Lasers Med Sci (2015) 30:685–694
DOI 10.1007/s10103-013-1397-z

ORIGINAL ARTICLE

# Susceptibility of multispecies biofilm to photodynamic therapy using Photodithazine®

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Received: 2 May 2013 / Accepted: 8 July 2013 / Published online: 3 August 2013


**Abstract** This in vitro study evaluated the effect of photodynamic therapy (PDT) on the multispecies biofilm of *Candida albicans*, *Candida glabrata*, and *Streptococcus mutans*. Standardized fungal and bacterial suspensions were cultivated appropriately for each species and inoculated in 96-well microtiter plates for mix-biofilm formation. After 48 h of incubation, the biofilms were submitted to PDT (P+L+) using Photodithazine® (PDZ) at 100, 150, 175, 200, or 250 mg/mL for 20 min and 37.5 J/cm$^2$ of light-emitting diode (LED) (660 nm). Additional samples were treated only with PDZ (P+L−) or LED (P−L+), or neither (control, P−L-). Afterwards, the biofilms were evaluated by quantification of colonies (CFU/mL), metabolic activity (XTT reduction assay), total biomass (crystal violet staining), and confocal scanning laser microscopy (CSLM). Data were analyzed by one-way ANOVA and Tukey tests ($p<0.05$). Compared with the control, PDT promoted a significant reduction in colonies viability of the three species evaluated with 175 and 200 mg/mL of PDZ. PDT also significantly reduced the metabolic activity of the biofilms compared with the control, despite the PDZ concentration. However, no significant difference was found in the total biomass of samples submitted or not to PDT. For all analysis, no significant difference was verified among P−L−, P+L−, and P−L+. CSLM showed a visual increase of dead cells after PDT. PDT-mediated PDZ was effective in reducing the cell viability of multispecies biofilm.

**Keywords** Antimicrobial photodynamic therapy · *Candida albicans* · *Candida glabrata* · *Streptococcus mutans* · Polimicrobial biofilm

## Introduction

In the human oral cavity, the *Candida* species are considered the main pathogens responsible for the development of common infections among the elderly such as oropharingeal candidosis (OPC) [1]. Some etiological factors such as poorly fitting dentures, poor oral hygiene, smoking habits, diabetes mellitus, and prolonged use of broad-spectrum antibiotic and immunosuppressive drugs can predispose the individuals to this opportunistic infection [2]. Although *Candida albicans* is by far the most commonly isolated species in these infections, a substantial proportion of non-*albicans* species, in particular *Candida glabrata*, has been reported in the oral Candidiasis development [3, 4]. This species has been associated with an increasing cause of fungaemia, especially in immunosuppressed patients [5].

Despite that the *Candida* species are considered important pathogens in the occurrence of OPC, bacteria may contribute to the colonization and proliferation of *Candida* strains in the oral cavity [6]. The fungal and the bacterial species are present on the oral microbiota living in harmony with each other and forming a polymicrobial biofilm [7, 8]. The microbial community is composed of microorganisms embedded in an extrapolymeric matrix and strongly attached to the biotic or abiotic surface. These complex structures have particular advantages that protect them from host defenses and promote mutually beneficial interactions [8]. An

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example of mutual cooperation is the phenomenon of coaggregation, which consists of a specific cell-to-cell recognition of genetically different cells [9]. Most of the early oral colonizers are species of *Streptococcus* [10], and according to Pereira-Cenci et al. [2] *Streptococcus mutans* increases *Candida* biofilm formation. These authors did not observe a difference in candidal counts between dual and single-species biofilm of *C. albicans* and *C. glabrata* [11, 12].

The antifungal agents are frequently used for OPC treatment [13], but the diluent effect of saliva and the cleansing action of the oral musculature tend to reduce the concentration of topical agents to sub-therapeutic levels. On the other hand, the systemic antifungal agents may show nephrotoxic or hepatotoxic side effects and cause the appearance of drug-resistant microorganisms. Moreover, when cells are organized such as biofilm, they show increased resistance to the conventional treatment [14, 15].

Thus, studies have been performed in order to search for new alternative therapies for treating biofilm-associated infections. One potential alternative approach is photodynamic therapy (PDT). Photodynamic therapy is an emergent process which requires an association of oxygen, visible light source, and (PS) [16]. When the PS is activated by the exposure of non-thermal visible light in an appropriate wavelength in the presence of oxygen, the PS is transformed from its ground state to its triplet excited state and it starts two oxidative mechanisms: the photochemical reaction generates free radicals (Type I) and/or singlet oxygen (Type II) [17]. These reactive oxygen species are responsible for causing irreversible damage in cellular targets [16, 17] such as membrane lyses and protein inactivation [17]. In general, PS is applied externally to the cell, thus the cell membrane is considered the initial target of the photodynamic process [18].

Investigations have shown that PDT has fungicidal activity against planktonic *C. albicans*, including resistant strains. However, complete killing of cells surrounded the biofilms is not usually observed [19, 20]. The association of methylene blue with low-power laser irradiation resulted in greater reductions in single species (2.32–3.29 $\log_{10}$) than the multispecies biofilms (1.00–2.44 $\log_{10}$) of *C. albicans*, *Staphylococcus aureus*, and *S. mutans* [21]. In another in situ study, the combination of toluidine blue together with red light promoted only a tendency of reduction in total *streptococci* and *mutans streptococci* counts in multispecies biofilms [22]. Previous studies have shown that PDT was efficient in reducing *C. albicans* count in a murine model of oral candidiasis [23] and for denture disinfection [4], employing a porphyrin and light-emitting diode (LED). Clinical studies showed that this combination promoted a reduction in candidal counts from dentures and palates of denture stomatitis [24, 25]. Although these investigations have evaluated in vitro and in vivo biofilms, few in vitro studies have assessed multispecies biofilms with bacteria and yeasts. In addition, research should be performed to find adequate parameters of PDT prior to clinical investigations and the search for new PSs remains an important goal.

The Photodithazine® (PDZ) is a second-generation photosensitizer, a chlorin e6 derivative, that has been considered interesting as a potential drug for PDT because it presents a high singlet oxygen quantum yield [26] and presents low toxicity [27]. Nonetheless, PDZ has been more investigated for anticancer PDT [28], and only a few studies have evaluated its antimicrobial effectiveness. PS has shown its photodynamic efficiency when it was associated with a visible light for inactivation of cell suspensions of *C. albicans* and *Candida guilliermondii* [18]. Soukos et al. [29] investigated the photodynamic effects of a conjugate of the chlorin e6 on the bacteria of natural dental plaque obtained from human subjects with chronic periodontitis. The conjugate showed 75 and 80 % killing of species when suspended in medium and phosphate-buffered saline (PBS), respectively. However, these microorganisms were exposed to light in suspension, and evaluations should be directed towards biofilms since they better resemble the in vivo conditions. Although Fontana et al. [30] had observed no damage on tissues of rat tongue submitted to PDT using PDZ and LED light, suggesting that PDT may be a safe in vivo procedure, assessment of antimicrobial PDZ-mediated PDT on multispecies biofilm has not yet been established. Thus, the aim of this study was to evaluate the effects of PDT mediated by PDZ on the inactivation of multispecies biofilm formed by *C. albicans*, *C. glabrata*, and *S. mutans*.

## Material and methods

### Microorganisms and biofilm production

American Type Culture Collection strains (ATCC; Rockville, MD, USA) of *C. albicans* (ATCC 90028), *C. glabrata* (ATCC 2001), and *S. mutans* (ATCC 25175) were used to produce multispecies biofilm. Prior to each experiment, *C. albicans* and *C. glabrata* were seeded on Sabouraud dextrose agar (Acumedia Manufacturers Inc., Baltimore, MD, USA) with 5 μg/mL of chloramphenicol and *S. mutans* on Mitis-Salivarius agar (MSB, Difco, Laboratories, Detroit, MI, USA) supplemented with 15 % sucrose and 0.2 IU/mL bacitracin, which were incubated at 37 °C for 48 h. All experiments with *S. mutans* were performed incubating it in candle jars. Subsequently, two loopfulls of each microorganism were inoculated into 20 mL of yeast nitrogen base (YNB, Himedia, Laboratories Pvt. Ltda, Mumbai, India) medium supplemented with 100 mM glucose for the *Candida* species and brain heart infusion (BHI, Himedia Laboratories Pvt Ltda, Mumbai, India) for *S. mutans*. All microorganisms were incubated at 37 °C overnight in appropriate conditions. Microbial cells were harvested, washed twice with PBS (pH 7.2) at 5,000×*g* for

5 min and re-suspended in BHI. *Candida* species suspensions were spectrophotometrically standardized at a concentration of $10^7$ and $10^8$ cells/mL for *S. mutans* [2]. Aliquots of 50 μl of each standardized cell suspension were inoculated in 96-well microtiter plates and incubated for 90 min at 37 °C in an orbital shaker at 75 rpm (adhesion phase) [14]. The non-adherent cells were removed by washing twice with 200 μL of PBS. For the biofilm formation, 200 μL of BHI medium was added in each well and the plates were incubated for 48 h at 37 °C in an orbital shaker at 75 rpm. The negative control groups consisted of BHI medium without microorganisms. All experiments were done in triplicate on three independent occasions.

### Photosensitizer and light source

PDZ (Moscow, Russia) was used as a PS for sensibilization of the biofilms. The PS were diluted in physiological solution (0.85 % NaCl) at concentrations of 100, 150, 175, 200, and 250 mg/L. Samples were exposed to LED light source in the red region, with a wavelength of 660 nm; the intensity of light emitted was 71 mW/cm$^2$ at a fluence of 37.5 J/cm$^2$. LED light was designed by Physical Institute of São Carlos (University of São Paulo, São Carlos, SP, Brazil). To calculate the exposure time, the following dosimetry formula was used: Fluence (J/cm$^2$)=intensity of light (W/cm$^2$)×exposure time (s).

PDT was performed by the administration of PDZ (100, 150, 175, 200, and 250 mg/L) and exposure to 37.5 J/cm$^2$ of LED light (660 nm) (P+L+group). Additional samples were treated either with PDZ (P+L−) or LED light only (P−L+). Positive control samples had neither light nor PDZ (P−L−). After biofilm formation, the wells were washed twice with PBS, and according to the described experimental groups, 200 μL of PDZ was added for groups P+L+and P+L−, and aliquots of 200 μL of physiological solution was added for groups P−L+and P−L−. Then, the microtiter plates were incubated in the dark for 20 min (pre-irradiation time). After this period, the P+L+and P−L+groups were illuminated for 9 min (37.5 J/cm$^2$).

All groups were evaluated by four methods:

1. Biofilm viability analyses
   At the end of the experimental conditions, to evaluate the cell viability, the biofilms were detached from the wells with a sterile swab (Johnson) and aliquots of 25 μL of serial dilutions were seeded presumptive in duplicate on CHROMAgar Candida (Difco, Laboratories, Detroit, MI, USA) and MSB for identification of *Candida* spp. and *S. mutans*, respectively. After incubation at 37 °C for 48 h, the colony forming unit per milliliter (CFU ml$^{-1}$) was determined and log-transformed ($\log_{10}$).
2. XTT reduction assay
   The metabolic activity of multispecies biofilm was measured for XTT reduction assay. After experimental conditions, 200 μL of XTT solution (containing 158 μL of PBS with 200 mM glucose, 40 μL of XTT ({2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hyadroxide}), and 2 μL of menadione) was placed in each well. The plates were incubated for 3 h in the dark at 37 °C and colorimetric measured in a microtiter plate reader at 492 nm [31].
3. Total biomass quantification
   The quantification of biofilm total biomass was performed by crystal violet (CV) staining. After being submitted to the experimental procedures, the biofilm was washed with PBS and then was fixed with 200 μL of methanol for 15 min. Methanol was removed, and the plates were allowed to dry at room temperature. After drying, 200 μL of CV (1 % *v*/*v*) was added in the wells and incubated for 5 min. The wells were washed with PBS, and 200 μL of acetic acid (33 % *v*/*v*) was added to dissolve the stain. The absorbance of the final solution was read using a microtiter plate reader at 570 nm [32].
4. Confocal scanning laser microscopy
   The viability of microorganisms were also evaluated by confocal scanning laser microscopy (CSLM) after applications of LED light (37.5 J/cm$^2$) in association with 175 and 200 mg/L of PDZ and compared with the positive control group. Multispecies biofilm of 48 h were grown on sterilized polystyrene coupons (10 mm diameter). After this period, the multispecies biofilm was washed twice with PBS and stained using the Live/Dead BacLight viability kit containing SYTO-9 and propidium iodide (PI) (Molecular Probes, Inc., Eugene, OR, USA). Biofilms were stained in the dark and incubated at room temperature for 15 min, according to the manufacturer's instructions. The maxima excitation/emission used for these stains are about 480/500 nm for SYTO-9 stain and 490/635 nm for PI [33]. Furthermore, it was evaluated if the microorganisms by themselves emitted fluorescence in the conditions described above that could be confused with those emitted by the stains. Due to the absence of fluorescence signal from the microorganisms, an image of transmittance mode was obtained to show the presence of the biofilm on the coupons.

### Statistical analysis

Homogeneity of the variance and normality was verified, respectively, by the Levene and Shapiro–Wilk tests. The results obtained were statistically evaluated using one-way analysis of variance (ANOVA) and the Tukey test for multiple comparisons. A significance level of 0.05 was used for all statistical tests.

## Results

The mean values and standard deviation of CFU/ml ($\log_{10}$) of three-species biofilms formed by *C. albicans*, *C. glabrata* and

*S. mutans* in different experimental conditions for all groups are shown in Fig. 1. PDT promoted a significant reduction of the three species evaluated compared with the control when 175 and 200 mg/mL of PDZ was associated with LED light. The highest reduction in the cell viability was detected in the group that used the concentration of 200 mg/L of PDZ in association with LED light for *C. albicans* (1.21 $\log_{10}$), *C. glabrata* (1.19 $\log_{10}$), and *S. mutans* (2.39 $\log_{10}$) when compared with the positive control group. Additionally, no statistical difference was found among the groups P−L−, P+L−, and P−L+.

The metabolic activity was measured by the application of the XTT reduction assay. The mean values and standard deviation of the absorbance values obtained in the XTT method for all groups are showed in Fig. 2. It can be seen that PDT provoked a slight interference in the metabolic activity of mixed biofilm. A significant reduction ($p<0.05$) in the cellular metabolism was observed when the biofilms were submitted to PDT (P+L+groups) compared with the control (P−L−). The highest reductions in the cellular metabolism were observed when the biofilms were exposed to the concentrations of 100, 150, 175, and 200 mg/L of PS and illuminated (no significant difference among them, $P>0.05$), but 250 mg/L of PDZ and light showed a significant difference ($p<0.05$) compared with samples treated with 100 and 150 mg/L of PDZ and light. Moreover, no significant difference among P−L−, P+L−, and P−L+was observed.

Figure 3 shows the mean values and standard deviation of the absorbance values obtained in the total biomass assay (CV staining) for all groups. Opposite to the other evaluations performed (CFU/mL and XTT), results obtained from the CV staining showed no significant differences among the groups.

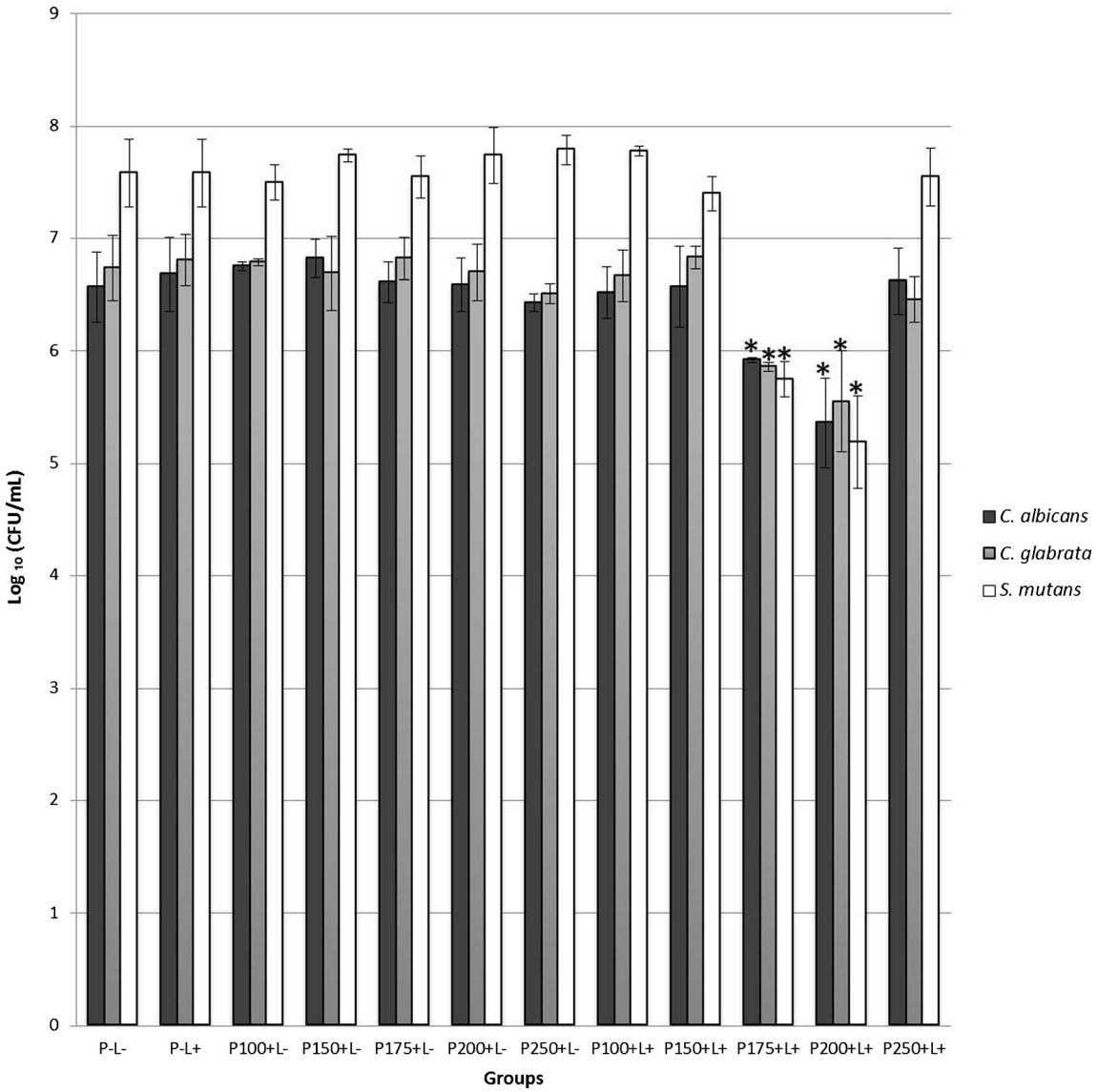


**Fig. 1** Mean values [$\log_{10}$(CFU/mL)] of cell viability (quantification of colonies) of *C. albicans*, *C. glabrata*, and *S. mutans*. *Error bars* standard deviation, *asterisks* significant difference ($p<0.05$) compared with the control (P−L−)

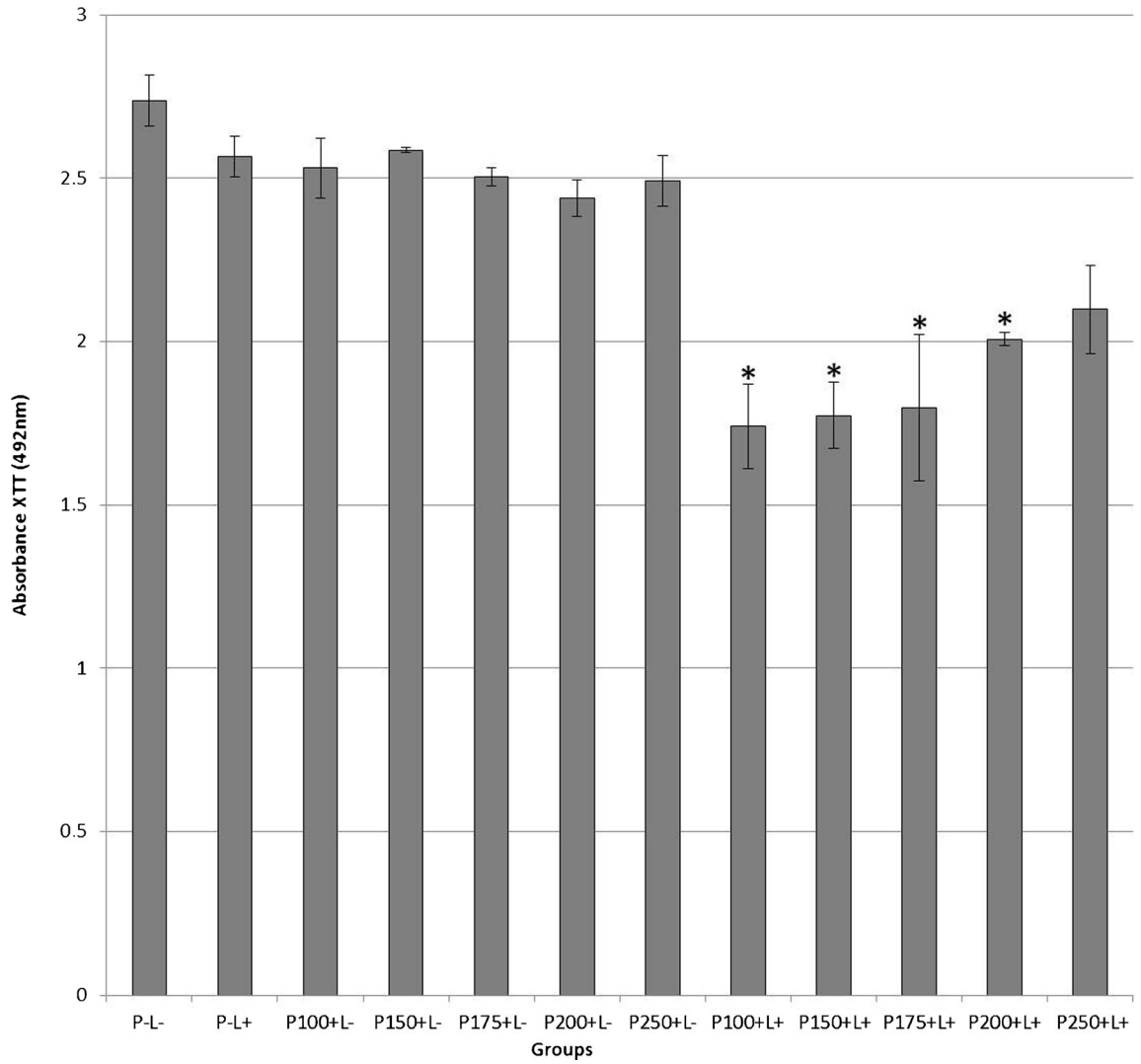


**Fig. 2** Mean values of metabolic activity (absorbance of XTT assay at 492 nm) of multispecies biofilm. *Error bars* standard deviation, *asterisks* significant difference ($p<0.05$) compared with the control (P−L−). &, significant difference ($p<0.05$) compared with P100+L+and P150+L+

The micrographs obtained from the CSLM (Fig. 4) showed that, in the absence of the SYTO-9 and PI, no fluorescence from biofilms was verified (Fig. 4a). The presence of microorganisms on the coupons was observed by transmittance mode (Fig. 4b). Cross section of samples showed a biofilm thickness of 19 μm (Fig. 4d, f, and h). An apparent increase of death cells of the biofilms submitted to PDT (Fig. 4e–h) was verified when compared with the control (P−L−, Fig. 4c, d). When PDZ at 175 mg/mL was associated with 37.5 J/cm$^2$, a visual increase of death cells was observed (Fig. 4e, f).

## Discussion

In the present investigation, the effect of PDT mediated by PDZ and LED light was evaluated by quantification of colonies (CFU/mL), metabolic activity (XTT assay), total biomass (CV staining), and CSLM. The conventional plating method (CFU/mL) has been considered labor-intensive and slow, and it requires the disruption of cell aggregates from the biofilm, which may affect cell viability. Hence, other model systems, such as the XTT and total biomass assays, have been described for biofilm quantification. On the other hand, as the XTT assay measures the metabolic activity of the cells, it would not enumerate the total cells, since the microorganisms within a biofilm may have restricted access to nutrients and oxygen, altering their metabolic activity. CV staining is another technique that allows biofilm–biomass quantification (matrix, dead, and alive cells) in the entire well of the microtiter plate. The results obtained from the CFU test revealed that the association of 175 and 200 mg/L of PDZ with LED light was able to significantly reduce the microbial viable counts when compared with the positive control group (P−L−). Although, there was no statistical difference between the groups that used 175 or 200 mg/L of PDZ, the concentration of 200 mg/L was able to promote the highest reduction in the cell viability which was

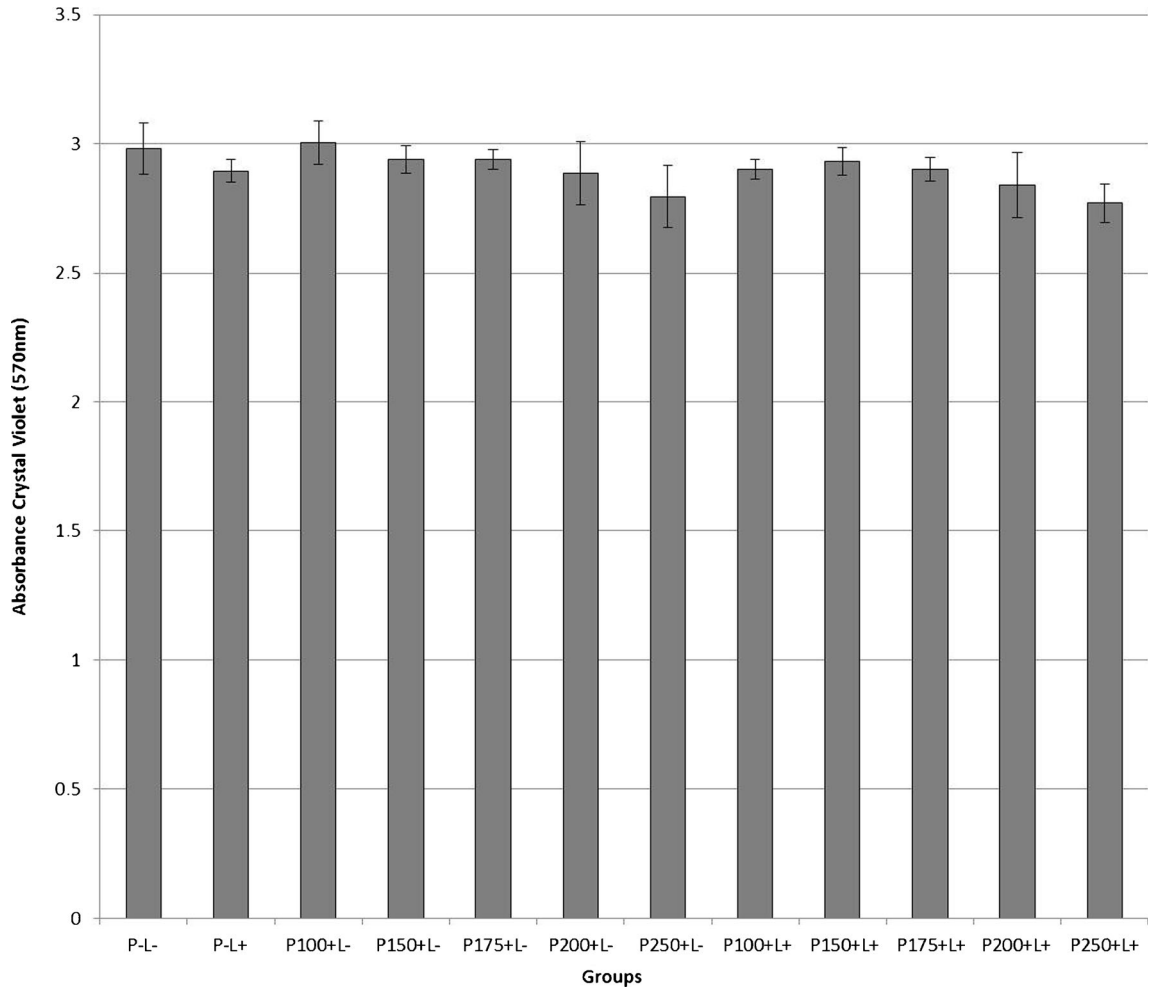


**Fig. 3** Mean values of the total biomass assay (absorbance of CV staining at 570 nm) of multispecies biofilm. *Error bars* standard deviation

equivalent to 1.21, 1.19, and 2.39 logs for *C. albicans*, *C. glabrata*, and *S. mutans*, respectively. To our knowledge, no study has evaluated the efficacy of PDZ-mediated PDT on polymicrobial biofilm. Therefore, no direct comparison is possible with the data currently available. Some reports evaluated a chlorin e6 derivative for microbial photoinactivation and showed effective photoinactivation of planktonic cultures [18, 29]. Strakhovskaia et al. [18] verified that planktonic culture of *C. guilliermondii* was 1.6 to 1.7 more photosensitive than *C. albicans* using PDZ. When biofilms of *Streptococcus pyogenes* cultivated on membranes were sensitized by Sn (IV) chlorin e6 and exposed to laser light in a confocal microscope, a progressive increase of cell death was observed in real time [34]. PDT mediated by chlorin e6 also resulted in a two-log reduction of bacterial biofilm in the root canals and reduced the bioluminescence signal of *Pseudomonas aeruginosa* and *Proteus mirabilis* by over 95 % [35]. When PDT was associated with conventional endodontic treatment, the bioluminescence signal of bacterial regrowth in the root canals after 24 h was significantly reduced compared with the control (no treatment) and with either single treatment.

Other investigations have also verified a significant reduction of cell viability of multispecies biofilm after PDT. Reductions from 1.00 to 2.44 were found when dual or three-species biofilms of *S. mutans*, *Staphylococcus aureus*, and *C. albicans* were treated by methylene blue and laser light [21]. Although a significant reduction of *S. mutans* mono-species biofilm was achieved in vitro, no significant effect was found in the viability of total *streptococci* and *mutans streptococci* in multispecies in situ biofilms treated with toluidine blue O and LED light [22]. When a cariogenic in vitro model of six-species biofilm (*Actinomyces naeslundii*, *Veillonella dispar*, *Fusobacterium nucleatum*, *Streptococcus sobrinus*, *Streptococcus oralis*, and *C. albicans*) was cultivated on bovine enamel disks and submitted to PDT (methylene blue and laser light), no significant difference was observed compared with the control (no treatment), with only a minimal effect on the cell viability of the biofilm (less than 1 $\log_{10}$ reduction) [36]. The outcomes of these studies show that multispecies biofilm is less susceptible to PDT, and this finding has been associated with the higher resistance of microbial cells when organized as biofilm and also with the protection of cells promoted by the polymeric extracellular matrix, which acts as a

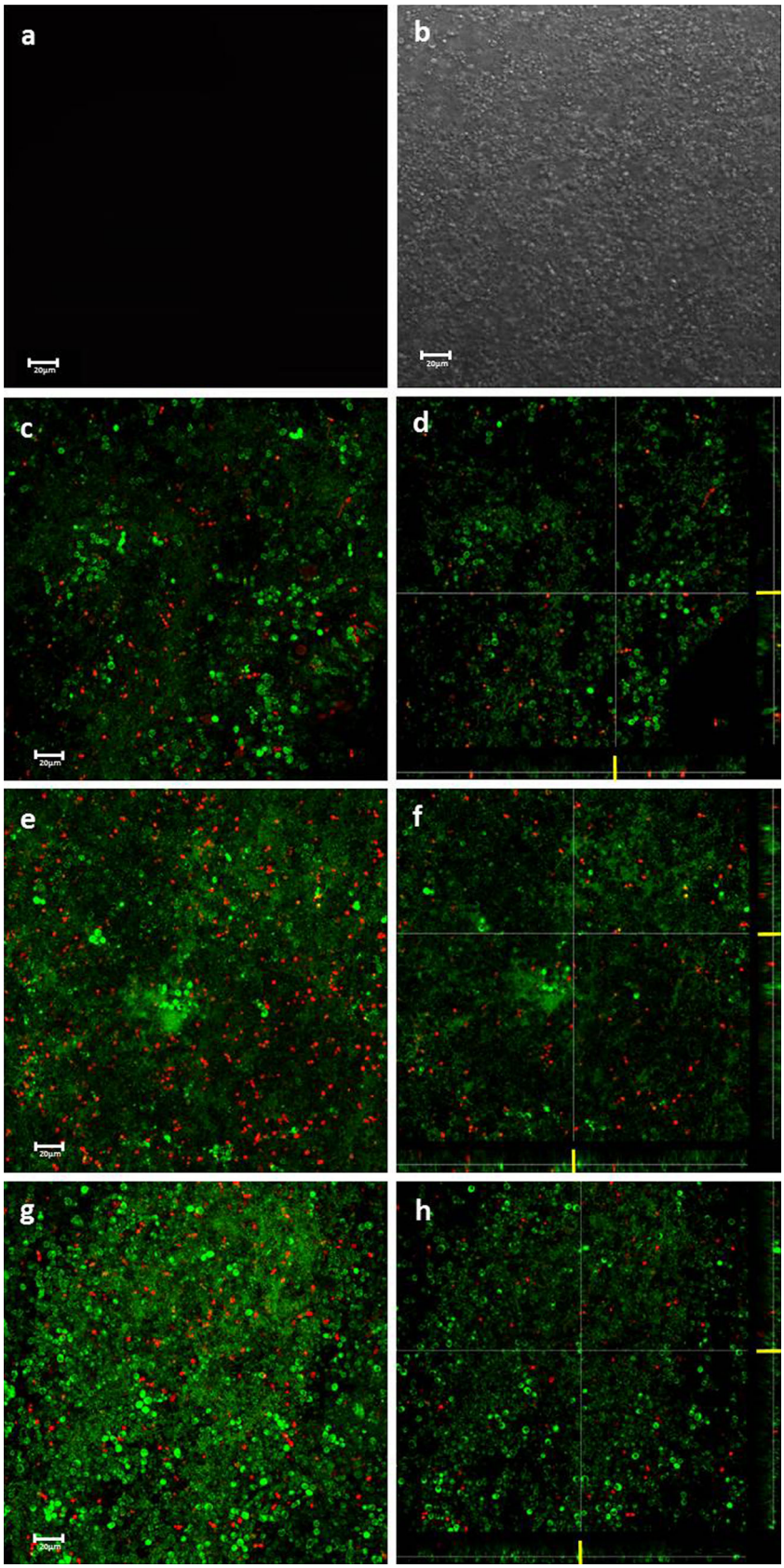


**Fig. 4** Viability and spatial arrangement in the multispecies biofilm. *C. albicans*, *C. glabrata*, and *S. mutans*. Biofilms were grown on coupons and stained with BacLight LIVE/DEAD and processed for CSLM. *Red cells* are considered dead (PI), while *green cells* are alive (SYTO-9). **a** Biofilm in the absence of the SYTO-9 and PI, **b** image of the transmittance mode of multispecies biofilm. **c** Image of biofilm from the positive control group. **d** Cross sections and side views of 19.0 μm (*yellow line*) thick biofilm from the positive control group. **e** Image of biofilm after PTD (175 mg/mL of PDZ and 37.5 J/cm$^2$ of LED light). **f** Cross sections and side views of 18.0 μm (*yellow line*) thick biofilm after PTD (175 mg/mL of PDZ and 37.5 J/cm$^2$ of LED light). **g** Image of biofilm after PTD (200 mg/mL of PDZ and 37.5 J/cm$^2$ of LED light). **h** Cross sections and side views of a 19.0 μm (*yellow line*) thick biofilm after PTD (200 mg/mL of PDZ and 37.5 J/cm$^2$ of LED light)

barrier for PS penetration. Accordingly, some studies have shown that the lethal photosensitization occurred predominantly in the outer layers of the biofilm [21].

With regard to the data obtained in this study, it is important to emphasize that PDT was more effective in killing bacteria than yeast cells. Similarly, Pereira et al. [21] evaluated the effectiveness of PDT mediated by methylene blue on the inactivation of mono, dual, and mixed species biofilms of *C.albicans*, *Staphylococcus aureus*, and *S. mutans*. The results showed that *S. aureus* and *S. mutans* were more susceptible to PDT when compared to *C. albicans* in mono and mixed biofilms. The vulnerability of the microorganisms to PDT can be due to the structural differences between the bacterial and fungal cells [14]. It appears that the presence of the nuclear membrane in the eukaryotic cell may work as an additional barrier for the penetration of the PS and for the reactive oxygen species generated after PDT. Moreover, the size/volume of *C. albicans* cells is about 25–50 times greater than the bacterial cells [37]. As a result, greater quantities of singlet oxygen per cell would be needed to inactivate the yeast cells [37, 38]. It has been suggested that photodynamic treatment against microbial cells is considered to depend on the mechanism of the singlet oxygen generation, which can act against a target molecule, such as membrane lipids, peptides, and nucleic acids [39].

In the present investigation, the XTT reduction assay was performed in order to assess the immediate effect of PDT on the multispecies biofilm as a whole. Since the XTT method is not able to evaluate the effect of the treatment on the cellular metabolism of each species involved, this test was performed as a complementary test to evaluate the efficacy of PDT. The use of the XTT method assay has been strongly correlated with other quantitative techniques, such as CFU, that are used to evaluate biofilm development [40]. This assay has been used to study microbial cell behavior in mono-species biofilms [19, 20, 41]. Moreover, unlike the method of quantification of colonies, in the XTT assay, the biofilm is evaluated intact, i.e., no mechanical disruption is performed after experimental conditions. The results obtained in the present investigation showed a significant reduction in the biofilm metabolic activity at concentrations of 100, 150, 175, and 200 mg/L, when compared with the positive control group (P−L−) with no significant difference among these concentrations. The mean value of reduction of the biofilm metabolic activity was equivalent to 36.4 %. The decrease of metabolic activity after PDT may be also verified in the CLSM micrographs in which an apparent increase of dead cells was observed after PDT. Previously, some studies evaluated the efficacy of treatments against the bacteria [42, 43] and yeast [19, 20] in mono-species biofilm and their planktonic counterparts by using the XTT method. When curcumin was used as the PS, the metabolic activity of *C. albicans* mono-species biofilm was proportional to the concentration of PS [19], and a significant reduction in the metabolic activity of mono-species biofilm of *C. albicans*, *C. glabrata*, and *C. dubliniensis* was observed after an increased incubation time with the PS [20].

The biofilm model employed here was also evaluated through quantification of total biomass by CV staining. The results showed that there was no significant difference among all groups evaluated when compared with the positive control group (P−L−). This finding contrasts with the other evaluations performed in the present investigation in which a significant reduction of the cell viability and metabolism after PDT was verified. The absence of a significant difference in the total biomass among the groups evaluated is probably attributed to the staining of the matrix as well as both living and dead cells within the biofilm; thus, the CV assay may not be so appropriate to evaluate the killing of the biofilm cells [40]. Moreover, when the potential of biofilm formation by multispecies of *Candida* was assessed by CV assay, a higher degree of slim production was verified with the association of *C. albicans* and *C. glabrata*, while *C. tropicalis* hampered the slim production when associated with other non-albicans *Candida* species [44]. Moreover, according to Silva et al. [32], the *C. glabrata* biofilm matrix presents a high content of carbohydrate and proteins, which are likely to adsorb more CV stain. Thus, it may be suggested that, although PDT promoted a significant reduction in cell viability and metabolism in the present investigation, no significant difference in total biomass was observed after PDT probably due to the composition and the high amount of slim produced by the association of *C. albicans* and *C. glabrata*. It seems that the production and interaction of matrix polymers produced by different microorganisms result in the increase of the matrix viscosity promoting the resistance of multispecies biofilms to disinfection methods [45]. Beyond the synergism between *C. albicans* and *C. glabrata*, Pereira-Cenci et al. [2] revealed that *S. mutans* increases *Candida* spp. biofilm development and inhibits hyphae formation of *C. albicans*. Since yeast forms are less susceptible to PDT than hyphae forms [46], it may be suggested that the presence of bacteria in a multispecies biofilm may promote the emergence of specific features that set lower susceptibility to PDT.

Based on the methodology employed in this study and the outcomes obtained, it may be concluded that PDT with the association of PDZ and red LED light was effective in decreasing cell viability of the multispecies biofilm evaluated. Nonetheless, further in vivo investigations for evaluation of this promising result are required.

**Acknowledgments** This work was supported by São Paulo Research Foundation (FAPESP) and Fundunesp, grants 2011/09054-0 and 878/11-DFP, respectively.

## References

1. Jose A, Coco BJ, Milligan S, Young B, Lappin DF, Bagg J, Murray C, Ramage G (2010) Reducing the incidence of denture stomatitis:

are denture cleansers sufficient? J Prosthodont 19(4):252–257. doi:10.1111/j.1532-849X.2009.00561.x
2. Pereira-Cenci T, Deng DM, Kraneveld EA, Manders EM, Del Bel Cury AA, Ten Cate JM, Crielaard W (2008) The effect of *Streptococcus mutans* and *Candida glabrata* on *Candida albicans* biofilms formed on different surfaces. Arch Oral Biol 53(8):755–764. doi:10.1016/j.archoralbio.2008.02.015
3. Li L, Redding S, Dongari-Bagtzoglou A (2007) *Candida glabrata*: an emerging oral opportunistic pathogen. J Dent Res 86(3):204–215
4. Ribeiro DG, Pavarina AC, Dovigo LN, Mima EG, Machado AL, Bagnato VS, Vergani CE (2012) Photodynamic inactivation of microorganisms present on complete dentures. A clinical investigation. Photodynamic disinfection of complete dentures. Lasers Med Sci 27(1):161–168. doi:10.1007/s10103-011-0912-3
5. Krcmery V, Barnes AJ (2002) Non-*albicans Candida* spp. causing fungaemia: pathogenicity and antifungal resistance. J Hosp Infect 50(4):243–260
6. Hsu LY, Minah GE, Peterson DE, Wingard JR, Merz WG, Altomonte V, Tylenda CA (1990) Coaggregation of oral *Candida* isolates with bacteria from bone marrow transplant recipients. J Clin Microbiol 28(12):2621–2626
7. Thein ZM, Seneviratne CJ, Samaranayake YH, Samaranayake LP (2009) Community lifestyle of *Candida* in mixed biofilms: a mini review. Mycoses 52(6):467–475. doi:10.1111/j.1439-0507.2009.01719.x
8. Peters BM, Jabra-Rizk MA, Scheper MA, Leid JG, Costerton JW, Shirtliff ME (2010) Microbial interactions and differential protein expression in *Staphylococcus aureus–Candida albicans* dual-species biofilms. FEMS Immunol Med Microbiol 59(3):493–503. doi:10.1111/j.1574-695X.2010.00710.x
9. James A, Beaudette L, Costerton W (1995) Interspecies bacterial interactions in biofilms. J Ind Microbiol 15:257–262
10. Banas JA (2004) Virulence properties of *Streptococcus mutans*. Front Biosci 9:1267–1277
11. Ramage G, Tomsett K, Wickes BL, López-Ribot JL, Redding SW (2004) Denture stomatitis: a role for *Candida* biofilms. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 98(1):53–59
12. Jarosz LM, Deng DM, van der Mei HC, Crielaard W, Krom BP (2009) *Streptococcus mutans* competence-stimulating peptide inhibits *Candida albicans* hypha formation. Eukaryot Cell 8(11):1658–1664. doi:10.1128/EC.00070-09
13. Pallasch TJ (2002) Antifungal and antiviral chemotherapy. Periodontol 2000(28):240–255
14. Chandra J, Mukherjee PK, Leidich SD, Faddoul FF, Hoyer LL, Douglas LJ, Ghannoum MA (2001) Antifungal resistance of candidal biofilms formed on denture acrylic in vitro. J Dent Res 80(3):903–908
15. Shapiro RS, Robbins N, Cowen LE (2011) Regulatory circuitry governing fungal development, drug resistance, and disease. Microbiol Mol Biol Rev 75(2):213–267. doi:10.1128/MMBR.00045-10
16. Bonnett R, Martínez G (2001) Photobleaching of sensitisers used in photodynamic therapy. Tetrahedron 57:9513–9547
17. Donnelly RF, McCarron PA, Tunney MM (2008) Antifungal photodynamic therapy. Microbiol Res 163:1–12
18. Strakhovskaia MG, Belenikina NS, Ivanova EV, Chemeris IK, Stranadko EF (2002) The photodynamic inactivation of *Candida guilliermondii* in the presence of photodithazine. Mikrobiologiia 71(3):349–353
19. Andrade MC, Ribeiro AP, Dovigo LN, Brunetti IL, Giampaolo ET, Bagnato VS, Pavarina AC (2012) Effect of different pre-irradiation times on curcumin-mediated photodynamic therapy against planktonic cultures and biofilms of *Candida* spp. Arch Oral Biol 12
20. Dovigo LN, Pavarina AC, Carmello JC, Machado AL, Brunetti IL, Bagnato VS (2011) Susceptibility of clinical isolates of *Candida* to photodynamic effects of curcumin. Lasers Surg Med 43(9):927–934. doi:10.1002/lsm.21110
21. Pereira CA, Romeiro RL, Costa AC, Machado AK, Junqueira JC, Jorge AO (2011) Susceptibility of *Candida albicans*, *Staphylococcus aureus*, and *Streptococcus mutans* biofilms to photodynamic inactivation: an in vitro study. Lasers Med Sci 26(3):341–348. doi:10.1007/s10103-010-0852-3
22. Teixeira AH, Pereira ES, Rodrigues LK, Saxena D, Duarte S, Zanin IC (2012) Effect of photodynamic antimicrobial chemotherapy on in vitro and in situ biofilms. Caries Res 46(6):549–554. doi:10.1159/000341190
23. Mima EG, Pavarina AC, Dovigo LN, Vergani CE, Costa CA, Kurachi C, Bagnato VS (2010) Susceptibility of *Candida albicans* to photodynamic therapy in a murine model of oral candidosis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 109(3):392–401. doi:10.1016/j.tripleo.2009.10.006
24. Mima EG, Pavarina AC, Silva MM, Ribeiro DG, Vergani CE, Kurachi C, Bagnato VS (2011) Denture stomatitis treated with photodynamic therapy: five cases. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 112(5):602–608. doi:10.1016/j.tripleo.2011.05.019
25. Mima EG, Vergani CE, Machado AL, Massucato EM, Colombo AL, Bagnato VS, Pavarina AC (2012) Comparison of Photodynamic Therapy versus conventional antifungal therapy for the treatment of denture stomatitis: a randomized clinical trial. Clin Microbiol Infect 18(10):E380-E388. doi:10.1111/j.1469-0691.2012.03933.x
26. Mazor O, Brandis A, Plaks V, Neumark E, Rosenbach-Belkin V, Salomon Y, Scherz A (2005) WST11, a novel water-soluble bacteriochlorophyll derivative; cellular uptake, pharmacokinetics, biodistribution and vascular-targeted photodynamic activity using melanoma tumors as a model. Photochem Photobiol 81(2):342–351
27. Kostenich GA, Zhuravkin IN, Zhavrid EA (1994) Experimental grounds for using chlorin e6 in the photodynamic therapy of malignant tumors. J Photochem Photobiol B 22(3):211–217
28. Wen LY, Bae SM, Chun HJ, Park KS, Ahn WS (2012) Therapeutic effects of systemic photodynamic therapy in a leukemia animal model using A20 cells. Lasers Med Sci 27(2):445–452. doi:10.1007/s10103-011-0950-x
29. Soukos NS, Mulholland SE, Socransky SS, Doukas AG (2003) Photodestruction of human dental plaque bacteria: enhancement of photodynamic effect by photomechanical waves in an oral biofilm model. Lasers Surg Med 33:161–168
30. Fontana CR, Lerman MA, Patel N, Grecco C, Costa CA, Amiji MM, Bagnato VS, Soukos NS (2013) Safety assessment of oral photodynamic therapy in rats. Lasers Med Sci 28(2):479–486. doi:10.1007/s10103-012-1091-6
31. Zamperini CA, Machado AL, Vergani CE, Pavarina AC, Giampaolo ET, da Cruz NC (2010) Adherence in vitro of *Candida albicans* to plasma treated acrylic resin. Effect of plasma parameters, surface roughness and salivary pellicle. Arch Oral Biol 55(10):763–770. doi:10.1016/j.archoralbio.2010.06.015
32. Silva S, Henriques M, Martins A, Oliveira R, Williams D, Azeredo J (2009) Biofilms of non-*Candida albicans Candida* species: quantification, structure and matrix composition. Med Mycol 47(7):681–689. doi:10.3109/13693780802549594
33. Fontana CR, Abernethy AD, Som S, Ruggiero K, Doucette S, Marcantonio RC, Boussios CI, Kent R, Goodson JM, Tanner AC, Soukos NS (2009) The antibacterial effect of photodynamic therapy in dental plaque-derived biofilms. J Periodontal Res 44:751–759
34. Hope CK, Wilson M (2006) Induction of lethal photosensitization in biofilms using a confocal scanning laser as the excitation source. J Antimicrob Chemother 57(6):1227–1230
35. Garcez AS, Ribeiro MS, Tegos GP, Núñez SC, Jorge AO, Hamblin MR (2007) Antimicrobial photodynamic therapy combined with conventional endodontic treatment to eliminate root canal biofilm infection. Lasers Surg Med 39(1):59–66

36. Müller P, Guggenheim B, Schmidlin PR (2007) Efficacy of gasiform ozone and photodynamic therapy on a multispecies oral biofilm in vitro. Eur J Oral Sci 115(1):77–80
37. Zeina B, Greenman J, Purcell WM, Das B (2001) Killing of cutaneous microbial species by photodynamic therapy. Br J Dermatol 144(2):274–278
38. Demidova TN, Hamblin MR (2005) Photodynamic inactivation of *Bacillus* spores, mediated by phenothiazinium dyes. Appl Environ Microbiol 71:6918–6925. doi:10.1128/AEM.71.11.6918- 6925.2005
39. Wainwright M (1998) Photodynamic antimicrobial chemotherapy (PACT). J Antimicrob Chemother 42:13–28
40. Peeters E, Nelis HJ, Coenye T (2008) Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J Microbiol Methods 72(2):157–165
41. Ramage G, Vandewalle K, Wickes BL, Lopez-Ribot JL (2001) Characteristics of biofilm formation by *Candida albicans*. Rev Iberoam Micol 18:163–170
42. Ribeiro AP, Pavarina AC, Dovigo LN, Brunetti IL, Bagnato VS, Vergani CE, Costa CA (2013) Phototoxic effect of curcumin on methicillin-resistant *Staphylococcus aureus* and L929 fibroblasts. Lasers Med Sci 28(2):391–398. doi:10.1007/s10103-012-1064-9
43. Mang TS, Tayal DP, Baier R (2012) Photodynamic therapy as an alternative treatment for disinfection of bacteria in oral biofilms. Lasers Surg Med 44(7):588–596. doi:10.1002/lsm.22050
44. Pathak AK, Sharma S, Shrivastva P (2012) Multi-species biofilm of *Candida albicans* and non-*Candida albicans Candida* species on acrylic substrate. J Appl Oral Sci 20(1):70–75
45. Skillman LC, Sutherland IW, Jones MV, The role of exopolysaccharides in dual species biofilm development (1998) J Appl Microbiol 85(Suppl 1):13–18. doi:10.1111/j.1365-2672.1998.tb05278.x
46. Chabrier-Roselló Y, Foster TH, Mitra S, Haidaris CG (2008) Respiratory deficiency enhances the sensitivity of the pathogenic fungus *Candida* to photodynamic treatment. Photochem Photobiol 84(5):1141–1148. doi:10.1111/j.1751-1097.2007.00280.x.330