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Scientific evidence in antimicrobial photodynamic therapy: An alternative approach for reducing cariogenic bacteria

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ABSTRACT

Several studies of antimicrobial photodynamic therapy (aPDT) have been performed to verify the efficiency of this treatment against caries-related microorganisms. Thus, the aim of this study is to describe the characteristics of aPDT and to review the literature regarding its effects on cariogenic microorganisms organized in biofilms and/or caries lesions. The literature was searched for reviews and original papers about aPDT and its outcomes against *Streptococcus mutans* as well as other caries-associated microorganisms or caries lesions. Moreover, research on photosensitizers and light sources are also reviewed. The publications were selected using PubMed, Web of Science, and manual search of references cited in key papers. The descriptors used were "dental caries" and "photodynamic therapy". The relative efficacy of aPDT to reduce the population of cariogenic bacteria in *in vitro* biofilms is demonstrated by large number of laboratory studies. Preclinical (*in situ* models) and clinical studies show a less pronounced bacterial reduction for aPDT than for *in vitro* models, especially in dentin carious lesions, since the bacteria in dentin caries may be less susceptible to this therapy due to the limited photosensitizer penetration as well as reduced diffusion of light along dentin structures. Although aPDT may be an efficient and less invasive complementary approach to disinfect deep caries lesions, there is insufficient scientific evidence of its efficacy to warrant a clinical recommendation for its use.

1. Introduction

Dental caries is a biofilm-sugar dependent disease associated with a complex etiology, including genetic, environmental and behavioral factors [1,2]. A wide variety of bacterial communities inhabit the oral cavity and form dental biofilm. This biofilm, called dental plaque, is formed on tooth surfaces bathed by saliva, and is composed by a diverse microbial community embedded in an extracellular polymeric substance (EPS), a virulence factor associated with dental caries [2–4]. This disease results in tooth demineralization by acids produced by cariogenic bacteria and can progress to cavitation, reaching the dentine and pulp tissues, at the extreme resulting in tooth loss [1,5].

Since dental caries is caused by the presence of multiple microorganisms in a collective biofilm [5,8], in which form microorganisms become much more resistant to antimicrobial treatments, harsh environmental conditions and host immunity, traditional antimicrobial therapies are generally not effective in treating caries [5].

The microbial biofilm cells have properties and gene expression patterns distinct from planktonic cells, including phenotypic variations in enzymatic activity, cell wall composition and surface structure, which increase the resistance to antibiotics and other antimicrobial treatments [3,6], demanding new strategies to treat biofilm-associated diseases [6]. The need for new approaches to attack biofilm-associated microorganisms has raised interest in antimicrobial photodynamic therapy (aPDT) as a promising candidate to control dental caries. Furthermore, aPDT, a non-antibiotic broad-spectrum antimicrobial treatment, is also active against multidrug-resistant microorganisms [3,7].

Antimicrobial photodynamic therapy based on the combination of a nontoxic photosensitizer (PS) and appropriate visible light wavelength, which in the presence of oxygen produces reactive oxygen species [7]. The occurrence of cell death induced by the interaction of light and chemicals has been recognized for nearly a century. However, only during World War II did this therapy become familiar in the English-speaking world [8].

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This technique's biocidal effect is based on a photophysical process that generates cytotoxic reactive oxygen species (ROS) that irreversibly damage cell components and modify metabolic activities, leading to rapid cell death. These highly reactive chemical species cause harm to proteins, lipids, nucleic acid and other cell components in microorganisms [3,7,9]. Cytotoxic ROS are highly potent against Gram-positive and Gram-negative bacteria, viruses and fungi, and they also are able to prevent and break down biofilms [10].

During aPDT, the photosensitizer must present the property of selective accumulation in infected tissues without causing any damage to healthy cells [11]. Light sources and drugs used in aPDT must be nontoxic, so only cells covered by the photosensitizer and that receive light should be affected by the photodynamic action. Several studies have presented promising results involving killing of caries-related microorganisms, including *Streptococcus mutans*, *Streptococcus sanguis*, *Lactobacillus acidophilus* and *Candida albicans* [4,12–15].

The aPDT process starts with PS linkage to the target cells, whereby the PS is preferentially absorbed by bacteria, accumulating inside them, in the cytoplasm membranes or in the vicinity. The PSs are activated by absorption of visible light of an appropriate wavelength to initially form the excited singlet state, followed by transition to the long-lived excited triplet state. This state can react with the endogenous oxygen to produce singlet oxygen and other free radical species, causing rapid and selective destruction of the target cells [16–18].

The PS in triplet state can react with biomolecules by two different mechanisms, named type I and type II processes. For the type I process, the excited state of PS undergoes electron transfer reactions that eventually form ROS. This mechanism can involve either acquisition or donation of electrons to form superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) (Fig. 1). The ROS generation *via* type II chemistry is mechanistically much simpler than *via* type I. The type II reaction involves energy transfer from the triplet state to the ground-state molecular oxygen to produce an electronically excited and highly reactive state known as singlet oxygen ($^1O_2$) (Fig. 1). Singlet oxygen is considered a highly reactive species, being more desirable in aPDT due to its high oxidative damage efficacy against prokaryotic cells. Nevertheless, the $^1O_2$ lifetime is very short (~10–320 ns), limiting its diffusion to approximate depth of only 10–55 nm in cells [17–23].

The two mechanisms are able to generate ROSs and operate in the target cells simultaneously, but their relative proportions depend on the type of PS. These reactive oxygen species are highly toxic and damage the microbial cells by targeting membrane lipids, proteins, nucleic acids and other cellular components, which may result in necrosis or apoptosis of the bacteria [17,18]. The type of cell death is related to several factors, such as the subcellular location of the PS in different organelles, PS concentration, light fluence and drug-light interval [17].

Considering that dental caries is a globally disseminated biofilm-dependent disease, and aPDT can be a promising alternative to control it, the aim of this article is to describe aPDT's characteristics and to review the literature regarding its effects on caries-related microorganism inhibition in biofilms and/or caries lesions. In aPDT, several factors related to light exposure and photosensitizer need to be understood and considered. Thus, we examine each component separately, with a focus on how each one affects aPDT mechanisms.

2. Photosensitizers (PSs)

Photosensitizers are molecules capable of producing chemical changes in other molecules through a photochemical process. A suitable PS agent for aPDT must have a specific effect on bacterial cells [13,24], since the aPDT's effectiveness depends not only on the PS concentration and contact time, but also the bacterial species involved [3]. Excessively high PS concentrations tend to form aggregates, decreasing the photodynamic activity of the therapy and also altering the absorption peak of light, thus reducing the light absorption. Important parameters for PS interaction include the relative solubility in water and lipids, constant ionization and other specific factors, such as light absorption characteristics and the efficacy in producing the excited state triplet and singlet oxygen [3,7,17,18].

Some PSs have been developed over the past two decades, among which the most used for oral bacteria are: toluidine blue O (TBO) [4,25], methylene blue (MB) [26,27], phthalocyanine (PT) [28,29], malachite green (MG) [30], indocyanine green (IG) [11], photodithazine (PDZ) [31], curcumin (CUR) [32,33], erythrosine (ERI) [34,35] and rose bengal (RB) [14,36] (Fig. 2).

The PSs used in aPDT have different classifications and mechanisms. Photodithazine and phthalocyanine have tetrapyrrole structures and tend to produce predominantly type II singlet oxygen, compared to the type I ROS (such as hydroxyl radicals) [17]. Phthalocyanines are examples of the earliest compounds, studied in the 1980s and 1990s, but this PS family must be chelated to chloroaluminum, zinc, copper or silicon-substituted substances to increase its phototoxicity [17,28,29]. The dyes toluidine blue O and methylene blue are phenothiaziniums, with absorption wavelength in the red region (630–660 nm), causing singlet oxygen production [17,18,37]. Rose bengal and erythrosine are members of the xanthene class and show high singlet oxygen generation

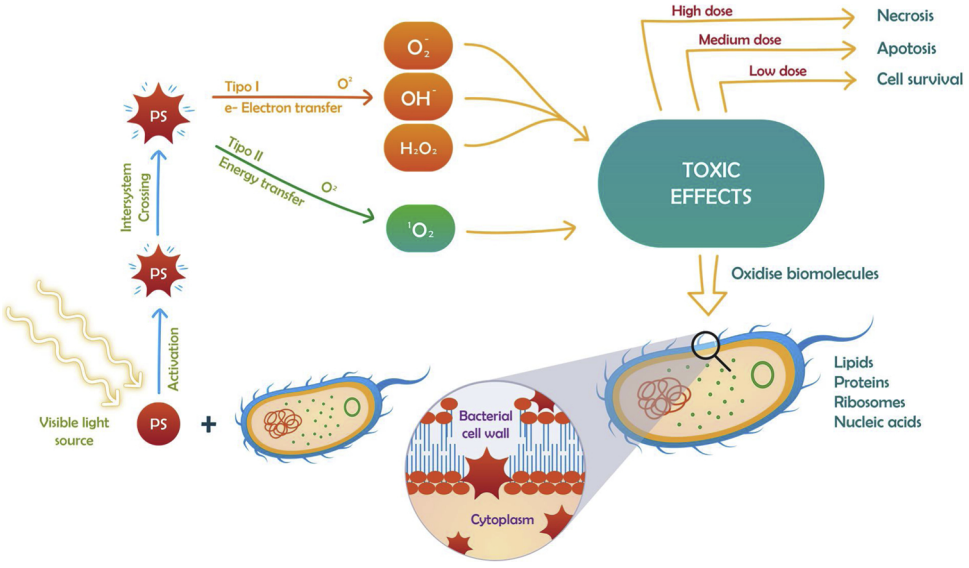

Fig. 1. Scheme of mechanism of action of aPDT.

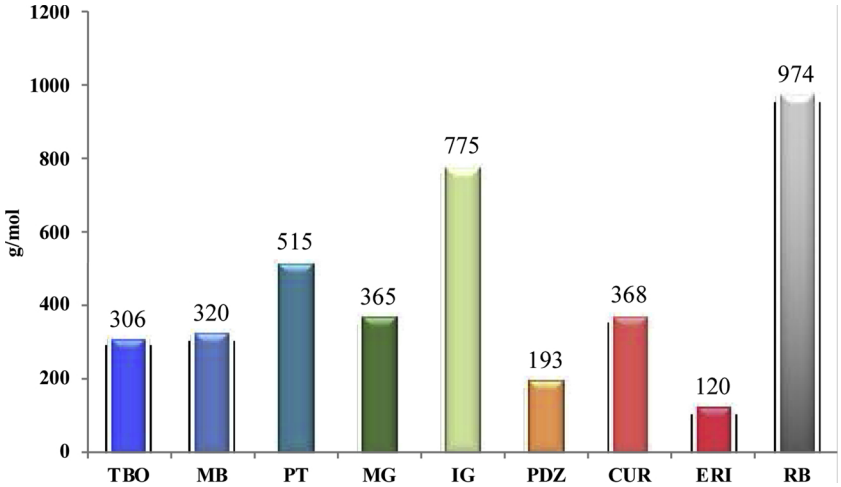

Fig. 2. Molecular weight of the photosensitizers according to PubChem (https://pubchem.ncbi.nlm.nih.gov): Toluidine blue O (TBO): CID 7083, methylene blue (MB): CID 6099, phthalocyanine (PT): CID 5282330, malachite green (MG): CID 11294, indocyanine green (IG): CID 11967809, photodithazine (PDZ): CID 151093, curcumin (CUR): CID 969516, erythrosine (ERI): CID 94176 and rose bengal (RB): CID 25474.

[14,37]. However, malachite green, which belongs to the triarylmethane family, seems not to produce singlet oxygen, indicating that the aPDT activity occurs *via* type I mechanisms [30]. In addition to the synthetic dyes mentioned, natural dyes such as curcumin have also been used against oral pathogens, and their effectiveness may be related to the capacity to induce ROS formation [17,38]. On the other hand, the antimicrobial action of indocyanine green, an organic dye, is mainly photothermal [11].

Many studies consider TBO to be a promising PS, since it is one of the most commonly used in dental aPDT [4,11,13,25,39]. Its high affinity for acidic molecular components, like DNA and RNA [40,41], is important to inhibit cell division and growth, leading to bacterial [40] and fungal [42] cell death.

In the presence of light and molecular oxygen, PS produces cytotoxic species capable of destroying bacterial cells. Consequently, a PS should not be toxic until being activated by an appropriate light wavelength [15,43]. A strong absorption peak of PS in the range of red to the near-infrared spectral region (between 650 and 800 nm) is preferable because absorption of single photons with wavelengths longer than 800 nm does not provide enough energy to excite oxygen to its singlet state [17]. Absorption spectrum analysis is widely used in studies involving aPDT, due to the need to determine the regions of higher PS absorption and the specific wavelengths of the light sources to be used, always aiming at greater efficacy and lower energy dispersion [14,40,44,45]. Fig. 3 shows the light absorption curves of the most frequently used PSs against cariogenic microorganisms in biofilms or dental caries.

Efficient PSs must have a noticeable cationic charge, connecting quickly to bacterial cells. Commonly, the more cationic the charge is, the more effective the PS will be, especially in targeting Gram-negative bacteria [17]. The production of cytotoxic species is directly related to the PS excitation itself from its ground state to its singlet state by light absorption in the 0.6-0.8 um region of a specific wavelength [15,39,46].

The wavelength used in the irradiation procedures should be chosen taking into account not only the ability of light penetration in the target tissue/substrate, but also the maximum PS absorption. The longer the wavelength, the higher the penetration obtained is and stronger are the effects are [15,46–48].

3. Light source

The basic requirement for aPDT light sources is that they match the activation spectrum of the used, thus generating adequate light potency at the proper wavelength. The efficiency of aPDT (reduction of bacterial viability) is directly related to the output power, energy density (amount of light dose) and time of exposure to the light. The output power and the irradiation time are important parameters to improve the microbial inactivation results [49], thus, the success of aPDT is dependent among other factors on the total light dose delivered in the target cells. With regard to light dose to be applied, the most important requirements for therapeutic photoirradiation systems employed in aPDT are that enough light must reach the target cells.

The first light sources used in photodynamic therapy (PDT) were polychromatic, non-coherent lamps designed to emit white light, causing heat in most cases. The light sources available for aPDT belong to three major groups: broad spectrum lamps, light-emitting diodes (LEDs) and lasers [7,50,51]. The spectra of these light sources can be found in Fig. 4.

Lasers are commonly used light sources in PDT that have been explored due to their healing, anti-inflammatory, anti-edematous and analgesic actions. Light emitted by lasers is unidirectional, monochromatic and coherent in time and space, which can be associated with photosensitizers with resonant absorption bands to the laser

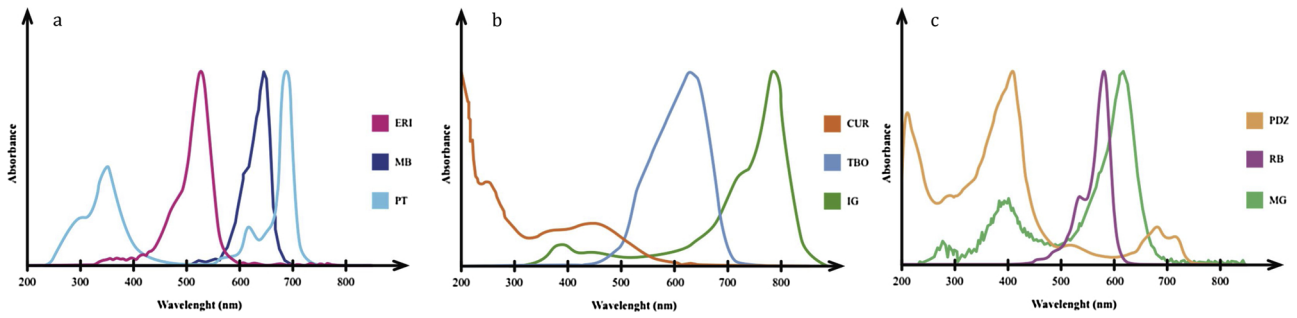

Fig. 3. Light absorption curves of PS used against cariogenic microorganisms in biofilms or dental caries: a) erythrosine (ERI), methylene blue (MB) and phthalocyanine (PT); b) curcumin (CUR), toluidine blue O (TBO) and indocyanine green (ICG); and c) photodithazine (PDZ), rose bengal (RB) and malachite green (MG).

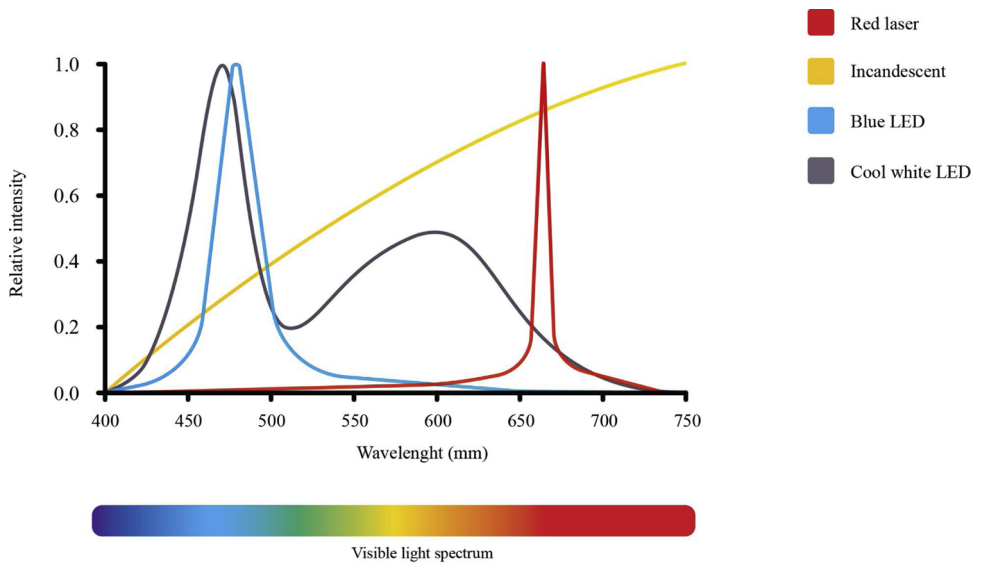

Fig. 4. Graph of light spectrum emission used in aPDT.

wavelength, thus being capable of absorbing the greatest part of the emitted radiation. Short wavelengths emit ultraviolet light (200–400 nm), while longer wavelengths can produce energy in the visible (400–700 nm) or near-infrared (700–1500 nm) spectrum [7,52,53].

Halogen lamps are another type of light source, which are cheaper and have high irradiance. This keeps the light constant, enabling aPDT to be performed with short exposure times. These lamps are advantageous because they can be spectrally filtered to the wavelength corresponding to any photosensitizer [14,15,37,51]. However, halogen lamps have drawbacks, such as the decrease in light production over time, as well as a strong thermal component and incoherence. Furthermore, although the light source is broadband, its power is less effective [51,31].

To overcome these drawbacks, plasma arc and light LED devices have begun to be used in light therapy. Plasma arc lighting devices exhibit high light luminance and radiance output. The development of LEDs has provided another alternative to therapy with halogen light. These light sources only recently become commercially available [31]. Among the main advantages of using LEDs are low cost, small size, and ease of configuration for different radiations. However, they do not present good collimation and coherence, resulting in wider emission bands and consequently light emission throughout bigger areas of the absorption spectrum [53–55].

Compared to lasers, LEDs are alternative light sources that are durable, and depending on the desired wavelength, can be relatively inexpensive [53–55]. Additionally, LED sources are already present in routine dental practice, commonly used for composite resin polymerization in restorations. Therefore, they can be used in aPDT without requiring the acquisition of new equipment [32,39,53,55].

In general, the most commonly used light sources in aPDT are lasers and LEDs at wavelengths near the visible blue and red-light spectra [4,11–13,15,54,56,57] (Fig. 4).

The ideal light source must generate low power located in the visible portion of the electromagnetic spectrum and specific wavelength. The wavelength depends on the dose and depth of action of the photosensitizer used [55].

4. Methods

4.1. Research strategy

The articles were found by searching the PubMed and Web of Science databases, combined with manual search of references cited in key papers. According to descriptors of Health Sciences (DeHS), the following ones were selected: “dental caries” and “photodynamic therapy”.

4.2. Types of study

Different types of studies such as *in vitro*, *in situ* and *in vivo* have demonstrated strong antibacterial action of aPDT, mainly on bacteria involved in the etiology of dental caries. Among these are species of the Streptococcus genus, such as *S. mutans,* the most studied bacterial agent for being one of the microorganisms responsible for the demineralization process of tooth surfaces [4,11,16,34,39,54].

In vitro biofilm models allow the analysis of different conditions due to easier control of experimental settings, by limiting variables as well as replicating samples [34,48]. In *in situ* models, realistic conditions of the oral environment such as the constant exposure to saliva, temperature, and the resident microbiota are achieved. These models have been widely used to measure caries processes and are invaluable for cariology research. However, they have limitations, such as high cost, difficulty of reproducibility and ethical issues [13,39,58]. *In vivo* studies are important because they confirm efficacy to enable use of aPDT in clinical practice [15].

Various biofilm models have been used to analyze aPDT efficacy against cariogenic biofilms (single, duo or multi-species biofilms) or microorganisms inside carious dentine. Table 1 summarizes studies of aPDT for the control of oral biofilms formed *in vitro* grown on microtiter plates and Table 2 summarizes the studies of aPDT for decontamination of biofilms or carious substrates formed *in vitro* and *in situ* when the microorganisms were seed over dental samples or biofilms which were found *in vivo* in dental caries lesions.

5. Discussion and conclusion

Dental caries is a very prevalent multifactorial disease in humans, and it is an important cause of oral pain and dental loss. Several studies have shown that cariogenic bacteria are susceptible to aPDT, reporting the efficiency in killing oral bacteria in biofilms [11,34,37,57]. This suggests that aPDT might be useful as a minimally invasive adjuvant therapy for dental caries control [15,26,43], by inactivating microorganisms involved in caries processes [59].

Studies of aPDT mediated by TBO dye and red laser light have resulted in the photoinactivation of *S. mutans* by 3.6–4 $\log_{10}$ [18] and

Table 1
Summary the studies of aPDT for the control of oral biofilms formed *in vitro* grown on microtiter plates.

Biofilm *in vitro*					
Photosensitizer	Light Source	Microorganisms Substrate	Protocol	Results	Authors
Erythrosine (Sigma Aldrich) (400 μM)	LED (MMOptics) 90 mW	*C. albicans* *S. sanguinis* **Single and dual-species biofilms**	Pre-irradiation for 5 min and irradiation for 3 min to an energy density of 16.2 J.	Reductions of 1.07 and 0.39 log_{10}, respectively, in biofilms of *C. albicans* alone and in association with *S. sanguinis*. Biofilms of *S. sanguinis* alone were more sensitive, with reduction of 4.48 log_{10}. When in association with *S. sanguinis*, a decrease of 2.67 log_{10} was observed.	[71]
Erythrosine (ERI) (VETEC) Methylene blue (MB) (Herzog) (100 and 250 μM)	Tungsten filament halogen (CL-K50 Kondortech) 232 W	*S. mutans* **Biofilms**	Pre-irradiation for 5 min and irradiation for a total of 2 min divided into 4 periods of 30 s with 30 s intervals to 0.6 J/cm^2 energy density.	At the concentration of 100 μM, only PACT with ERI + MB showed statistically significant reduction (3.2 log_{10}) relative to untreated control. At the concentration of 250 μM, all groups demonstrated statistically significant reductions. ERI, MB and ERI + MB showed significant reduction of 4.5 log_{10}, 4.3 log_{10}, and 5.3 log_{10}, respectively.	[37]
Methylene blue (Sigma Aldrich) (100 μM)	LED 473 mW	*S. mutans* **Biofilms**	Pre-irradiation for 10 min and irradiated for 60, 240, 360, 390, 450, 480 s with 10, 40, 60, 65, 75, 80 J/cm^2 of radiant exposure.	Biofilm was completely inactivated by aPDT after 6 min of irradiation. However, the presence of glucose delayed the complete inactivation of the biofilm, being observed only following 8 min of irradiation.	[75]
Toluidine blue O New methylene blue (Sigma Aldrich) (10 and 50 μM)	Laser 100 mW	*S. mutans* **Biofilms**	Pre-irradiation for 30 min and then irradiated with 5 J/cm^2 (39 s) and 50 J/cm^2 (6 min (25 s) of light.	The photosensitizers were not able to reduce the biofilm with 10 μM of each dye and 5 J/ cm^2 while 50 μM with 50 J/cm^2 of light dose photoinactivated 3.6–4 log_{10}.	[18]
Toluidine blue O (Sigma-Aldrich) (100 g/mL^{-1})	LED (Laserbeam) 40 mW	*S. mutans* **Biofilms**	Pre-irradiation time of 5 min, following this time, the biofilms were exposed for 15 min to a 55 J/ cm^{-2} energy density.	Significant decreases were observed ($p > 0.05$), with median viable counts of $1.40 \times 10^4 \pm 1.27 \times 10^4$ (SD) and log_{10} reductions of up to 5.48.	[39]
Toluidine blue O (Sigma-Aldrich)(100 μg/mL)	LED (Laserbeam) 0.04 W Red noncoherent light (LumaCare) 2.24 W	*S. mutans* **Biofilms**	Pre-irradiation time for 5 min, which was followed by two different red lights. Laserbeam (900 sec with a 56.6 J/cm^2 energy density) and LumaCare (56.6 J/ cm^2 for 22 sec, 149 J/ cm^2 for 60 sec, 317.05 J/ cm^2 for 120 sec and 475.58 J/ cm^2 for 180 sec.	Laserbeam and LumaCare, with equal energy density, did not show significant statistical difference with each other. Therefore, due to the difference in the irradiation time (from 15 min to 22 sec), LumaCare may be a better light source for clinical use. The LumaCare group caused reductions of up to 2.38 log_{10} and 2.27 log_{10} after 120 and 180 sec of irradiation, respectively.	[73]
Toluidine blue O (Sigma-Aldrich) (0.4 mg/mL) Indocyanine green (IG) (Sigma-Aldrich) (4 mg/mL)	Laser (Konftec) 200 mW Laser (A.R.C.laser) 250 mW	*S. mutans* **Biofilms**	Irradiation time of 30 s, with an energy density of 17.18 and 15.62 J/cm^2 for TBO and IG, respectively, and a room temperature (25 ± 2 °C).	Significant differences in reducing biofilm formation was seen at the concentration of 100 μg/ml TBO (63.87%) and 1000 μg/ml IG (67.3%) compared with other groups ($p < 0.05$).	[11]

Table 2
Summary of studies of aPDT applied on biofilms or caries lesions performed *in vitro*, *in situ* and *in vivo*.

Caries models					
In vitro					
Photosensitizer	Light Source	Microorganism Substrate	Protocol	Results	Authors
Curcumin (0.75, 1.5, 3.0, 4.0, and 5.0 g/l)	LED (Edixeon) 19 and 47.5 mW	*L. acidophilus* *S. mutans* **Dual-species biofilms**	Pre-irradiation time of 5 min and irradiation for 5 min or 2 min at fluence of 5.7 J/cm^2.	Both light intensities required 5.0 g/l of curcumin for significant bacterial reduction ($p < 0.05$).	[32]
Curcumin (Sigma-Aldrich) (300 μM)	LED (Biotable) 40 mW	Microcosm (saliva as inoculum) **Multi-species biofilms**	Pre-irradiation time of 2 min and energy density (75 J/cm^2) determined by the mean time variation of 11 min.	Reduction of total microorganisms (1.32 log_{10}), total streptococci (1.2 log_{10}), total lactobacilli (0.5 log_{10}) and mutans streptococci counts (0.19 log_{10}). After 24 h of incubation, biofilms presented a tendency of increasing CFUs, with a non-significant decrease of lactobacilli counts	[69]
Curcumin (Sigma-Aldrich) (600 μmol/L^{-1})	LED (Biotable) 40 mW	Microcosm (saliva as inoculum) **Multi-species biofilms**	Pre-irradiation time of 2 min of light exposure (935 s or 1.870 s), corresponding to total energy of 37.5 and 75 J/cm^2, respectively.	At 37.5 J/cm^{-2} energy density, count reductions of 1.89, 1.71, 1.68 and 1.69 log10 were observed. When using 75 J/cm^{-2}, reductions of 2.00, 1.52, 1.70 and 2.33 log10 were observed in total microorganisms, total streptococci, mutans streptococci and lactobacilli, respectively.	[70]
Methylene blue (Aptivalux) (0.025 g/mL)	LED (MM Optics)40 mW	*S. mutans* **Biofilms**	Pre-irradiation time of 5 min, irradiation for 1 min and energy density of 60 J/cm^2.	The aPDT significantly reduced the bacterial load on the surfaces of caries-like affected dentin discs by 81.01% (mean reduction of log_2 1.010 ± 0.1548; $p = 0.0029$).	[67]
Methylene blue and phosphoric acid (FGM) (37%)	Laser (DML) 5 W	*S. mutans* **Biofilms**	Pre-irradiation and irradiation 15 s each, with energy density of 4 J/cm^2.	The aPDT showed a relative reduction in *S. mutans* counts of 55.22% ($p < 0.0001$). However, significant reductions in *S. mutans* counts were also observed in the groups using only light source or dye.	[66]
Methylene blue (MB) Silver Nanoparticles (Ag NPs)	Laser 200 mW	*S. mutans* **Biofilms**	Pre-irradiation time of 5 min (group MB) followed by irradiation for 3 min for both photosensitizers.	The aPDT treated with Ag NPs and MB recorded the greatest reduction percentage of CFU/ml (95.28%) compared to the control specimens.	[76]
Photoditazine® (PDZ) (0.6 mg/mL) Fotoenticine® (FTC)(0.6 mg/mL) Methylene blue (MB) (1 mg/mL)	Laser 0.035 W	*S. mutans* **Biofilms**	Pre-irradiation followed by irradiation for 480 s with energy density of 15 J/cm^2.	The three photosensitizers significantly reduced the number of *S. mutans*. Reductions of 4 and 6 log were observed in the MB and PDZ, respectively, while complete elimination of the *S. mutans* biofilm occurred in the FTC.	[69]
Methylene blue (MB) (Merck) (2%) Indocyanine green (IG) (Merck) (0.2%)	Laser 40 mW (MB) 100 mW (IG)	*S. mutans* **Biofilms**	The teeth in the MB group were irradiated with density of 2.4 J/cm^2 for 60 s, while the teeth in the IG group were irradiated with density of 6 J/cm^2 for 60 s.	The two experimental groups led to reduction in the number of *S. mutans* ($p = 0.0001$). *S. mutans* bacteria were completely eradicated, the final number of colonies was zero.	[77]
Toluidine blue O (Sigma-Aldrich) (100 g/mL^{-1})	LED (Laserbeam) 40 mW	*S. mutans* **Dentin caries**	Pre-irradiation time of 5 min and irradiation for 5, 10 or 15 min, with incident energy dose of 47, 94, or 144 J/cm^2, respectively.	The groups treated with aPDT for 10 or 15 min presented statistically higher log reduction values when compared to the respective control groups. The log reduction values ranged from 0.39 to 5.80, with the highest value for 15 min irradiation.	[25]
Toluidine blue O (Pharmácia Específica) (200 mM)	LED (Biotable) 40 mW	Microcosm (saliva as inoculum) **Multi-species biofilms**	Pre-irradiation for 2 min (1.5 ml of TBO), then irradiated for 468 s, 935 s or 1870s, which correspond to 1.88 J, 3.75 J, or 7.50 J of total light energy.	The highest aPDT mean reductions of the viability were detected with 75 J/cm2, varying from 2.15 to 2.37 log10 CFU/mL. However, significant statistical differences were observed in only total microorganisms ($P < 0.0001$) and total streptococci ($P < 0.0001$).	[63]
Toluidine blue O (Sigma-Aldrich) (0.1 mg/mL)	LED (MMO) 80 mW	*S. mutans* **Dentin caries**	Pre-irradiation for 5 min, then irradiation for 4.2 min or 6.5 min, which correspond to 166 or 249 J/cm^2 of total light energy	The mean lesion depth produced by the microbiological model used in this experiment was 253.7 ± 40.66 μm. The log reduction values ranged	[21]

(*continued on next page*)

Table 2 (*continued*)

Caries models					
In vitro					
Photosensitizer	Light Source	Microorganism Substrate	Protocol	Results	Authors
Toluidine blue O (Sigma-Aldrich) (0.1 mg/mL $^{-1}$)	LED (Laserbeam) 32 mW	*S. mutans* *S. sobrinus* *S. sanguinis* **Biofilms**	Pre-irradiation time of 5 min, after which the biofilms were exposed for 7 min to 85.7 J/ cm^{-2} energy density.	from -1.63 to 2.43 and the aPDT using 249 J/cm2 light energy exhibited the highest log reduction. A significant reduction (≈ 95%) in viability was observed for *S. mutans* and *S. sobrinus* biofilms following photosensitization, with a > 99.9% reduction in the viability of *S. sanguinis* biofilms.	[4]
In situ					
Photosensitizer	**Light Source**	**Protocol**	**Microbiological methods**	**Results**	**Authors**
Toluidine blue O (Sigma-Aldrich) (100 g/mL^{-1})	LED (Laserbeam) 40 mW	Pre-irradiation time of 5 min (50 µl of TBO), following this time, the biofilms were exposed for 15 min to a 55 J/ cm^{-2} energy density.	Culture to determine total streptococcus and mutans streptococci viability. **Biofilms**	The aPDT showed a slight reduction in the microbiological counts for both total streptococci and mutans streptococci. However, there was no statistical significance between the control group ($p > 0.100$).	[39]
Toluidine blue O (Sigma-Aldrich) (100 g/mL^{-1})	LED (Laserbeam) 40 mW	Pre-irradiation with 5 µl of TBO for 5 min and light source for 5 min (47 J/ cm^{-2}) or 10 min (94 J/ cm^{-2}).	Culture of mutans streptococci, total streptococci, lactobacilli, and total microorganisms. **Dentin caries**	The PACT resulted in a significant decrease in the viability of total streptococci ($P < 0.0004$), mutans streptococci ($P < 0.0223$), lactobacilli ($P < 0.0092$), and total microorganisms ($P < 0.0004$). However the treatment for 10 min with 94 J /cm^{-2} of irradiation had a significant effect on bacterial reduction in dentin caries, even in the absence of sensitizer.	[13]
Toluidine blue O (TBO) Chlorin e6 (Ce6) (100 µg ml^{-1})	VIS + wIRA radiator (Hydrosun Medizintechnik GmbH) 200 mW	Pre-irradiation for 2 min followed by light irradiation for 5 min.	CFU quantification of the stained photoinactivated microorganisms. **Biofilms**	TBO- and Ce6-mediated aPDT yielded significant decreases of up to 3.8 and 5.7 $\log_{10}$ CFU for initial and mature oral biofilms, respectively. The Ce6 presented elevated permeability and higher effectiveness in eradication (89.62%) compared to TB (82.25%).	[58]
In vivo					
Photosensitizer	**Light Source**	**Protocol**	**Microbiological methods**	**Results**	**Authors**
Methylene blue (Aptivalux) (100 mg/L)	Halogen light (3MEspe) 260 mW	Pre-irradiation time of 5 min and irradiation for fractionated 1 min (20s- two of 30 s.).	Culture (*Streptococcus* and *Lactobacillus)* and real-time PCR for *S. mutans*.	The PACT resulted in mean log10 reductions in CFU of between 2.5 ± 0.6 (for the superficial dentin), 1.9 ± 0.9 (for deep dentin directly irradiated), and 2.3 ± 0.8 (for deep not directly irradiated).	[15]
Methylene blue (MB) (Hyrofarma) (0.01%) Toluidine blue O (TBO) (0.1 mg/mL)	LED (MMO) 100 mW Laser (DMC) 100 mW	Pre-irradiation time of 5 min of light exposure 60 s (LED/TBO) or 90 s (Laser/MB), corresponding to a total energy of 30 and 320 J/cm^2, respectively	The number of *S. mutans*, *S. sobrinus*, *Lactobacillus casei*, *Fusobacterium nucleatum*, *Atopobium rimae*, and total bacteria were established by quantitative polymerase chain reaction.	All therapies were effective in reducing the number of microorganisms, except for *S. sobrinus*. No statistical differences were observed among the protocols used	[64]
Toluidine blue O (Merck,) (0.1 mg/mL)	LED (CMS Dental) 2000 mW	Pre-irradiation for 1 min and irradiated 150 s with the total energy density was 300 J/cm2.	Saliva samples were collected (1 h and 7 days after treatment) and *S. mutans* counts were performed.	The counts of *S. mutans* in saliva decreased significantly after 1 h ($P < 0.001$). However, the difference in reduction of *S. mutans* counts in saliva was not significant between the baseline and 7 days after treatment ($P > 0.05$)	[65]
Toluidine blue O (Merck) (0.1 mg/mL)	Laser (Mustang 2000) 20 mW	TBO solution was kept in the mouth for 5 min (pre-irradiation) followed by irradiation on the tongue mucosa and buccal and lingual tooth surfaces for 5 min (continuous wave mode).	Salivary samples were collected before and after aPDT. Samples were cultured on mitis salivarius agar, and after incubation, the colonies were counted.	Bacterial count significantly decreased on days 1 and 3, and 1 and 2 weeks after the second intervention. Bacterial count also decreased following the use of TBO and laser separately, but these reductions were not significant ($P > 0.05$)	[60]
Toluidine blue O (Sigma-Aldrich) (100 µg/mL^{-1})	LED (MM Optics) 150 mW	Pre-irradiation time by 5 min (10 µl of TBO) and the total energy 94 J/cm^{-2}.	Samples of dentin were collected before and immediately after treatments for analysis of total viable bacteria, mutans streptococci and *Lactobacillus* spp. counts	The aPDT caused significant reductions in mutans streptococci (1.08 ± 1.20 log), *Lactobacillus* spp. (1.69 ± 1.37 log), and total viable bacteria (1.07 ± 1.01 log) compared to the control.	[62]
Toluidine blue O (Sigma-Aldrich) (100 µg/mL)	LED (Laserbeam) 40 mW	Pre-irradiation with 10 µl of TBO for 5 min and light source for 10 min (94 J/ cm^2).	Before and after treatments, dentin samples were analyzed with regard to the counts of total viable microorganisms, total streptococci, mutans streptococci, and lactobacilli.	Log reductions ranged from -0.12 to 2.68 and significant reductions were observed for aPDT when compared to the other groups for total streptococci and mutans streptococci.	[61]

decreases of bacterial count in salivary samples [60]. Besides lasers, studies have also evaluated TBO effects associated with red LED light on the viability of oral microorganisms in biofilms formed *in vitro*, caries models (*in vitro* and *in situ*) and clinical studies (*in vivo*) [3,4,13,21,39,61,62,78], and have shown promising antibacterial effects. *In vitro* biofilm studies have observed significant *S. mutans* decreases, with $\log_{10}$ reductions of up to 5.48 [39]. Significant results were also observed for *S. mutans* biofilms exposed to TBO and indocyanine green, with concentrations of 100 (63.87%) and 1000 μg/ml (67.3%), respectively. These results have been attributed to the detachment of the biofilms by the disintegration of the interaction among bacteria, owing to ROS exposure following aPDT [11].

Several studies have shown bacterial photoinactivation by aPDT performed with TBO and LED, in caries lesions in demineralized slices of dentin. These caries lesions can be formed *in vitro* or *in situ* [3,4,13,21,25]. *In vitro* studies have demonstrated significant reductions of *S. mutans* and *S. sobrinus* biofilm viability (≈ 95%) and a 99.9% reduction in the *S. sanguinis* biofilm viability, after exposure to TBO and LED irradiation [4]. It has been suggested that this photosensitization decreased the *S. mutans* cell diameter [3], reducing *S. mutans* counts, when compared to the respective control groups, with $\log_{10}$ reduction of 5.80 for 15-min irradiation time [25]. The microbial pool from microcosm also was tested, but significant statistical differences were observed only for total microorganisms and total streptococci [63].

The *in situ* models are based on multispecies biofilm accumulation and sucrose exposure and are considered as a proper tool for testing the effect of aPDT on microorganisms involved in dental caries processes [13,39]. Investigation of *in situ* caries models has demonstrated that TBO associated to LED is able to decrease microbial load present in dentine caries and may be a useful technique to eliminate bacteria from dentine carious lesions before restoration [13]. However, TBO and LED are not so effective in killing oral streptococci present in multispecies biofilms grown *in situ*. The inefficiency of aPDT in *in situ* biofilms tested may be related to the biofilm thickness (1000 μm) present in this caries model [39].

Studies have also evaluated the *in vivo* effects of TBO associated with LED on carious dentin and the results showed that aPDT is effective in reducing the number of *S. mutans*, *Lactobacillus casei*, *Fusobacterium nucleatum*, *Atopobium rimae* and total viable bacteria compared to the control, except for *S. sobrinus* [61,62,64]. In saliva, the counts of *S. mutans* were significantly decreased at 1 h after aPDT treatment [65]. Some mechanisms can increase TBO efficacy, such pre-irradiation time and pH values higher than 6, since alkaline pH increases the singlet oxygen production. Likewise, the bacterial destruction degree is related to the PS diffusion through the membranes and light energy: the higher the light energy, the greater the log reduction is [4,21,55,56]. In addition, a study also noted that *S. mutans* biofilm reduction is inversely proportional to the TBO concentration, because the count decreased as TBO concentration increased [11].

Similar to TBO, MB dye is a phenothiazinium used in aPDT that reduces the bacterial load of *S. mutans* and *Lactobacillus* spp., among others [14,66,67]. However, TBO is more commonly used, produces more singlet oxygen and is more phototoxic, causing greater damage to the bacterial cells compared to MB [18]. When compared to PDZ, MB was also considered less effective since aPDT performed using MB achieved microbial reductions around 4 $\log_{10}$ while with PDZ, the *S. mutans* reduction was about 6 $\log_{10}$ [68].

The use of curcumin together with blue LED in aPDT protocols showed phototoxic effects by decreasing the number of viable bacteria compared to the control groups [12]. The photodynamic effects of curcumin on *S. mutans* and *L. acidophilus* grown in biofilm phases and in dentinal carious lesions were investigated, and the results showed curcumin in combination with blue LED had a significant phototoxic effect. All curcumin concentrations tested (0.75, 1.5, 3.0, 4.0, and 5.0 g/l) demonstrated beneficial results, and the lowest curcumin concentration (0.75 g/l) was sufficient to kill 97.5% of the microorganisms. Conversely, when aPDT was applied to microorganisms in dentinal carious lesions, the results showed that these bacteria were more resistant to aPDT. This is possibly due to light penetration and drug diffusion impairment [24]. Furthermore, studies have shown that the phototoxic effect is dependent on the curcumin concentration [32,33]. However, the microcosm model, which uses saliva as inoculum, constituting an artificial caries protocol by multi-species biofilm, was also used to evaluate the aPDT activity. Reductions were observed of total microorganisms, total streptococci, mutans streptococci and lactobacilli [69,70].

Some studies have focused on the potential effects of erythrosine associated with white light or LED, demonstrating a significant decrease *in vitro S. mutans* biofilm (reduction of 3.7 $\log_{10}$ CFU/mL) [34,35,37]. The antimicrobial effects of ERI associated with LED were demonstrated by reductions in microbial counts of *C. albicans* and *S. sanguinis* grown alone or in dual-species biofilms. Biofilms of *S. sanguinis* alone were more sensitive, with reduction of 4.48 $\log_{10}$, whereas when the microorganisms were grown in association, the *S. sanguinis* decrease was only of 2.67 $\log_{10}$, indicating that aPDT is less effective against more complex biofilms [71].

Similar promising results of aPDT protocols mediated by erythrosine associated with three different light sources commonly used in dental practice (halogen, LED, and, plasma arc) were observed when applying the same amount of energy on planktonic and biofilm populations of *S. mutans* [72]. However, the outcome was different when comparing the planktonic and biofilm states, where the planktonic state was much more susceptible to bacterial viability reductions [72].

Considering that aPDT efficacy depends on the photosensitizer and the light used and that white light has a broad emission spectrum, covering the wavelength of various dyes [37], the antimicrobial effect of the combination of the erythrosine/methylene blue activated by a white halogen light device on *S. mutans* biofilm showed better results for the PS combination regardless of the dye concentration [37]. Consequently, the combination of different photosensitizers and white lights can be a promising approach to treat biofilm-dependent diseases like dental caries, due to higher ROS production caused by broad coverage of the light absorption and light emission spectrum combination [14].

Clinical and *in vitro* studies have evaluated aPDT mediated by aluminum-chloride-phthalocyanine against cariogenic bacteria in caries lesions and showed that bacterial cells compared to eukaryotic dental pulp cells preferentially absorbed aluminum-chloride-phthalocyanine. The process was efficient in the reduction of microbial load from bacterial cultures. In addition, the clinical study showed a mean reduction of 82% for total bacteria in the treated cavities after aPDT application. Taken together, the results showed that aPDT mediated by aluminum-chloride-phthalocyanine is safe for clinical application and is effective in reducing bacterial load in caries lesions [28].

Most studies that have evaluated aPDT have used LED, since it is a durable light source that is neither coherent nor collimated, besides being inexpensive and easy to install. Moreover, the LED sources are already present in dental routine and can be used in aPDT, without requiring the purchase of new equipment [4,26]. The ideal light source for aPDT should be located in the visible portion of the electromagnetic spectrum and the specific wavelength of dye absorption [46].

With regard to safety, since aPDT is applied using light sources that are able to produce pulpal temperature increases, a study showed LED combined with TBO is a safe and effective approach for dentine caries disinfection because temperature rises lower than 2 °C were observed for both pulp and periodontium sites [25]. In addition, more recent study compared the effect of aPDT using TBO associated with two different red lights (Laserbeam® and LumaCare) on *in vitro S. mutans* biofilms. The two red light sources, with equal energy density, did not show significant statistical difference to each other [73] and also demonstrated that this therapy is minimally invasive and harmless to tooth structures, with safe pulpal temperature increases [73].

As a phototherapeutic agent, the PS must have high molar absorption in the spectral region between 600–950 nm, known as the "phototherapeutic window". Irradiation by light sources at these wavelengths provides a therapeutic effect because the cell membrane has considerable transparency to electromagnetic radiation [17,18,74].

Despite the extensive investigation, many dentists lack confidence to use novel approaches for the treatment of caries. This is one of the biggest issues working against the introduction of aPDT in dental practice [16,57]. Besides that, another limitation to using this approach is the need for long exposure times to attain significant inhibitory action against microorganisms related to oral diseases [59]. However, results showed that a high intensity dental light source applied for a short period of time effectively caused the death of *S. mutans* [59].

This review showed that aPDT is a potential antimicrobial therapy capable of inducing death of microorganisms by oxidative damage. This suggests that aPDT is complementary to other therapies, such as the use of chlorhexidine [59] or selective caries removal [78]. It should be noted that there have been no reports of the development of bacterial resistance after the application of photodynamic therapy, unlike chlorhexidine [59].

Most of the studies found used dentin caries models *in vitro*, which are not the best ones to yield strong scientific evidence [11,12,14,50,54]. Although they have made a substantial contribution to advances in the study of the oral biofilm, *in vitro* models only allow for the study of a small number of species interactions in controlled laboratory conditions, which may not accurately reflect the physiological situation of the oral cavity, such as salivary flow [4,13,39,52]. In general, antimicrobial photodynamic therapy is satisfactory when applied to oral biofilms, showing positive results, so it can be used as adjunct therapy to conventional clinical methods.

5.1. Perspectives

Microbiological control is the main purpose of aPDT. Therefore, this therapy is applicable to dental caries treatment and the results are promising so far. aPDT has good perspectives in clinical practice, with inclusion of lasers, LEDs and novel technologies in modern dentistry. The intention here is to provide information for researchers and to a wider readership, as well as to serve as a tool to promote appropriate discussion on the ideal use of the components of this therapy.

Conflicts of interest

The authors reported no conflicts of interest related to this study.

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