeatability represents advantages in relation to mechanical debridement.¹⁴

In vitro studies have shown PACT to completely elimination of *Streptococcus mutans*.^{15,16} Furthermore, this novel antimicrobial treatment have demonstrated a high rate of photosensitization on biofilm structures formed by *Candida albicans*, *A. actinomycetemcomitans* and cariogenic bacteria.¹⁷⁻²¹ In addition, similar reductions of bacterial biofilms involved with peri-implantitis were achieved when compared the antimicrobial effects of a chlorhexidine regimen with those of photodynamic application.²²

In this field, there are several PS options. Among of them, the most used are the phenotiazinium dyes (toluidine blue O and methylene blue),²³⁻²⁶ porphyrin derivatives (Photogem® and Photosan®)^{24,27} and xanthene (Rose Bengal).²⁸ However, limitations regarding the process of purification and possibility of tooth and restoration staining become difficult the PACT applicability in the dental practice.^{24,29}

Some studies have proposed curcumin as a feasible PS to be applied over fungi²¹ and cariogenic planktonic³⁰ and on its biofilm counterparts²¹ due to its remarkable characteristics such as easy handling, low cost and great effectiveness.^{21,31} Curcumin has been used for centuries as a medicine, dietary pigment, and spice.^{32,33} The drug has a variety of traditional pharmaceutical applications, including treatment of liver diseases, wounds, and blood purification.^{34,35} Also, antitumor, anticancer, antioxidants

and antimicrobial properties can be enhanced under proper light illumination by lipid peroxidation of outer membrane.^{36,37}

In summary, the photodynamic effects mediated by curcumin over *S. mutans* and *Candida* species have been attested by some previous investigations.^{21,30,31,38} However, none of these studies have focused on the comparison between the effects of curcumin-PACT on cariogenic biofilm and chlorhexidine, considered the “gold standard” antimicrobial oral decontaminant.

Due to these facts, the aim of this present study was to compare the efficacy of PACT mediated by curcumin and chlorhexidine on a mature *S. mutans* biofilm.

MATERIALS AND METHODS

Photosensitizer and light source

To the present study, curcumin (C) was used as photosensitizer (PS) (Sigma Aldrich, St Louis, MI, USA) and dimethyl sulfoxide (DMSO; Sigma Aldrich, St Louis, MI, USA) was used as solvent to obtain a stock solution of this PS at 50mM. On the day of the experiment, this solution was diluted in deionized water at 2.5mM (keeping 10% of DMSO at final concentration) and involved in an aluminum foil until the experimental phase.

As a light source was used a light-emitting diode in a blue wavelength (Blue LED – BL) (Prototype, Project Finep/ Gnatus LED Edixeon, Edison Opto Corporation, New Taipei City, Taiwan) at central spectra absorption at 450 nm $\pm$ 30 with 240.1 mW.cm⁻² of power density. The dosimetry (light fluency) tested was 48J/cm² (200 seconds) and to achieve this desired parameter the work distance was kept at 5 mm (distance between the light source and biofilm surface) following a previous published equation.³⁸

In vitro biofilm formation and PACT treatment

Biofilms of *S. mutans* UA 159 (ATCC 700610) were formed on saliva coated hydroxyapatite sterile discs (HA) (diameter 1 cm, Clarkson Chromatography Products Inc., South Williamsport, PA) in a horizontal position in batch culture at 37°C and 5%CO₂. The biofilms were grown in buffered tryptone yeast extract broth (TSBYE) containing 1% of sucrose and this culture medium was changed every 24 h for 5 days.^{39,40} After the 5th day, the biofilms were submitted to treatment as follows: kept in the dark into curcumin solution for 1 minute (pre irradiation time – PIT) and exposed to blue LED (PACT group: C+BL+), exposed to curcumin alone for 1 minute (PIT) in the dark (C+BL-), irradiated just with blue LED (C-BL+) and not exposed to PS nor light (control group at 10% of DMSO: C-BL-). Additional discs were submitted to immersion in 0.12% of chlorhexidine solution for 1 minute (CHX group). After the treatments, the biofilms were gently scraped with a sterile spatula, transferred to 5 ml of phosphate buffered saline at 1 X (PBS) and subjected to sonication using three 15-s pulses at an output of 6W with interval at same amount of time (Branson Sonifier 150; Branson Ultrasonics, Danbury, CT, USA).³⁹ The volume of 1 ml of the homogenized suspension was transferred to a preweighed microtube and dried in a Speed Vac concentrator and used for the determination of dry weight. An aliquot of the homogeneized suspension (100 uL) was used for ten-fold serial dilutions and 50 uL were plated onto 5% defibrinated sheep blood agar (Sigma Chemicals Co.) and incubated at 37°C, 5%CO₂ for 48 h to verify the microorganism viability. The results were expressed as colony-forming units (CFU) per milligram of dry weight biofilm and transformed into logarithmical scale (log₁₀).

Confocal laser scanning microscopy (CLSM)

The groups submitted to PACT treatment (C+BL+) and 0.12% of chlorhexidine solution (CHX group) were selected to confirm the quantitative analysis through confocal laser scanning microscopy images (CLSM). To achieve this goal, that groups were compared with control group images (C-BL-) as well.

The viability of bacteria within the biofilms was determined by staining the biofilms with LIVE/DEAD *BacLight* Bacterial Viability kit (Molecular Probes, Inc., Eugene, OR, USA) which includes two fluorescent nucleic acids stains: green-SYTO 9 and red-propidium iodide. The biofilms were treated according to the manufacturer's instructions, being stained immediately after the treatments, kept in the dark and protected from light until the analysis. Stained biofilms were examined with a confocal scanning fluorescence microscope using specific filters (488/507 nm) for detection of SYTO 9 and a propidium iodide filter (503/615 nm) for detection of red stain (Leica TCS SP 5 II Confocal microscope, Leica Microsystems, Wetzlar, Germany).

Environmental scanning electron microscopy (ESEM)

Aiming to verify whether the application of PACT and CHX was able to modify the topography/morphology of the biofilms, samples were analyzed by environmental scanning electron microscopy (ESEM) with an EVO® 50 Series microscopy (Carl Zeiss, AG, Germany). This microscopy is able to obtain images without the need of samples pre-treatment, keeping the natural aspect and characteristics of biofilms right after the application of the treatments. The acquisition of these images was performed by the combination of vacuum and pressure at different magnifications.

Statistical analysis

The assays of PACT effect were performed in duplicate for each group and the procedure was repeated two times on different days ($n = 6$). The mean and the standard deviation (SD) of the numbers of surviving microorganism/mg of dry weight biofilm for each treatment was calculated. Colony-forming units were transformed into $\log_{10}$ in order to stabilize variance heterogeneity. A Mann-Whitney test followed by t-student test with equal variances was applied to verify the differences among all the studied groups. The statistical significance cutoff level was set as $p < 0.05$.

RESULTS

The application of PACT over *S. mutans* biofilms demonstrated a significant bacterial reduction when compared to control group and chlorhexidine solution (CHX group) ($p < 0.05$) attesting the efficacy of photodynamic treatment with no statistical difference ($p > 0.05$) between these last studied groups (C-BL- and CHX group). On this same way, the effect of light source (C-BL+) and PS alone (C+BL-) produced slight bacterial decrease on bacterial viable counts with no statistical difference when compared to C-L- ($p > 0.05$). These findings are illustrated on Figure 1.

INSERT FIGURE 1

The confocal images revealed the efficacy of PACT by high proportion of dead cells represented by red fluorescence (Figure 2). Contrarily, the biofilms submitted to chlorhexidine treatment (Figure 3) showed green fluorescence with no difference when compared to control group images (Figure 4), which attest a substantial number of viable cells.

INSERT FIGURES 2,3 AND 4

The ESEM images showed no differences among the studied groups with absence of modification on biofilms morphology after the treatments with PACT and CHX when compared to ESEM control images. Two different magnifications were performed to each treatment, which attested no modifications at closer views. The Figures 5, 6 and 7 represent the ESEM images of PACT, CHX and control group, respectively.

INSERT FIGURES 5, 6 AND 7

DISCUSSION

The present study was designed to compare the antimicrobial effects of curcumin-PACT and chlorhexidine solution over *S. mutans* mature biofilms. The results demonstrated that photodynamic treatment based on the application of a blue light at 48 J/cm² in the presence of 2.5mM of curcumin achieved a substantial photoinactivation rate when compared to chlorhexidine treatment. Furthermore, there was no statistical difference between control group and CHX ($p > 0.05$) indicating that this substance, due to its protocol used, did not present any antimicrobial effects. Aiming confirm these results, CLSM is a reasonable tool to assess the therapy effectiveness.¹¹ The CHX confocal images (Figure 3) confirmed these findings, where a great number of viable cells marked in green fluorescence was verified.

It has been clearly demonstrated that PACT is able to photoinactivate microorganisms activity such as fungi,²¹ virus⁴¹ and bacteria.³⁰ In this study, a viable bacterial reduction of 2.13 log₁₀ was achieved after a single application of PACT in

the presence of a solution of curcumin. Similar studies have corroborated with our findings.^{21,30,31,38} Dovigo et al.²¹ achieved a significant decrease of *Candida* biofilms when exposure to 5.28 J/cm² in the presence of 20µM of curcumin. This same dye at 1.5 g/L was used by Araujo et al.³¹ that reduced the number of *S. mutans* present in the saliva of 13 volunteers when irradiated with a blue LED device (20.1 J/cm²) for 5 minutes. CLSM images revealed the PACT efficacy by the proportion of red cells representing a high bacterial inviability (Figure 2). This investigation represents the first approach applying curcumin over an *in vitro* mature biofilm formed by *S. mutans*. Additionally, ESEM images aimed to verify the behavior of biofilm morphology after PACT application. Even though the PACT images (Figure 5) revealed no substantial damages to biofilm polymeric matrix when compared to controls and without differences at magnifications studied, this analysis was able to elucidate that curcumin-PACT did not act directly to biofilm bulk and point out other ways of photodynamic way of action.

Bevilacqua et al.¹⁵ were the first investigators to demonstrate a lethal photosensitization of planktonic *S. mutans* to toluidine blue O (100 µg/mL) exposure to a red LED (2.18 J/cm²). In this same way, Mattiello et al.⁴² achieved a mortality of 84.33% of *S. sanguinis* using 0.01% of TBO activated with a diode laser (AlGaAsP) at 10 J/cm² whereas Williams et al.⁴³ noted ~ 99.9% death of *S. mutans* in a planktonic suspension, using a diode laser emitting at 633 ± 2 nm with fluences ranging from 0.4–4.8 J with TBO as PS. Overall, our results attested that PACT was less effective than in planktonic studies. In comparing biofilm with planktonic effects, a degree of reduced efficacy would be expected of any penetrant molecular substance.⁴⁴ Incomplete bacterial killing by photodynamic therapy is not limited to curcumin. In a previous study, a conjugate between chlorine6 and poly-L-lysine failed to eradicate microorganisms completely in dental plaque scrapings.⁴⁵ Recently,

incomplete elimination of *S. mutans* in an artificial biofilm model was verified after laser-induced photodynamic exposed to diode laser at 660 nm (100 mW/cm²) for 2 minutes in the presence of phenothiazine chloride (Helbo, Wels, Austria).⁴⁶ Explanations regarding to the lowered PACT effect in biofilms may be related to the distinct role of polysaccharide matrix that provides protection to the bacteria and limits the diffusion and distribution of external agents such as dyes or antimicrobials. Yet, bacteria organized in biofilms express phenotypic changes, what corroborate with this last characteristic.⁴⁷ Another important issue is related to reduction of dye penetrability.⁴⁴ Studies regarding confocal microscopy analysis attest this fact. Investigation of Fontana et al.⁴⁴ using the same reagents utilized in the present study (SYTO 9 and propidium iodide) verified no fluorescent signal below 200 µM-depth of biofilms provided by dental plaque samples of patients with chronic periodontitis. In accordance, confocal images of O'Neill et al.⁴⁸ attested a substantial photosensitization of the outer layers of biofilms submitted to toluidine blue and subsequently exposure to proper light source. It can be speculated that due to diameter of water channels (20 - 600 µm)⁴⁹ presenting in the biofilm matrix allied to the high molecular weight of curcumin³⁸ can prevent its access to the interior of cell clusters,⁵⁰ and, consequently, reducing the contact and, consequently, therapy effectiveness.

Throughout the world, there have been reports of natural agents used in the treatment and prevention of different oral conditions including caries and gingivitis.⁵¹ Positive antimicrobial effects against cariogenic bacteria using natural products have been investigated.⁵¹ The PS used in this present study was a yellow pigment extracted from the rhizomes of *Curcuma longa* L (turmeric plant). This dye is well known for its medicinal properties and, more recently, the role in PACT has been highlighted by some studies.^{21,30,31,38} Low costs (US \$5/micrograms) making possible

its production at affordable prices, efficiency of ROS generation and easy handling represent curcumin advantages.^{21,30,31} Moreover, the concentration of curcumin (2.5 mM) was based on a previous study that determined a safe concentration in terms of damage to the mucosa and discoloration of the teeth, important aspects to clinical applicability.⁵² However, as a natural product, the solubility represent its main limitation. In this investigation, dimethyl sulfoxide (DMSO) was used to dissolve curcumin and its final concentration was kept at 10%. DMSO is the most commonly used vehicle to evaluate the phototoxic effects of PACT in *in vitro* studies and it has become a control to evaluate several types of drug solvents because of its excellent solvent propriety.^{53,54} In the present study, dilution of curcumin in DMSO increased its solubility in deionized water, improving its bioavailability and promoting bacterial photokilling. Even though in this study DMSO not generated any toxic effects to *S. mutans* (data not shown), this solvent is not the ideal for *in vivo* applications due to its capacity of increase membrane permeability and may cause tissue damages.⁵⁴ Therefore, the study of different vehicles as efficient as DMSO that, at same time, produce high curcumin photokilling efficacy without side effects should be investigated.

The PS and the visible light - essential components of PACT - are able to alter cell functions. However, in the present study, just a slight difference, without statistical significance, were observed between the groups exposed only to curcumin (C+BL-) or blue LED light (C-BL+) when compared with the control (C-BL-). Curcumin generated significant reduction on viable cell numbers only when used in combination with visible light due to ROS formation. The results of this investigation are in agreement with previous findings that achieved no significant reductions on the viability of *S. mutans* using the same blue LED and dye.^{30,31}

The photokilling rate exerted by curcumin-PACT application is not directly related to high productions of ROS or singlet oxygen ($^1\text{O}_2$).²¹ An investigation stated that once superoxide anion radical O_2^- (a ROS specie) was formed, other reactive species, such as H_2O_2 could be produced and then amplify the photosensitizing activity of curcumin.⁵⁵ Dovigo et al.²¹ investigated the possible involvement of $^1\text{O}_2$ by adding NaN_3 before LED illumination. They verified that *C. albicans* photokilling was not related to that oxygen specie, indicating that this reactive specie was not essential in promoting the photodynamic effect. Still, Dahl et al.⁵⁶ suggested the involvement of hydrogen peroxide in the photokilling of bacteria with no evidence of formation $^1\text{O}_2$. Still, Hauvik et al.⁵⁴ measured the oxygen quantum yield in different curcumin preparations and found very limited $^1\text{O}_2$ formation. Additionally, however the relative production of ROS by curcumin was not determined in the present study, Rolim et al.⁵⁷ have demonstrated that a PS irradiated by blue light sources presented lower amount of ROS than the other PS irradiated by red lights. On this way, from the point of view of bacteria and PS interaction, the effectiveness of PACT is mostly related to PS capability of interacting with the bacterial membrane and its ability of penetration and action inside the cell rather than the high quantities of reactive oxygen species or free radicals.⁵⁸ Therefore, the PACT mechanism regarding the production of other reactive components and its relation to exert bacterial sensitization still need to be determined in future investigations.

Chlorhexidine 0.12% was chosen because it appears to be the most effective chemical agent in plaque control.⁵⁹ The positive adjunctive effect of chlorhexidine to routine mechanical plaque control has also been well documented in short-term as well as long-term clinical trials.^{60,61} Reports have proved that daily rinses of 0.12% CHX (60 s) solution for at least 7-11 days are able to achieve significant improvements in gingival health and plaque accumulation.⁵⁹ These effects

appear to be related to the high substantivity in the oral cavity.⁶² However, concerning related to calculus deposition and extrinsic tooth staining are the main expectable side effects of CHX mouthrinse when used in long-terms or without specific supervision or responsible prescription.^{60,63} The main objective of this present study was compare the efficacy of both antimicrobial therapies by a single application over a mature cariogenic biofilm. The results showed that PACT presented a high photokilling bacterial reduction in comparison to CHX group. Since CHX was just applied once for a limited period of time, its main antibacterial activity was not achieved, resulting, consequently, in a high number of viable cells confirmed by quantitative and confocal analysis (Figure 6). Thus, PACT emerges as a suitable tool in controlling plaque accumulation without that undesirable treatment consequences.

Even though this present study demonstrated a success in PACT application to exert a high photosensitization on a mature *S. mutans* biofilm, no investigation has focused the daily curcumin-PACT application aiming elucidate whether the increase of interventions is able to inhibit the formation of polysaccharide matrix as acts the actual chlorhexidine mouthrinse protocol regimen. Still, the use of hydroxyapatite discs and the constant exposure to a rich sucrose medium without simulate the challenges present in the oral environment represents limitations of this report.

Regarding the obtained results, curcumin in combination with a blue LED represent a potential approach against cariogenic biofilm formed by *S. mutans*. A single application of PACT was sufficient to control bacteria involved with caries lesions in comparison with a chlorhexidine solution. Applications focusing *in situ* models that mimic the natural adversity encountered in the oral cavity should be encouraged aiming determine appropriate dosage parameters to future *in vivo* application and, additionally, contribute with the knowledge related to mechanism of photodynamic action.

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FIGURE SUBTITLES

Figure 1. Mean of viable cells ($\log_{10}$) after PACT application. Data represent mean values ($n = 6$) and error bars represent standard deviation. Data followed by different letters differ statistically ($p < 0.025$). *For the studied groups consider blue LED (BL) at 48 J/cm^2 and curcumin (C) at 2.5 mM .

Figure 2. 3. and 4. CLSM images of biofilms after 5 days in batch culture at 1% of sucrose. Viable and affected bacteria are represented by green and red fluorescence, respectively. **2.** Biofilms submitted to PACT at 2.5 mM of curcumin exposed at 48 J/cm^2 of blue LED light. **3.** Biofilms submitted to chlorhexidine rinse for 60 s. **4.** Control group submitted to 10% DMSO for 60 s.

Figure 5. ESEM image of PACT group (C+BL+). Consider curcumin (C) at 2.5 mM and blue LED (BL) at 48 J/cm^2 . **5.1.** Magnification at $3000 \mu\text{m}$. **5.2.** Magnification at $500 \mu\text{m}$.

Figure 6. ESEM image of 0.12% chlorhexidine group (CHX group). **6.1.** Magnification at $3000 \mu\text{m}$. **6.2.** Magnification at $500 \mu\text{m}$.

Figure 7. ESEM image of control group (C-BL-) at 10% DMSO. **7.1.** Magnification at $3000 \mu\text{m}$. **7.2.** Magnification at $500 \mu\text{m}$.

FIGURES

Figure 1.

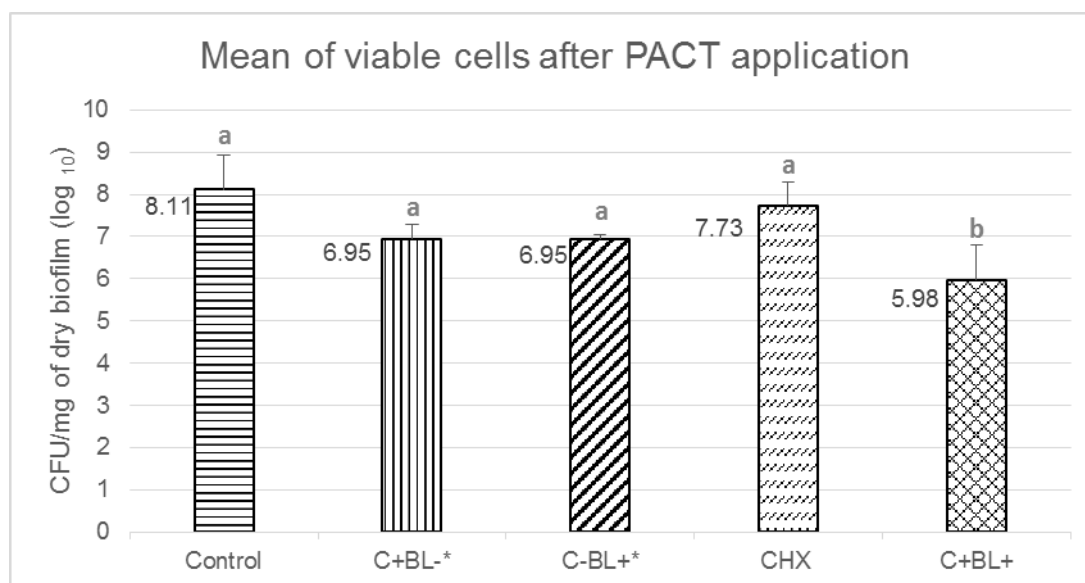


Figure 2.

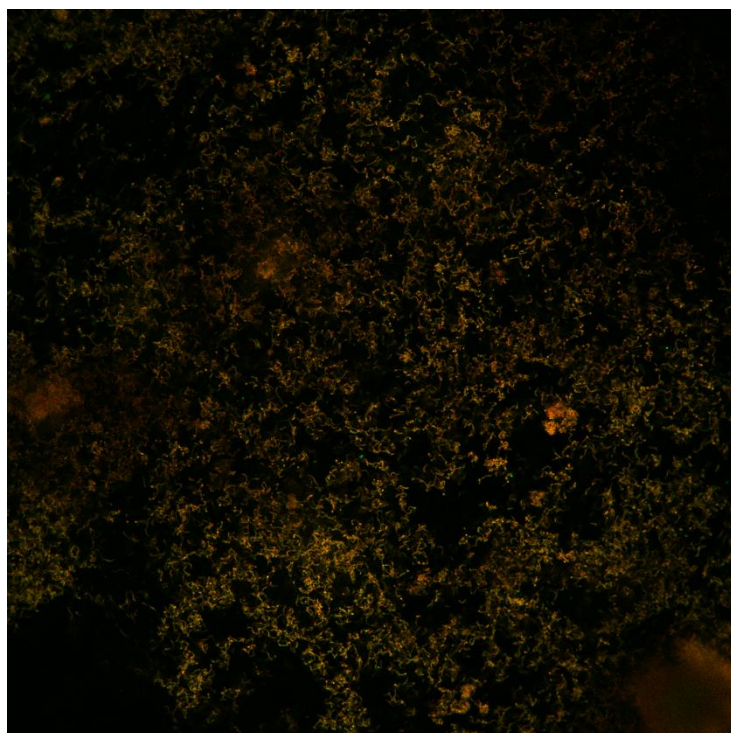


Figure 3.

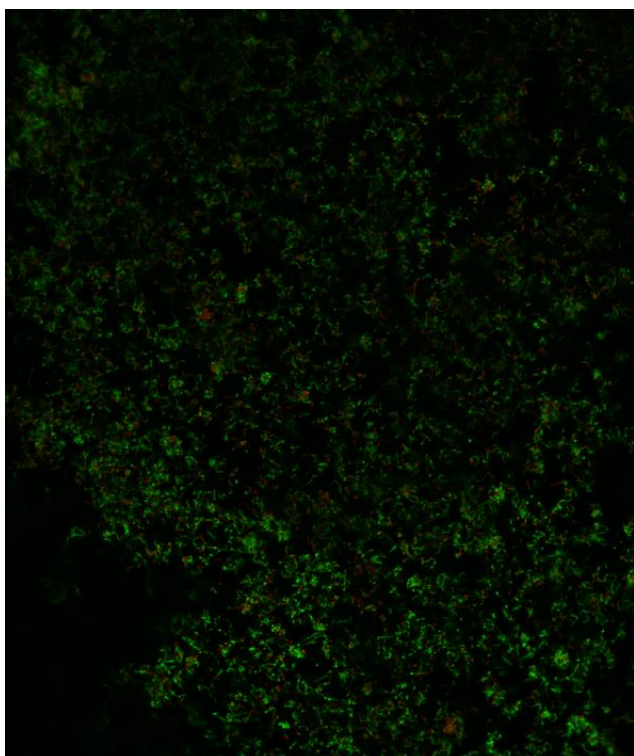


Figure 4.

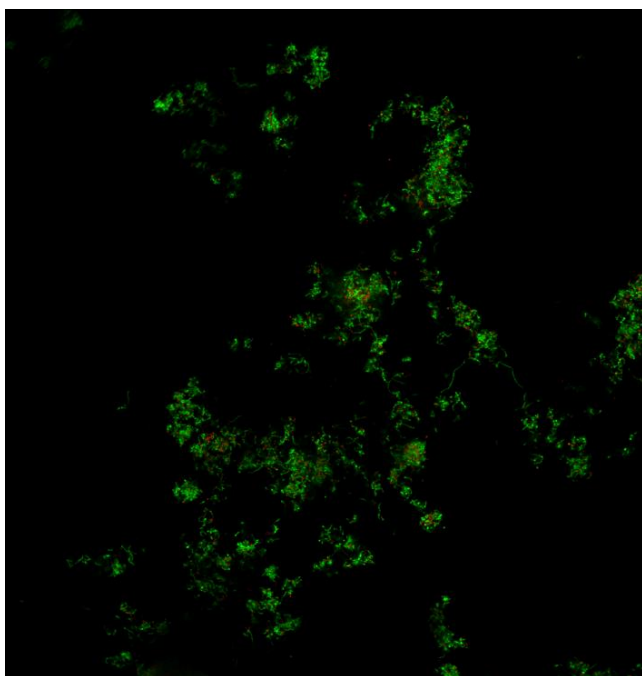


Figure 5.

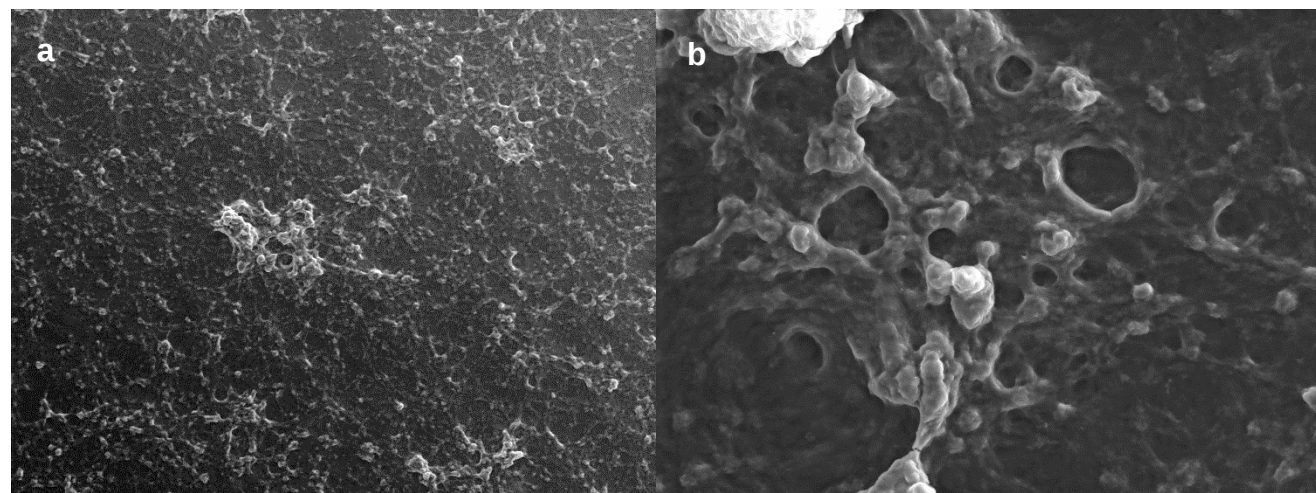


Figure 6.

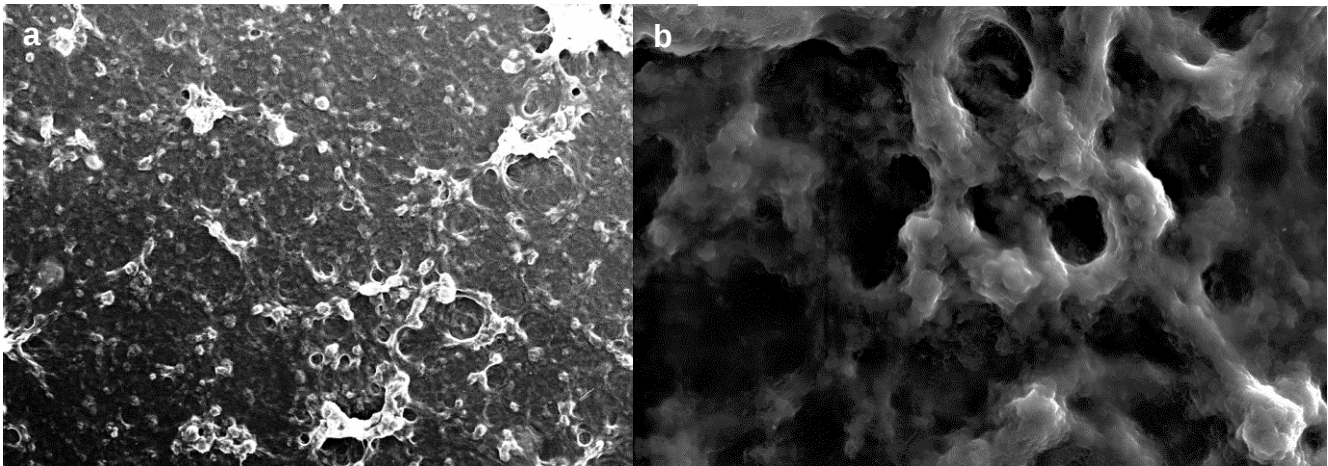
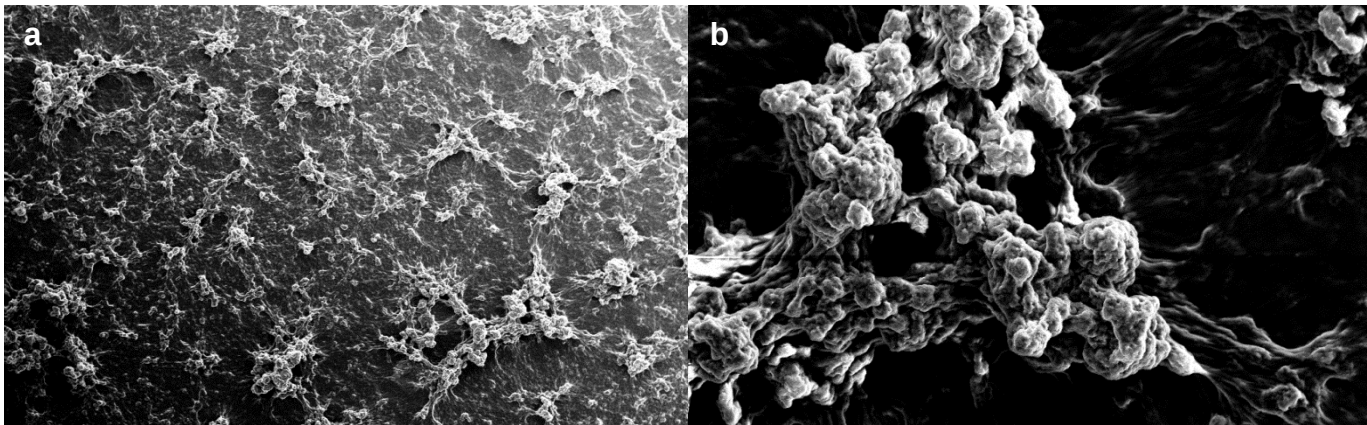


Figure 7.



3 . 3 Capítulo 3*

* Artigo formatado segundo as normas do periódico *Photomedicine and Laser Surgery*

***Streptococcus mutans* photoinactivation with a broad-spectrum visible light
in the presence of curcumin and toluidine blue**

**Running title: *Streptococcus mutans* photoinactivation with a broad-
spectrum visible light**

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ABSTRACT

Objective: The goal of this study was to investigate the antimicrobial effect of a novel single source of a non-coherent light that delivers high power intensity at a short exposure time over suspensions of *Streptococcus mutans*. **Background data:** Long illumination times to activate photosensitizers (PS) and the use of high concentrations of these drugs in photodynamic antimicrobial chemotherapy (PACT) are limitations of its application as an antimicrobial technology to dental practice. The search of new light sources providing high power intensity at short exposure, with low concentrations of PS, are highly desired. **Materials and Methods:** Planktonic suspensions of *S. mutans* were standardized and submitted to PACT treatment at low concentrations of curcumin (C) and TBO (T) exposed to 42 J/cm² of a white light (WL) (C+WL+ and T+WL+: PACT groups); isolated effect of both C (C+WL-) and T concentrations (T+WL-); effect of light source (C-WL+ and T-WL+) and suspensions neither submitted to PS nor LED illumination (control groups: C-WL- and T-WL-). Aliquots of each group were diluted and cultured on blood agar plates and incubated at 37°C, 5% CO₂ for 24 h. The number of Colony Forming Units (CFU)/mL was recorded, transformed into log₁₀ and analyzed by ANOVA and Tukey's test at cut-off value at 0.05. **Results:** The groups submitted to PACT presented a substantial photobactericidal rate (~99.9%) in comparison to control groups ($p < 0.05$). PS or LED lights used alone demonstrated no effect on the number of viable bacterial counts. **Conclusion:** PACT using the novel LED device, which needs a short illumination exposure time and allows the use of a single source of light, in combination with low concentrations of photosensitizers is potentially an effective antimicrobial *in vitro* approach to reduce number of microorganisms related to dental caries process. Future studies need to be done to test this novel LED source on biofilms.

INTRODUCTION

Nowadays, there are several options for plaque removal aiming the control of dental caries.^{1,2} Among of the feasible methods, the use of toothbrushes of different shapes and designs, dental floss and wood sticks represent the most common ways of controlling the plaque development.¹ However, in industrialized countries the average person brushes for less than 1 minute, which may be not adequate for achieving an optimal level of plaque removal.² Also, interdental areas are difficult to achieve total cleaning.² Yet, the effectiveness of plaque disorganization is under dependence of factors such as maturity, compliance and dexterity of the subjects.³⁻⁵ Thus, the need for novel antimicrobial techniques that act in combination with the mechanical debridement are essential.

Several chemical products for plaque inhibition are available on the market.¹ The bisbiguanide compounds, which includes chlorhexidine gluconate, are considered the most effective antimicrobial agents used.¹ However, side effects including lack of taste, staining and potential toxicity still tend to discourage its long-term use.^{6,7}

For these reasons, alternatives that might effectively remove cariogenic biofilm from the hard dental tissue surfaces are being explored. Photodynamic antimicrobial chemotherapy (PACT) could become a novel method of antibacterial treatment and may be used in combination to mechanical cleaning for the control of biofilm accumulation, consequently preventing dental caries.⁸

PACT involves the combination of visible light, usually laser or light-emitting diodes (LEDs) and a photosensitizer (PS).⁹ The PS is a compound that is capable of absorbing light of specific wavelength and transform it into useful energy.¹⁰ This energy is in the form of free radicals of singlet oxygens and is capable of damaging virtually all types of biomolecules and of killing microbial cells

by reacting with cellular components such as proteins, nucleic acids, and lipids.¹¹ Light and drug are non-toxic by themselves; hence, only cells binded by PS and receiving light are affected by “photodynamic action”.¹² PACT treatments of bacteria including *Streptococcus mutans*,^{13,14} *Streptococcus sanguinis*,¹⁵ *Porphyromonas gingivalis*¹⁶ and *Actinobacillus actinomycetemcomitans*^{15,17} have shown positive outcomes. In addition, PS such as methylene blue (MB), toluidine blue (TBO), phthalocyanine, haematoporphyrin in combination with proper light sources provide success to this antimicrobial therapy.¹⁸⁻²⁰ The photodynamic approach to killing bacteria is clearly a rapidly emerging alternative to current antimicrobial regimens. Significantly, it is unlikely that bacteria could develop resistance to the photodynamic action of cytotoxic singlet oxygen or free radicals, as it has been reported with conventional antimicrobials and antibiotics.^{21,22}

TBO has been shown to have bactericidal effects against Gram-positive and Gram-negative bacteria.^{18,19} Characteristics as: low toxicity to host cells associated with hydrophilicity; simple molecular structure; and efficacy on singlet oxygen production make this compound the most studied dye in the PACT field.²³⁻²⁵ Although this agent is very promising, its use is subject to lengthy clinical and legislative assessment. Furthermore, potential to staining teeth, resin-based restorations and toxicity represent clinical limitation.¹⁹

Therefore, more immediate benefit could be attained from PS that potentially have less side effects.²⁶ Several investigations have highlighted the use of curcumin as a feasible PS in PACT application.^{14,27-29} Curcumin has been used as spice in the traditional Indian cuisine and medicine as well.^{30,31} Besides, curcumin is the major constituent of turmeric powder exhibiting a wide range of pharmacological effects, including anti-inflammatory, anti-carcinogenic, and anti-infection activities.^{32,33} Its effective use in oral disinfection and prosthetic

decontamination have been attested by recent studies.^{28,34} Additionally, antimicrobial property by lipid membrane peroxidation could be enhanced by light in a proper wavelength.^{35,36}

The photodynamic effectiveness also involves the rationale use of a visible light source, which includes its potency, wavelength, tested light fluency, and distance from the sample. Thus, the use of a suitable light source that could fulfill these characteristics is desired.

Some reports have shown the use of a broad-visible light (400 – 700 nm) (e.g. halogen lamps, tungsten filament lamp and white LED) to photoinactivate some microorganisms involved with endodontic infections,³⁷ dental caries³⁸ and mycoses.³⁹ Overall, these studies used long times of incubation and exposure as well. Investigation of Gonzales et al.³⁹ photosensitized samples of *Metarhizium anisopliae* and *Aspergillus nidulans* after an incubation time of 30 minutes in methylene blue and TBO and subsequently exposure to a 300 W halogen lamp at 50 mW.m⁻² for 30 and 60 min, respectively. A 2-log₁₀ and 4-log₁₀ reduction was achieved after exposure of *Escherichia coli* and *Enterococcus faecalis* to irradiation for 30 min of a white LED (15 mW/cm²; 13 J/cm²) in the presence of TBO (62.5 µM).³⁷ A tungsten filament lamp (400 W; 22.5 – 22.7 mW/cm²) in combination with different PS at 22 µM achieved a bacterial reduction of 48 h-old *S. mutans* biofilms ranging from 1.5-log₁₀ to 2.2-log₁₀ after irradiation for 15 minutes.⁴⁰

Even though the use of a non-coherent broad visible light is claimed to be a limitation due to its long exposure time to photoactivate the studied PS, none of the previous reports have investigated the PACT effect of a white light in the presence of curcumin and TBO aiming to photoinactivate cariogenic microorganisms. In addition, in this study we used a novel LED light source that delivers high power intensity at a short exposure time.

Therefore, the aim of this study was to verify the antimicrobial effect of PACT mediated by curcumin and TBO exposed to a novel broad-visible light device at high power intensity on planktonic suspensions of *S. mutans*.

MATERIALS AND METHODS

Bacterial strain

The microorganism used in this study was *S. mutans* UA 159 (ATCC 700610). An aliquot was inoculated in buffered tryptone yeast extract (TSBYE) containing 1% (wt/vol) glucose and grown overnight, at 37° C, 5% CO₂. Then, the bacterial suspension was centrifuged at 4000 RPM for 5 min and the supernatant discarded. The cell pellet was re-suspended in 20 ml of sterile phosphate buffered saline (PBS). The cell numbers were adjusted at an optical density (OD) of 1.5 at 540 nm, equivalent to approximately 2×10^9 colony forming units (CFU / mL).

Photosensitizers

To this present investigation, curcumin (C) (Sigma Aldrich, St Louis, MI, USA) was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution at 750 µM. On the day of the experiment, this solution was diluted in deionized water to obtain final concentrations at 0.075 µM; 0.75 µM and 7.5 µM (keeping the final concentration of DMSO at 0.25%). In this same way, a 1000µM stock solution of toluidine blue (T) (Sigma Aldrich, St Louis, MI, USA) was prepared in deionized water and subsequently dissolved at concentrations of 0.25 µM; 2.5 µM and 25 µM. On the day of the experiment, both PS were diluted in deionized water and kept in the dark until its use.

Light Source

A single source of a non-coherent light that produces the full spectrum of visible light was used (LumaCare LC-122 A, LumaCare Medical Group, Newport Beach, CA, USA). This light offers interchangeable probes at specific frequencies that are connected with a simple interlocking connection. Previous pilot tests (data not shown) used different concentrations of curcumin and TBO exposure to different dosimetries of lights at specific wavelengths (blue and red LED, respectively) with different dosimetries established by published works^{14,41} aiming to define the protocol to be applied by white light. This light probe presented a wide spectrum from 400 to 700 nm at 550 nm (± 150 nm) of central wavelength with a power intensity of 3410 mW/cm². The dosimetry tested was 42 J/cm² and the work distance to activate both PS was fixed at 5mm (distance between the light source and cell line surface) do not generate thermal effect (LumaCare™ manufacturer instruction).

Photodynamic therapy application

Study design

S. mutans suspensions were incubated with concentrations of the tested photosensitizers (PS). Curcumin solution was kept in the dark with *S. mutans* suspension for 60 sec (pre-irradiation time)¹⁴ whereas TBO was kept for 5 minutes.¹³ After the incubation time, the solution (PS/bacterial suspension) was irradiated with the white light for 12.2 s to achieve the tested dosimetry (42 J/cm²). PACT groups were treated with both PS and light (C+WL+ and T+WL+, where C - curcumin; T – TBO; WL – white light). To determine whether PS alone induced any toxic effects on bacterial viability, bacterial suspensions were exposed to PS under identical conditions to those described above, but no exposure to light (treated only

with C or T: Group C+WL- and T+WL-). Exposing cells to irradiation determined the isolated effect of white light with no previous exposure to PS (treated only with white light: Group C-WL+ and T-WL+). *S. mutans* suspensions with no exposure to neither PS nor white light (C-WL- and T-WL-) were used as control groups. Figure 1 illustrates the application of PACT.

INSERT FIGURE 1

After the treatment application, aliquots of the treated suspensions were used to perform ten-fold serial dilutions and the diluted samples were plated onto 5% defibrinated sheep blood agar (Sigma Chemicals, Co.) and then incubated at 37°C, 5% CO₂ for 48 h to investigate the number of viable microorganisms. After incubation, the total number of CFU was determined and number of CFUs *per* millimeter of suspension (CFU/mL) was obtained and transformed into logarithm ($\log_{10}$).

Statistical Analysis

In order to verify the differences among the studied groups, the variable reduction in viable bacterial colony counts was analyzed by two-way ANOVA and Tukey's test. The P value was considered as <0.05 to statistical significance. The BioEstat 5.0 software for Windows (Sociedade Civil Mamiraua, Manaus, AM, Brazil) was used for data analysis.

RESULTS

Curcumin at the concentration of 0.75 μM exposed to 42 J/cm^2 (12.2 s) of white light resulted in lethal photosensitization ($\sim 99.9\%$) when compared to control group ($p < 0.05$).

The exposure of 42 J/cm^2 of the white light in the presence of TBO at 25 μM concentration (T+WL+) also achieved a substantial photokilling rate ($p < 0.05$). The Figures 2 and 3 demonstrate the effect of white light-PACT mediated by curcumin and TBO, respectively. The application of light (C-WL+ and T-WL+) and both PS (C+WL- and T+WL-) itself did not reduced the CFU when compared to control group ($p > 0.05$).

INSERT FIGURES 2 AND 3

DISCUSSION

The present study investigated the effects of a new broad visible light (white light) at high power intensity to activate two different photosensitizers. The results showed that low concentrations of curcumin and TBO exposed to a short white light illumination time (C+WL+ and T+WL+) caused a lethal photosensitization ($\sim 99.9\%$) of *S. mutans* in suspension, which was different than the control group (C-WL- and T-WL-). The groups submitted to light illumination alone (C-WL+ and T-WL+) did not produce bacterial reduction ($p > 0.05$), indicating there is no thermal effect. These results reaffirm PACT intervention as an alternative option as an antimicrobial treatment in the dental field and corroborates with previous reports that achieved optimal outcomes when this procedure was tested *in vitro* and *in vivo*.

approaches over cariogenic microorganisms.^{14,19,20} However, the illumination time used in the other reports are higher than the one used in this work.^{14,19,20}

In this investigation, a $5.41 \log_{10} (\pm 0.26)$ and $5.37 \log_{10} (\pm 0.22)$ bacterial reduction was achieved when TBO and curcumin, respectively, were exposed to only 12.2 s (42 J/cm^2) of a novel source of white light. This is not the first investigation to use a white light;³⁷ however, the time in the presence of curcumin and TBO is much shorter than the ones reported in the literature referent to the white light.³⁷⁻³⁹ The current light source used (LumaCare™ system) presents high power intensity (3410 mW/cm^2) provided by the current tested light (white light), and it is highly absorbed by the substrates due to its broad spectrum with a low percentage of reflection, allowing a more relevant interaction with superficial tissue layer; short illumination times are known to be more efficient in ROS generation allied to a low photobleaching rate (photodegradation of PS).²⁷

The results based on the use of white light here are in agreement with previous reports that achieved a notable photokilling rate using the same broad spectrum. Wood et al.⁴⁰ verified that erythrosine (a dental plaque disclosing agent) at $22 \mu\text{M}$ showed a bactericidal efficacy of $1 - 2 \log_{10}$ of *S. mutans* biofilms when irradiated for 15 min using a 400 W white light source. In this same year, Metcalf et al.,²⁶ using this same light source and PS cited above, fractionated the light dose (6.75 J/cm^2) into 5 intervals of 1 minute each, that achieved $3 \log_{10}$ of bacterial viable *S. mutans* biofilm reduction. Still, further fractionation of the light dose into 10 X 30 s doses separated by 2 min recovery periods resulted in $3.7 \log_{10}$ of cell killing, an improvement of $1.7 \log_{10}$ compared with the continuous irradiation protocol. They concluded that short illumination times followed by dark periods were able to redistribute the PS and avoid photobleaching, allowing the equilibrium of the photosensitizer concentration gradient. In the present study we were able to

achieve a remarkable photokilling rate of *S. mutans* suspensions after an extremely short exposure time, 12.2 seconds, and by low concentrations of TBO and curcumin, which can indicate that PACT mediated by either curcumin or TBO illuminated by this novel source of white light is a promising intervention against oral microorganism. The next step is to study these therapies against this bacterium organized as biofilm.

The correlation between ROS species and efficacy of the PACT have been questioned by previous reports.^{13,27} It is expected light sources that provide energy at around PS peak absorption are able to generate more oxygen singlet and free radicals. However, even though in the present investigation we used a broad-visible light and two different photosensitizers, which are absorbed by lights that cover the red and blue spectrum, the data show a lethal photoinactivation (~99.9%) of *S. mutans* after PACT mediated by the white light. This source of light presents a central wavelength of 550 nm ($\pm$ 150 nm) and a high power intensity delivered by only 12.2 s. The absorption peak of curcumin and TBO are close to 430 nm and 636 nm, respectively, so the white light most likely covered both peaks (400 – 700 nm). Furthermore, other compounds in addition to ROS could be responsible for the effect of photodynamic application. The effect of photodynamic process is dependent on the conjugation of PS and target cells, since ROS present high reactivity and limited diffusion power.⁴⁰ *Streptococcus mutans* is a Gram-positive bacteria that present a thick membrane which is relatively permeable, and is intimately associated with lipoteichoic and negatively charged teichuronic acids.^{41,42} This membrane displays a relatively high degree of porosity, since various macromolecules, such as glycopeptides and polysaccharides with a molecular weight in the 30.000 – 60.000 range, were found to readily diffuse to the inner plasma membrane.⁴² Thus, in this class of bacteria, the outer wall does not act as a

permeability barrier for the most commonly used PS. Curcumin binds preferentially to lipid membrane and some cell proteins whereas TBO, a positively charged cationic phenothiazinium dye, acts to membrane Gram-positive peptidoglycan cell walls, generating strand breaks in the organism's nucleic acid, with resultant genetic mutation and photodamage. These interactions are due to molecular weight (TBO - $305.83 \text{ g. mol}^{-1}$ and curcumin - $368.38 \text{ g mol}^{-1}$) that allow the diffusion of PS to outer walls of studied microorganism allied to capacity of solubility.^{14,38} In this experiment, curcumin was dissolved in 0.25 % DMSO to increase its solubility, due to its high hydrophobicity, property not presented by TBO. A vehicle control was used to monitor the effect of 0.25 % DMSO in the cells, and the data shows there was no toxic effect using the DMSO at this concentration. Future studies need to be done to improve curcumin solubility in water. Moreover, low concentrations of dyes were able to produce high photodynamic effects.^{14,28} Some authors have shown that high concentrations could serve as "barrier" to light diffusion, due to optical quenching by excess dye in solution.^{14,43,44} Factors related to type of microorganism and its organization (suspension or biofilm), concentration of dyes and type of light source, could be directly related to PACT effectiveness.

This present investigation is the first report to demonstrate a high bactericidal rate at short illumination time of a broad visible light in the presence of low concentrations of curcumin and TBO. It gives the basis for future studies aiming the application of this approach to reduce bacteria related oral infections. The parameters tested are feasible for the dental practice, since it uses a visible light in a very short exposure time. Apart of PS limitations, such as staining potential (TBO) and hydrophobicity (curcumin), further investigations on biofilms and more information regarding side effects on host cells are needed to ensure the potential of this new light source to be clinically used.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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Figure subtitles

Figure 1. **a.** Light source used in the present study. **b.** Close view of PACT application.

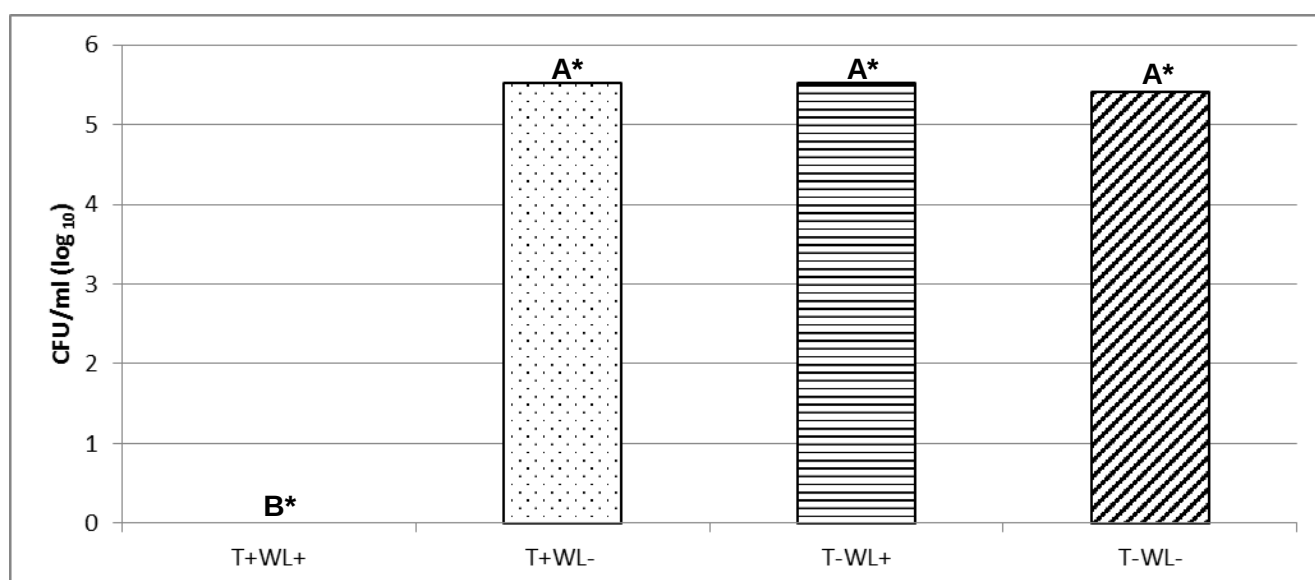
Figure 2. Treatment effects considering TBO at 25 μM (T) and white light at 42 J/cm^2 (L). (PACT group: T+WL+), with T and without WL (PS group: T+WL-), without T and with L (Light group: T-WL+) without T and without L (control group: T-WL-) on the viability of *S. mutans* suspensions.

Figure 3. Treatment effects considering curcumin at 0.75 μM (C) and white light at 42 J/cm^2 (L). (PACT group: C+WL+), with C and without WL (PS group: C+WL-), without C and with L (Light group: C-WL+) without C and without L (control group: C-WL-) on the viability of *S. mutans* suspensions.

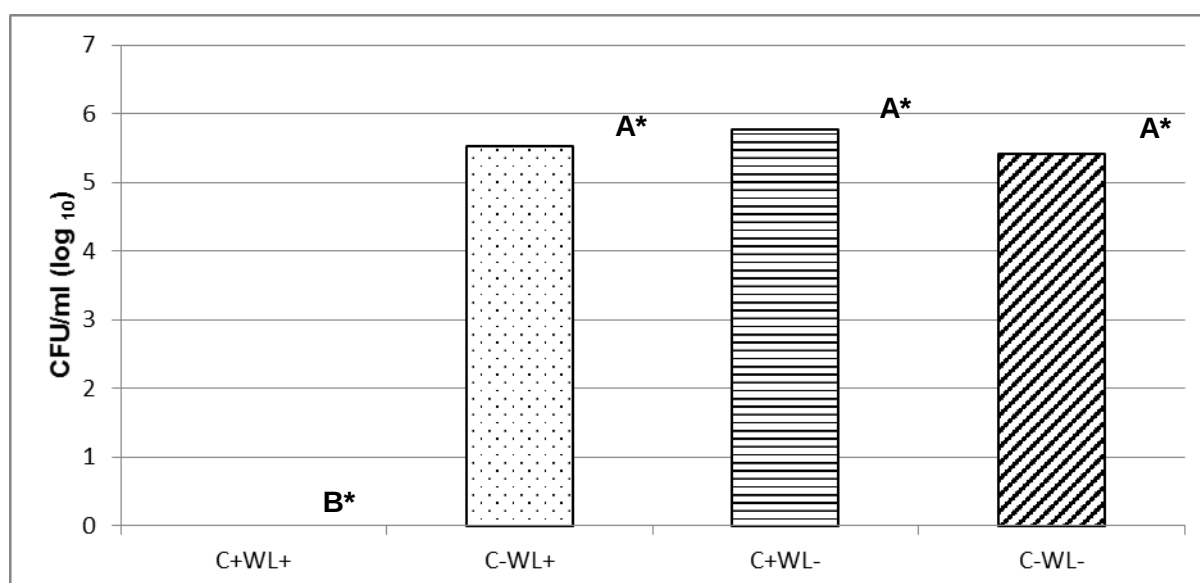
Figure 1.



Figure 2.



* Data followed by different letters differ statistically ($p < 0.05$).

Figure 3.

* Data followed by different letters differ statistically ($p < 0.05$).

4 Conclusão

4 CONCLUSÃO

A partir dos três capítulos desenvolvidos, pode-se concluir que:

- O fotossensibilizador natural estudado, curcumina, apresentou potencial antimicrobiano fotodinâmico sobre biofilme maduro formado por *Streptococcus mutans*;
- A terapia fotodinâmica antimicrobiana (PACT) mediada por curcumina apresentou eficácia antimicrobiana superior a solução de clorexidina a 0.12%, quando aplicados por uma única sessão.
- A fonte de luz de amplo espectro (luz branca) de alta intensidade de potência apresentou efeito antimicrobiano letal na presença de baixas concentrações de azul de toluidina e curcumina por um período de exposição extra-curto.

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