

# Antimicrobial Photodynamic Therapy: Photodynamic Antimicrobial Effects of Malachite Green on *Staphylococcus*, Enterobacteriaceae, and *Candida*

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## Abstract

**Objective:** This study investigated *in vitro* the photodynamic antimicrobial effects of the photosensitizer malachite green on clinical strains of *Staphylococcus*, Enterobacteriaceae, and *Candida*. **Materials and Methods:** Thirty-six microbial strains isolated from the oral cavity of patients undergoing prolonged antibiotic therapy, including 12 *Staphylococcus*, 12 Enterobacteriaceae, and 12 *Candida* strains, were studied. The number of cells of each microorganism was standardized to  $10^6$  cells/mL. Twenty-four assays were carried out for each strain according to the following experimental conditions: gallium-aluminum-arsenide laser and photosensitizer ( $n = 6$ , L+P+), laser and physiologic solution ( $n = 6$ , L+P–), photosensitizer ( $n = 6$ , L–P+), and physiologic solution ( $n = 6$ , L–P–). Next, cultures were prepared on brain–heart infusion agar for the growth of *Staphylococcus* and Enterobacteriaceae, and on Sabouraud dextrose agar for the growth of *Candida*, and incubated for 48 h at 37°C. The results are reported as the number of colony-forming units (CFU/mL) and were analyzed with analysis of variance and the Tukey test. **Results:** The *Staphylococcus*, enterobacterial, and *Candida* strains were sensitive to photodynamic therapy with malachite green (L+P+). A reduction of  $\sim 7 \log_{10}$  for *Staphylococcus*,  $6 \log_{10}$  for enterobacteria, and  $0.5 \log_{10}$  for the genus *Candida*. Significant statistical differences were observed between the L+P+ groups and the control groups (L–P–). **Conclusion:** The *Staphylococcus*, Enterobacteriaceae, and *Candida* strains studied were sensitive to photodynamic therapy with malachite green.

## Introduction

PHOTODYNAMIC ANTIMICROBIAL THERAPY consists of the combination of a photosensitizer and visible light, which is able selectively to destroy microbial cells. The antimicrobial effects of this therapy have been known for >100 years. However, only recently has this therapy begun to be studied in detail in the search for alternative treatments for antibiotic-resistant pathogens. Numerous *in vitro* studies have demonstrated that photodynamic therapy is highly effective in the destruction of viruses and protozoans, as well as gram-positive and gram-negative bacteria and fungi.<sup>1–4</sup>

In photodynamic antimicrobial therapy, the photosensitizer is activated by exposure to light of a specific wavelength in the presence of oxygen. The transfer of energy from the activated photosensitizer to available oxygen results in the formation of toxic oxygen species, such as singlet oxygen and free radicals. These reactive chemical species destroy proteins, lipids, nucleic acids, and other cell components.

Photodynamic therapy exerts no genotoxic or mutagenic effects, a fact preventing the development of microbial resistance.<sup>5</sup>

Photosensitizers possess structures similar to those of chlorophyll and hemoglobin (*i.e.*, the molecules contain a heterocyclic ring). In addition, these agents should be biologically stable, photochemically active, and minimally toxic to tissues of the organism. The photosensitizers used include hematoporphyrin derivatives, phenothiazines (toluidine blue and methylene blue), cyanines, phytotherapeutic agents, and phthalocyanines.<sup>6</sup> Phenothiazine dyes have been extensively studied and have been shown to be effective photodynamic agents for the reduction of bacteria<sup>7–12</sup> and fungi.<sup>13–16</sup> Studies investigating new photosensitizers are currently being conducted. Garcez et al.<sup>17</sup> tested azulene to eliminate *Enterococcus faecalis* from root canals. Prates et al.<sup>18</sup> reported the use of malachite green as a photosensitizer in photodynamic therapy. This dye is a member of the triarylmethane family, like crystal violet, and shows strong absorption of red light in

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the visible spectrum. These authors demonstrated the efficacy of this photosensitizer against *Aggregatibacter actinomycetemcomitans*.

Microorganisms such as *Staphylococcus*, Enterobacteriaceae, and *Candida* are found in the oral cavity and are usually present in small numbers. However, prolonged systemic administration of antimicrobials or immunosuppressive therapy may result in an increase of the numbers of these microorganisms.<sup>19,20</sup> According to Smith et al.,<sup>21</sup> the genus *Staphylococcus* is commonly isolated from the oral cavity in a specific group of subjects, such as children, elderly individuals, patients with end-stage systemic diseases, patients with rheumatoid arthritis, and patients with malignant hematologic diseases. Colonization of the oropharynx with methicillin-resistant *S. aureus* strains is frequently observed in many of these patients.

Isolation of species of the family Enterobacteriaceae from the oral cavity has been demonstrated in various studies. Barbosa et al.<sup>22</sup> evaluated the subgingival occurrence of enteric bacilli and *Pseudomonas* in patients with periodontal disease, with these microorganisms being detected in 31.2% of the patients studied. The most frequently isolated species were *P. aeruginosa* and *S. marcescens*. Baydas et al.<sup>23</sup> identified enterobacteria in the saliva from 19 (76%) of 25 patients with the habit of nail biting and in nine (26.5%) of 34 control patients. The isolates were identified as *E. coli*, *E. aerogenes*, *E. cloacae*, and *E. gergoviae*.

Oral infections with yeast of the genus *Candida* are highly frequent in patients infected with HIV. Oropharyngeal candidiasis is the most common manifestation of HIV infection and is observed in 84% of patients.<sup>14</sup> Although *C. albicans* is the main etiologic factor of fungal infections, nonalbicans species are also common, with the most important species being, in decreasing order, *C. parapsilosis* (20–40% of infections), *C. tropicalis* (10–30%), *C. krusei* (10–35%), and *C. glabrata* (5–40%). Currently emerging species include *C. lusitanae* and *C. guilliermondii*.<sup>24</sup>

Many studies have demonstrated the efficacy of phenothiazine dyes such as methylene blue and toluidine blue in photodynamic therapy for the reduction of *Staphylococcus*, Enterobacteriaceae, and *Candida*.<sup>6,8,13,15,26</sup> However, the use of malachite green as a photosensitizer has been investigated only with *Aggregatibacter actinomycetemcomitans*.<sup>18</sup> Therefore, the objective of the present study was to evaluate *in vitro* the sensitivity of *Staphylococcus*, Enterobacteriaceae, and *Candida* strains isolated from the human oral cavity to photodynamic antimicrobial therapy by using malachite green as photosensitizer.

## Materials and Methods

### Microorganisms

Thirty-six microbial strains, including 12 *Staphylococcus*, 12 Enterobacteriaceae, and 12 *Candida* strains, were studied. The staphylococcal strains included *S. aureus* ( $n=3$ ), *S. epidermidis* ( $n=3$ ), *S. schleiferi* ( $n=3$ ), *S. capitis* ( $n=1$ ), *S. haemolyticus* ( $n=1$ ), and *S. lentus* ( $n=1$ ). The following enterobacteria were selected: *Enterobacter cloacae* ( $n=3$ ), *Klebsiella pneumoniae* ( $n=3$ ), *Klebsiella oxytoca* ( $n=3$ ), and *Escherichia coli* ( $n=3$ ). For the genus *Candida*, *C. albicans* ( $n=3$ ), *C. tropicalis* ( $n=3$ ), *C. parapsilosis* ( $n=2$ ), *C. krusei* ( $n=2$ ), and *C. glabrata* ( $n=2$ ) were used.

All strains were obtained from the Laboratory of Microbiology and Immunology, São José dos Campos Dental School, UNESP. The strains were isolated from the oral cavity of patients undergoing prolonged antibiotic therapy for a minimum period of 45 days for the treatment of pulmonary tuberculosis.

Twenty-four assays were carried out for each strain, according to the following experimental conditions: gallium-aluminum-arsenide laser and photosensitizer ( $n=6$ , L+P+), laser and physiologic solution as control with light ( $n=6$ , L+P-), photosensitizer ( $n=6$ , L-P+), and physiologic solution as control ( $n=6$ , L-P-), for a total of 864 assays.

### Preparation of the microbial suspension

Microbial suspensions containing  $10^6$  cells/mL were prepared. For this purpose, *Staphylococcus* strains were seeded onto Mannitol agar (Difco, Detroit, MI), enterobacterial strains were cultured on MacConkey agar (Difco), and *Candida* strains were seeded onto Sabouraud dextrose agar (Difco). All strains were incubated for 24 h at 37°C. Next, the *Staphylococcus* and enterobacterial strains were cultured in brain–heart infusion broth (Difco) for 18 h at 37°C, and the *Candida* strains were cultured in Sabouraud broth (Difco) for 16 h at 37°C.

The cultures were centrifuged at 1,300  $g$  for 10 min, and the supernatant was discarded. The sediment was resuspended in 5 mL sterile physiologic solution (0.85% NaCl). This procedure was repeated, and the number of viable cells in suspension was determined with a spectrophotometer (B582; Micronal, São Paulo, Brazil).

### Photosensitizer

Malachite green (4-dimethylaminophenol) was used as photosensitizer for the sensitization of the microorganisms studied. The malachite green solution at a concentration of 0.1% was prepared by dissolving the powder (Synth, São Paulo, Brazil) in physiologic solution (0.85% NaCl) and was filtered through a sterile membrane (0.22- $\mu$ m pore diameter; MFS, Dublin, CA).

### Low-power laser

The light source used was a gallium-aluminum-arsenide laser (Easy Laser; Clean Line, Taubaté, Brazil) with a wavelength of 660 nm (visible red) and output power of 35 mW. The wavelength of the laser corresponds to the high-absorption spectrum of the photosensitizers used. The laser beam irradiated an area of 0.38 cm<sup>2</sup>, and the irradiation time was 4.45 min, resulting in an energy dosage of 26 J/cm<sup>2</sup> and energy of 10 J. The temperature monitoring at the bottom of the well was made with an Infrared Thermometer (MX4; Raytek, Sorocaba, Brazil), and no increase of temperature was observed after the laser irradiation.

### In vitro photosensitization

An aliquot of the microbial suspension (0.1 mL) and 0.1 mL of the photosensitizer or physiologic solution were added to 96-well flat-bottom microtiter plates. Next, the plates containing the strains were shaken for 5 min in an orbital shaker (Solab, Piracicaba, Brazil), and the content of each well was then irradiated according to the experimental

groups described earlier. Three samples per time were prepared, shaken and irradiated, resulting in the time interval of 13.35 min between the first-irradiated and the last-irradiated well. The strains were irradiated under aseptic conditions under a laminar-flow hood.

After irradiation, serial dilutions were prepared for each strain, and 0.1-mL aliquots of the dilutions were seeded in duplicate onto plates containing brain-heart infusion agar (Difco) for the growth of *Staphylococcus* and enterobacteria, and Sabouraud dextrose agar (Difco) for the growth of yeast. The plates were incubated for 48 h at 37°C. The number of colony-forming units (CFU/mL) was then determined, and the results were submitted to statistical analysis.

The experiment was carried out in the dark, and the plates were covered with a matte black screen with an orifice whose diameter corresponded to the size of the well entrance to prevent the spreading of light. Only 24 wells per microtiter plate were used to avoid light scattering at the well walls, resulting in an overdose at the other already irradiated wells.

#### Statistical analysis

Statistical analysis was performed with the Minitab program by using analysis of variance and the Tukey test, with the level of significance set at 5%.

#### Results

Figures 1–3 show the results of descriptive analysis, analysis of variance, and Tukey test obtained for the 12 *Staphylococcus*, 12 Enterobacteriaceae, and 12 *Candida* strains studied, respectively. Similar results were observed for the *Staphylococcus* and enterobacterial strains. Figures 1 and 2 show that the strains studied were sensitive to photodynamic therapy with malachite green (L+P+). Comparison of the photosensitizer alone (L–P+) showed microbial reduction of *Staphylococcus* and enterobacteria mediated by malachite green. In addition, none of the species was sensitive to the use of laser therapy alone (L+P–).

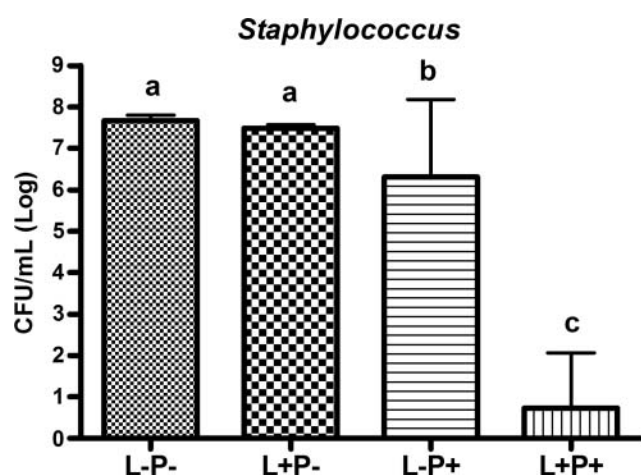


FIG. 1. Mean CFU/mL of *Staphylococcus* submitted to the following treatments: physiologic solution as control (L–P–), laser and physiologic solution (L+P–), photosensitizer (L–P+), and laser and photosensitizer (L+P+). Different letters (a, b, c) indicate a significant difference between groups (Tukey test,  $p < 0.05$ ).

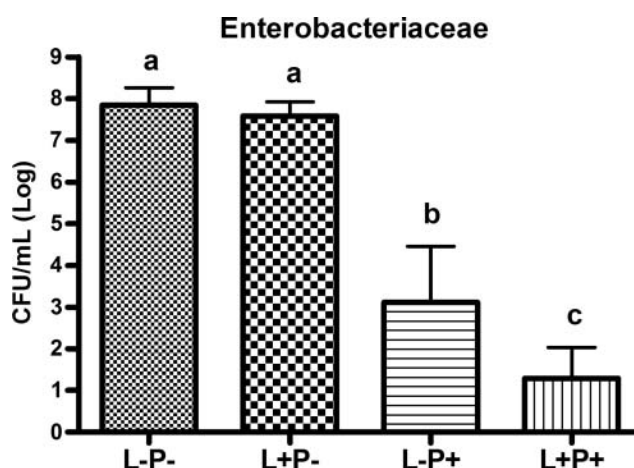


FIG. 2. Mean CFU/mL of Enterobacteriaceae submitted to the following treatments: physiologic solution as control (L–P–), laser and physiologic solution (L+P–), photosensitizer (L–P+), and laser and photosensitizer (L+P+). Different letters (a, b, c) indicate a significant difference between groups (Tukey test,  $p < 0.05$ ).

Results different from those obtained for *Staphylococcus* and enterobacteria were observed for the *Candida* strains studied. As shown in Fig. 3, all laser-treated *Candida* groups (L+P+ and L+P–) had lower mean CFU counts than did the groups not submitted to laser therapy (L–P+ and L–P–). However, a significant difference was observed only for the L+P+ group when compared with the L–P+ and L–P– groups. Similar results were observed for the L–P+ group when compared with the control group (L–P–), suggesting that the use of a photosensitizer alone has no toxic effect on clinical *Candida* strains.

Mean CFU counts (log) obtained by analysis of the photosensitivity of each *Staphylococcus*, enterobacterial, and *Candida* species studied are shown in Tables 1–3, respectively. As

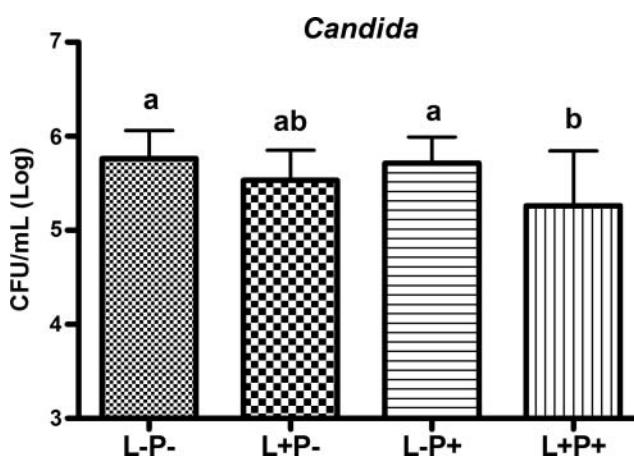


FIG. 3. Mean CFU/mL of *Candida* submitted to the following treatments: physiologic solution as control (L–P–), laser and physiologic solution (L+P–), photosensitizer (L–P+), and laser and photosensitizer (L+P+). Different letters (a, b) indicate a significant difference between groups (Tukey test,  $p < 0.05$ ).

TABLE 1. MEAN COUNTS (LOG CFU/mL) OF *STAPHYLOCOCCUS* SPECIES

<i>Staphylococcus</i> species	Treatment			
	L-P-	L+P-	L-P+	L+P+
<i>S. aureus</i>	7.65 ± 0.05	5.43 ± 3.07	7.49 ± 0.17	0.58 ± 1.69
<i>S. epidermidis</i>	7.66 ± 0.05	7.50 ± 0.13	7.31 ± 0.35	2.31 ± 2.66
<i>S. schleiferi</i>	7.68 ± 0.25	7.54 ± 0.10	6.37 ± 1.62	0.05 ± 0.23
<i>S. capitis</i>	7.69 ± 0.05	7.58 ± 0.01	4.00 ± 0.00	0.00 ± 0.00
<i>S. lentus</i>	7.52 ± 0.03	7.44 ± 0.07	6.98 ± 0.02	0.00 ± 0.00
<i>S. haemolyticus</i>	7.59 ± 0.10	7.47 ± 0.14	7.29 ± 0.09	0.00 ± 0.00

The species were submitted to the following treatments: physiologic solution as control (L-P-), laser and physiologic solution (L+P-), photosensitizer (L-P+), and laser and photosensitizer (L+P+).

shown in Table 1, *S. epidermidis* was the staphylococcal species least sensitive to photodynamic therapy with malachite green. Species of the family Enterobacteriaceae showed similar results under the different experimental conditions (Table 2). Analysis of the genus *Candida* showed that all the species had the same sensitivity to photodynamic therapy (Table 3).

## Discussion

The present results demonstrate that photodynamic therapy with malachite green was effective in microbial reduction of all *Staphylococcus* and enterobacterial strains, and of most *Candida* strains. Comparison of the mean number of CFUs between the L+P+ and control (L-P-) groups showed a reduction of  $\sim 7 \log_{10}$  for *Staphylococcus* and of  $6 \log_{10}$  for enterobacteria, whereas the reduction observed for the genus *Candida* was  $< 1 \log_{10}$ . These data indicate that bacteria were more sensitive to malachite green-mediated photosensitization than were yeast species.

According to Demidova and Hamblin,<sup>25</sup> gram-positive bacteria are more susceptible to photodynamic therapy than are gram-negative bacteria. This fact can be explained by the structure of the cell wall of these bacteria. Gram-negative bacteria possess a cell wall consisting of two lipid bilayers, whereas the cell walls of gram-positive bacteria consist of only one lipid bilayer and is therefore more permeable to photosensitizers. Yeast are more resistant to photodynamic therapy than bacteria because of their larger cell size and the presence of a nuclear membrane, which represents an additional barrier to the penetration of the photosensitizer.<sup>3</sup>

In the case of the *Staphylococcus* and enterobacterial strains, the number of CFU/mL was reduced by  $\sim 6$  to

$7 \log_{10}$  after application of photodynamic therapy with 0.1% malachite green. Prates et al.<sup>18</sup> also observed a reduction in bacteria of the species *Aggregatibacter actinomycetemcomitans* given combined treatment with 0.01% malachite green and a diode laser. However, the reduction in CFU/mL was only 2 to  $3 \log_{10}$ .

The results obtained with malachite green as a photosensitizer in photodynamic therapy were similar to those reported in studies using phenothiazine dyes, such as methylene blue and toluidine blue. Usacheva et al.<sup>26</sup> evaluated *in vitro* the interaction between phenothiazine dyes and lipopolysaccharides (LPSs) of some gram-negative bacteria, including *E. coli*, *P. aeruginosa*, *K. pneumonia*, and *S. marcescens*, and observed a greater interaction between toluidine blue and LPS when compared with methylene blue. Souza et al.<sup>15</sup> reported a significantly lower number of log CFU/mL of *C. albicans* (ATCC 18804) treated with a gallium-aluminum-arsenide laser and methylene blue (4.68) when compared with the control group (5.69).

However, the results obtained in the photodynamic therapy with malachite green were lower than the results obtained with the cationic phthalocyanine zinc.<sup>27</sup> Mantareva et al.<sup>27</sup> evaluated the photodynamic activity of two new water-soluble phthalocyanines, one cationic tetrakis-(3-methylpyridyloxy) and one anionic tetrakis-(4-sulfophenoxy)-phthalocyanine zinc with the strains of *S. aureus*, *P. aeruginosa*, and *C. albicans*. The cationic photosensitizer completely inactivated *S. aureus* and *C. albicans*. In the case of *P. aeruginosa*, a reduction of  $6 \log_{10}$  was seen. In contrast, the anionic photosensitizer with the same drug concentration ( $6 \mu\text{M}$ ) was not sufficient to photoinactivate the gram-negative *P. aeruginosa*. For the yeast *C. albicans*, the

TABLE 2. MEAN COUNTS (LOG CFU/mL) OF ENTEROBACTERIAL SPECIES

Enterobacterial species	Treatment			
	L-P-	L+P-	L-P+	L+P+
<i>E. cloacae</i>	8.27 ± 0.29	7.78 ± 0.97	3.82 ± 0.29	1.13 ± 1.33
<i>E. coli</i>	8.11 ± 0.33	7.89 ± 1.83	2.94 ± 0.28	1.74 ± 1.12
<i>K. oxytoca</i>	7.30 ± 0.23	7.21 ± 0.94	1.76 ± 0.27	0.62 ± 0.90
<i>K. pneumoniae</i>	7.71 ± 0.38	7.44 ± 0.93	3.99 ± 0.28	1.69 ± 0.98

The species were submitted to the following treatments: physiologic solution as control (L-P-), laser and physiologic solution (L+P-), photosensitizer (L-P+), and laser and photosensitizer (L+P+).

TABLE 3. MEAN COUNTS (LOG CFU/mL) OF *CANDIDA* SPECIES

<i>Candida species</i>	<i>Treatment</i>			
	<i>L–P–</i>	<i>L+P–</i>	<i>L–P+</i>	<i>L+P+</i>
<i>C. albicans</i>	5.67 ± 0.31	5.41 ± 0.19	5.65 ± 0.18	5.32 ± 0.61
<i>C. tropicalis</i>	5.49 ± 0.04	5.33 ± 0.13	5.46 ± 0.10	4.72 ± 0.59
<i>C. parapsilosis</i>	6.12 ± 0.07	5.79 ± 0.05	5.97 ± 0.00	5.54 ± 0.39
<i>C. krusei</i>	5.96 ± 0.28	5.83 ± 0.60	5.93 ± 0.32	5.73 ± 0.55
<i>C. glabrata</i>	5.75 ± 0.38	5.46 ± 0.37	5.72 ± 0.38	5.19 ± 0.31

The species were submitted to the following treatments: physiologic solution as control (L–P–), laser and physiologic solution (L+P–), photosensitizer (L–P+), and laser and photosensitizer (L+P+).

photoinactivated cells were only 1–2 log<sub>10</sub>. *S. aureus* was deactivated <4 log<sub>10</sub>.

The application of laser therapy alone had an antimicrobial effect only on the *Candida* strains studied. The effect of low-level laser therapy on *Candida* has also been reported by Maver-Biscanin et al.,<sup>28</sup> who studied the effect of this therapy on *C. albicans* in two patients with palatal inflammation caused by the use of complete dentures. The patients were irradiated with different wavelengths for different periods of time (830 nm for 5 min and 685 nm for 10 min). Regression of the inflammatory process and a reduction in the number of *Candida* colonies collected from the palate were observed after therapy.

In the present study, application of 0.1% malachite green alone also resulted in the microbial reduction of *Staphylococcus* and enterobacteria, suggesting that malachite green at the concentration tested exerted a bactericidal effect on the microorganisms studied. However, Prates et al.<sup>18</sup> observed no reduction in the number of *Aggregatibacter actinomycetemcomitans* when malachite green was evaluated separately.

Individual analysis of the results obtained for each microbial species studied showed that *S. epidermidis* is the staphylococcal species least sensitive to photodynamic therapy with malachite green. Evaluating the photodynamic effects of merocyanine on the biofilms of two different *S. epidermidis* strains (RP62A and 1457), Sbarra et al.<sup>29</sup> observed significant inactivation of cells when biofilms were exposed to photosensitizer and laser simultaneously. However, the strain 1457 was more susceptible to photodynamic therapy than the strain RP62A.

The present results demonstrated the efficiency of malachite green as a photosensitizer in photodynamic therapy against strains of *Staphylococcus*, Enterobacteriaceae, and *Candida*. The possibility of eliminating pathogenic microorganisms by using a low-power laser in combination with specific dyes makes photodynamic therapy a promising alternative in dentistry in view of its low cost, high efficiency, and short time of application. However, further studies are necessary so that other dyes can be regulated as photosensitizers and commercialized for dental use.

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### Author Disclosure Statement

No competing financial interests exist.

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