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Photodynamic inactivation of clinical isolates of *Candida* using Photodithazine®

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This study evaluated the photodynamic inactivation (PDI) mediated by Photodithazine® (PDZ) against 15 clinical isolates of *Candida albicans*, *Candida glabrata* and *Candida tropicalis*. Each isolate, in planktonic and biofilm form, was exposed to PDI by assessing a range of PDZ concentrations and light emitting diode fluences. Cell survival of the planktonic suspensions was determined by colony forming units (CFU ml^{-1}). The antifungal effects of PDI against biofilms were evaluated by CFU ml^{-1} and metabolic assay. Data were analyzed by non-parametric tests ($\alpha = 0.05$). Regardless of the species, PDI promoted a significant viability reduction of planktonic yeasts. The highest reduction in cell viability of the biofilms was equivalent to 0.9 $\log_{10}$ (CFU ml^{-1}) for *C. albicans*, while 1.4 and 1.5 $\log_{10}$ reductions were obtained for *C. tropicalis* and *C. glabrata*, respectively. PDI reduced the metabolic activity of biofilms by 62.1, 76.0, and 76.9% for *C. albicans*, *C. tropicalis*, and *C. glabrata*, respectively. PDZ-mediated PDI promoted significant reduction in the viability of *Candida* isolates.

Keywords: photodynamic therapy; *Candida* spp.; biofilms

Introduction

Although *Candida* species are considered commensal yeasts of the human oral cavity, these microorganisms can induce the development of oropharyngeal candidosis (OPC), a common infection among the elderly (Jose et al. 2010). Some etiological factors for this pathologic condition have been established and include the use of dental prostheses, smoking habits, diabetes mellitus, prolonged use of broad-spectrum antibiotic and immunosuppressant drugs (Samaranayake & Yaacoub 1990; Allen 1994; Samaranayake & Samaranayake 2001). Denture-induced stomatitis is one of the most frequent clinical presentations of OPC, which develops secondary to long-standing occlusion of the oral mucosa by a dental prosthesis (Wilson 1998). This infection is characterized by erythema of the upper denture-bearing oral mucosa and it can also be related to oral pain, altered sense of taste, and nutritional compromise, especially when associated with a compromised immune system (Jose et al. 2010). The continuous swallowing or aspiration of microorganisms from OPC lesions, particularly in the immunocompromised host or medicated elderly, can increase the risks of unexpected systemic infections (Nikawa, Hamada, et al. 1998).

Candida albicans is the most commonly isolated species in denture stomatitis, but an increasing proportion of other *Candida* species, such as *Candida glabrata* and *Candida tropicalis*, are being implicated in the development of such infection (MacPhail et al. 1993; Scully et al. 1994; Ribeiro et al. 2012). *Candida* colonization and subsequent biofilm formation on denture materials and oral mucosa are associated with the development of denture stomatitis and the resistance to treatment (Costerton 1999). The complex structure of *Candida* biofilms provides survival advantage for the yeast and elevated resistance to antifungal drugs, when compared with their planktonic counterparts (Chandra et al. 2001; Seneviratne et al. 2008, 2009). The increased use of antifungal drugs to prevent and to treat oral candidiasis, combined with several cases of treatment failures, has drawn attention to the problem of clinical antifungal drug resistance (White et al. 1998).Therefore, attention to this problem has stimulated the search for new alternative therapeutic methods to inactivate *Candida* species and subsequently, to treat oral candidosis (Malik et al. 1990; Powderly 1994). One possible approach is the photodynamic treatment.

Photodynamic inactivation (PDI) of microorganisms is based on a combination of a sensitizing drug, called photosensitizer (PS), which is activated by exposure to light of a specific wavelength (Konopla & Goslinski 2007; Donnelly et al. 2008). In the presence of molecular oxygen, it produces toxic forms of oxygen species, such as singlet oxygen ($^1O_2$) and free radicals, which are responsible for killing microbial cells (Konopla & Goslinski 2007; Bouillaguet et al. 2008; Donnelly et al.

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2008). When compared with other therapies, PDI has several advantages, such as high target specificity, few undesired side effects, and the unlikelihood of the development of antimicrobial resistance (Donnelly et al. 2008). Different compounds have been evaluated for PDI, including porphyrins (Bliss et al. 2004; Dovigo, Pavarina, Mima, et al. 2011), phenothiazine dyes (Paardekopper et al. 1992; Zeina et al. 2001), chlorins (Strackhovskaya, Zhukhovitskii, Mironov, et al. 2002; Copley et al. 2007), and phthalocyanines (Bertoloni et al. 1992; Mantareva et al. 2011). Porphyrins are the first generation of PS that can act in promoting an initial limited alteration of the cytoplasmic membrane, allowing penetration into the microbial cell and causing damage to intracellular targets (Bertoloni et al. 1989; Zeina et al. 2001; Strackhovskaya, Belenikina, Ivanova, et al. 2002; Bliss et al. 2004; Copley et al. 2007). However, these compounds have a limited ability to act against yeast cells and to penetrate into the deeper layers of biofilms, due to their hydrophilic characteristic and large molecular size (Zoladek et al. 1997; Carré et al. 1999). Recent investigations have shown that different PSs with adequate effectiveness against *Candida* biofilms are still needed (Calzavara-Pinton et al. 2012).

Chlorins are second-generation PSs, with shorter photosensitization periods, absorption of light of longer wavelengths, (which penetrate deeper within tissues), and higher $^1O_2$ yields (Ferreira et al. 2008). Photodithazine® (PDZ) is a water-soluble chlorin e6 derivative that has shown efficacy against viruses, bacteria, and yeasts, thus making this PS an interesting candidate for PDI (Donnelly et al. 2008). Its effects against *Candida* species have been suggested elsewhere, but only against a planktonic suspension of *C. albicans* and *Candida guilliermondii* (Strackhovskaya, Belenikina et al. 2002; Strakhovskaya, Zhukhovitskii et al. 2002). Thus, the purpose of this study was to evaluate the efficacy of PDZ for photo-inactivating clinical isolates of *C. albicans*, *C. glabrata*, and *C. tropicalis*.

Materials and methods

Candida *strains and growing conditions*

A total of 15 clinical isolates of *Candida* comprising *C. albicans*: Ca1, Ca2, Ca3, Ca4, and Ca5; *C. glabrata*: Cg1, Cg2, Cg3, Cg4, and Cg 5; and *C. tropicalis*: Ct1, Ct2, Ct3, Ct4, and Ct5 were used in this study. The research protocols for using clinical isolates were approved by the Ethics Committee of the Araraquara Dental School, UNESP – Univ. Estadual Paulista. The strains were isolated from denture stomatitis patients, identified and frozen during a previous investigation (Mima et al. 2012). Isolates were maintained in microtubes with solid Yeast-Peptone-Glucose medium at −70 °C. Before each experiment, yeast isolates were aerobically cultured at 37 °C for 24 h on Sabouraud's Dextrose Agar (SDA).

For the experiment with planktonic cultures, yeasts were individually inoculated in 5 ml of Tryptic Soy Broth and grown aerobically overnight at 37 °C for 16 h. Each culture was harvested after centrifugation at 2,000 rpm for 10 min, washed twice with sterile distilled water, and resuspended in 5 ml of sterile saline to a turbidity of 10^6 cells ml^{-1} (McFarland standard).

For the experiment with biofilms, yeast isolates were individually inoculated in 5.0 ml of RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) and incubated in an orbital shaker (AP 56, Phoenix Ind Com Equipamentos Científicos Ltda, Araraquara, SP, Brazil) at 37 °C for 16 h. After incubation, the yeasts were washed twice with 5.0 ml of phosphate-buffered saline (PBS), and suspended to a turbidity corresponding to 10^7 cells ml^{-1}. Biofilm formation was allowed to occur in pre-sterilized polystyrene, flat-bottomed 96-well microtiter plates. Aliquots of 100 µl of the standard cell suspension were transferred into each well, and the plate incubated at 37 °C for 1.5 h in an orbital shaker at 75 rpm (adhesion phase). After the adhesion phase, the cells of each well were carefully aspirated and washed twice with 150 µl of PBS to remove the remaining non-adherent cells. In order to allow biofilm growth, 150 µl of fresh RPMI 1640 medium were transferred into each well, and the plates were incubated at 37 °C for 48 h in an orbital shaker at 75 rpm.

PS and light source

A chlorin e6 derivative (PDZ) produced in Russia was used as the PS. PDZ was excited by a light emitting diode (LED) light in the red region of the spectrum. The LED device, denominated Dual Table, was designed by the 'Physics Institute of São Carlos, USP' (Univ. de São Paulo, São Carlos, SP, Brazil). This system is composed of red LEDs (LXHL-PR09, Luxeon® III Emitter, Lumileds Lighting, San Jose, California, USA) uniformly distributed throughout the device. The LED device spectrum was centered at 660 nm, with 20 nm bandwidth, delivering 25 mW cm^{-2}. The doses tested in this study were 37.5, 25.5, and 18.0 J cm^{-2}.

Photodynamic treatment against planktonic cultures

After preparation of each standardized *Candida* suspension, aliquots of 100 µl were individually transferred to separate wells of 96-well microtiter plates. An equal volume of PDZ was added to each well to give final concentrations of 25, 50, and 75 mg l^{-1}. After incubation in the dark for 20 min (pre-irradiation time), each plate was placed on the LED device and illuminated for 12, 17, and 25 min (to obtain doses of 18, 25.5 and 37.5 J cm^{-2}, respectively) resulting in nine experimental PDI groups (P25L18, P50L18, P75L18, P25L25.5,

P50L25.5, P75L25.5, P25L37.5, P50L37.5, and P75L37.5). Samples were not washed prior to illumination, therefore, the PS remained in contact with the cells during the PDI. To determine whether PDZ alone had any effect on cell viability, additional wells containing the yeast suspensions were exposed to PDZ under identical conditions to those described above, but not exposed to LED light (groups P25L0, P50L0, and P75L0). The effect of LED light alone was tested by exposing cells to light without being previously exposed to PS (groups P0L18, P0L25.5, and P0L37.5). Suspensions not exposed to PS nor LED light acted as overall control (P0L0), resulting in a total of 16 experimental groups. To determine the cell survival, aliquots of the contents of each well were serially diluted 10-fold in sterile saline and duplicate samples were spread onto the surfaces of SDA plates. All plates were aerobically incubated at 37 °C for 48 h. After incubation, yeast colony counts on each plate were quantified using a digital colony counter (CP 600 Plus, Phoenix Ind Com Equipamentos Científicos Ltda, Araraquara, SP, Brazil) to determine the colony forming unit per milliliter (CFU ml^{-1}).

Photodynamic treatment against biofilm

Based on the results from planktonic samples, the most effective concentration of PDZ (75 $mg\,l^{-1}$) was selected to evaluate its effectiveness against *Candida* biofilms. In addition, two higher concentrations (100 and 125 $mg\,l^{-1}$) of PDZ were also tested. The isolates were individually inoculated in 5.0 ml of RPMI 1640, and incubated overnight in an orbital shaker (AP 56, Phoenix Ind. Com Scientific Equipment Ltda, Araraquara, SP, Brazil) at 120 rpm, 37 °C for 16 h, for *in vitro* biofilm formation. After this period, the fungal cells were centrifuged at 5000 rpm for 7 min, and washed twice with 5.0 ml of PBS. The cells were suspended to 10^7 cells ml^{-1} in 1 ml of PBS and the optical density of the suspension was adjusted to 0.38 at 520 nm. Aliquots of 100 ml of the standard cell suspensions were transferred to 96-well microtiter plates. The plates were incubated at 37 °C for 1.5 h in an orbital shaker at 75 rpm (adhesion phase). After this time, each well was washed twice with 150 µl of PBS to remove non-adherent cells. In order to allow biofilm formation, 150 µl of RPMI-1640 were added to each well, and the plates were incubated at 37 °C, at 75 rpm for 48 h. After biofilm formation, the wells were carefully washed twice with 200 µl of PBS to remove remaining non-adherent cells. Then, 100 µl of PS solution (75, 100, and 125 $mg\,l^{-1}$) were added to each well. The plates were incubated in the dark for 20 min, and then illuminated (37.5, 25.5, and 18.0 $J\,cm^{-2}$). Biofilms were not washed prior to illumination; therefore, the PS remained in contact with the cells during the PDI. The 16 groups evaluated (P75L18, P100L18, P125L18, P75L25.5, P100L25.5, P125L25.5, P75L37.5, P100L37.5, P125L37.5, P75L0, P100L0, P125L0, P0L18, P0L25.5, P0L37.5, and P0L0) were subjected to similar procedures as described previously for planktonic culture evaluation. PDI mediated by PDZ against *Candida* biofilms were evaluated by a metabolic assay based on the reduction of XTT (Sigma-Aldrich, St Louis, MO), a tetrazolium salt. To prepare the XTT solution, the salt was dissolved in PBS (1 $mg\,ml^{-1}$) and stored at −70 °C. For each experiment, 158 µl of PBS with glucose, 40 µl of XTT plus 2 µl of menadione (Sigma-Aldrich, St Louis, MO) were mixed and transferred to each well. The plates were incubated in the dark at 37 °C for 3 h. After this, 100 µl of the reacted XTT salt solution were transferred to a new 96-well microtiter plate and this was analyzed by proportional colorimetric changes and light absorbance measured by a microtiter plate reader (Thermo Plate – TP Reader) at 492 nm (Dovigo, Pavarina, Carmello, et al. 2011).

Since the protocol of using 125 $mg\,l^{-1}$ of PDZ with 37.5 $J\,cm^{-2}$ showed the highest reduction in metabolic activity (XTT) assay, additional experiments were performed using these parameters in order to evaluate the viability of yeasts cells after PDI. *Candida* biofilms were grown identically to those described previously. The PDI protocol of 125 $mg\,l^{-1}$ of PDZ with 37.5 $J\,cm^{-2}$ was used and viability was assessed by means of colony count (CFU ml^{-1}).

Statistical analysis

Each experimental treatment, with the various PDZ concentrations in the presence of different light illumination doses, was independently repeated three times for each isolate ($n = 3$). The results from the five isolates of the same species were combined to obtain the general response of the species, resulting in 15 samples for each species. For the purpose of analysis, CFU ml^{-1} values were transformed into logarithm ($\log_{10}$). The results collected after the evaluation of planktonic suspensions (log (CFU ml^{-1})) and biofilm cultures (XTT and log (CFU ml^{-1})) did not fit the ANOVA assumptions. Therefore, the data were evaluated by the non-parametric Kruskal–Wallis and Dunn *post hoc* test. Differences were considered statistically significant at $p < 0.05$.

Results

PDI of C. albicans *isolates*

Exposure of planktonic *C. albicans* cultures to PDZ or to light alone resulted in statistically similar log (CFU ml^{-1}) values to those of the P0L0 group ($p > 0.05$). On the other hand, exposure of cultures to PDI promoted a significant reduction in cell viability for all the associations of PDZ and light ($p < 0.001$). Figure 1A shows the behavior of the species in the different groups. The

susceptibility of the five clinical isolates varied, but the combined treatment of 50 and 75 mg l^{-1} with illumination at 37.5 J cm^{-2} resulted in complete killing of the five strains of *C. albicans*. In addition, the use of 75 mg l^{-1} of PDZ and 25.5 J cm^{-2} was also effective for completely inactivating the samples. In these cases, a 6.4 (CFU ml^{-1}) log reduction was observed.

With respect to the analysis of the susceptibility of biofilms, it was also observed that all the combined treatments of PDZ and light resulted in significant reductions in cellular metabolism (XTT) in comparison with P0L0. Almost all PDI groups were considered statistically similar, with the exception of the groups photosensitized with 100 and 125 mg l^{-1} and illuminated 37.5 J cm^{-2} (P100L37.5 and P125L37.5), which resulted in the lowest cellular metabolism values (Figure 1B). In these two groups, the reduction in biofilm metabolism was ~59.2 and 62.1% in comparison with Group P0L0, respectively. *C. albicans* cells within biofilms showed reduced viability with exposure to PDI (P125L37.5) when compared to P0L0 (Figure 2A) by ~0.9 log (CFU ml^{-1}). The use of PDZ or light alone caused no significant alteration in the cell metabolism and viability of *C. albicans* biofilms ($p > 0.05$).

PDI of C. glabrata *isolates*

The results obtained from evaluating the planktonic cultures of *C. glabrata* are presented in Figure 3A. All associations of PDZ with light promoted significant reductions in fungal viability ($p < 0.001$). Nevertheless, total inactivation of *C. glabrata* was not observed for three clinical isolates of this species, even with the use of the highest concentration of PDZ and doses of light. In general, the association of 75 mg l^{-1} with 25.5 J cm^{-2} and associations of 50 and 75 mg l^{-1} with 37.5 J cm^{-2} resulted in the lowest log (CFU ml^{-1}) values. The reduction observed in these groups was 3.6, 3.9, and 4.3 log (CFU ml^{-1}), respectively. Statistical analysis showed a significant increase in log values of P25L0, P50L0, and P75L0 groups when compared to P0L0, but this raise was <0.30log in all cases.

Significant reductions in biofilm metabolism were also observed (Figure 3B). The association of 125 mg l^{-1} of PDZ and illumination with 37.5 J cm^{-2} resulted in the lowest values of XTT, in comparison with the control group (P0L0). The percentage reduction in viability in this case was ~76.9%. Also, *C. glabrata* biofilms had their viability reduced with the exposure to PDI (P125L37.5) when compared to P0L0 (Figure 2B). The reduction was ~1.5log (CFU ml^{-1}). Although the use of PDZ alone in the planktonic samples caused an increase in the CFU ml^{-1} values, this effect was not observed for the biofilm samples, in which the use of PDZ or light alone did not lead to any alteration in cell metabolism and viability ($p > 0.05$).

PDI of C. tropicalis *isolates*

Figure 4A shows the results obtained in the evaluation of PDI against planktonic cultures of *C. tropicalis*. Application of PDI resulted in a significant reduction in the log (CFU ml^{-1}) values regardless of the PDZ concentration and the light fluence, when compared with the P0L0 group. The association of both 50 and 75 mg l^{-1} with 37.5 J cm^{-2} was the most effective to photoinactivate *C. tropicalis* isolates, resulting in a 4.6 and 5.6 log of reduction, respectively. Complete inactivation occurred in four of the five clinical isolates of *C. tropicalis* in P75L37.5 group. Statistical analysis also showed a significant increase in log values of P25L0, P50L0, and P75L0 groups when compared to P0L0, but this was <0.30log in all cases.

A significant reduction in biofilm metabolism was also observed in all PDI groups (Figure 4B). Although the use of 125 mg l^{-1} associated with 25.5 J cm^{-2}, as well as the associations of 75, 100, and 125 mg l^{-1} with 37.5 J cm^{-2} were the most effective in reducing the XTT values, the highest percentage reduction of metabolis was found in the P125L37.5 group (~76.0%). For this group, a significant decrease in log (CFU ml^{-1}) values was also observed (Figure 2C). The use of PDZ or light alone was not capable of promoting significant alterations in the metabolism or the viability of *C. tropicalis* biofilms.

Discussion

Antimicrobial photodynamic therapy has been suggested as a powerful method for microbial inactivation and, therefore, for the treatment of local infections such as oral candidiasis (Mima et al. 2011, 2012; Tsai et al. 2011). At present, different PSs are being investigated in order to establish effective protocols for microbial photoinactivation. PDZ is a chlorin e6 derivative that has been applied most extensively against tumor cells and the studies suggest this PS as a novel and efficient drug, capable of requiring a lower dose and shorter illumination time (Tian et al. 2008). However, evaluations of this PS against *Candida* cells are scarce.

In the present study, the effectiveness of PDI mediated by PDZ and red LED light was assessed for killing clinical isolates of *C. albicans*, *C. glabrata*, and *C. tropicalis*. Initially, the susceptibility of planktonic suspensions was investigated. The results showed that PDI promoted significant reductions in the viability of *C. tropicalis* and *C. glabrata* (reduction of 5.6 and 4.3 logs, respectively), as noted by the lower colony counts found when the yeast suspensions were exposed to the therapy. In addition, the five strains of *C. albicans* were completely killed after PDI (reduction of 6.4 logs). These findings partially agree with those described by Dovigo

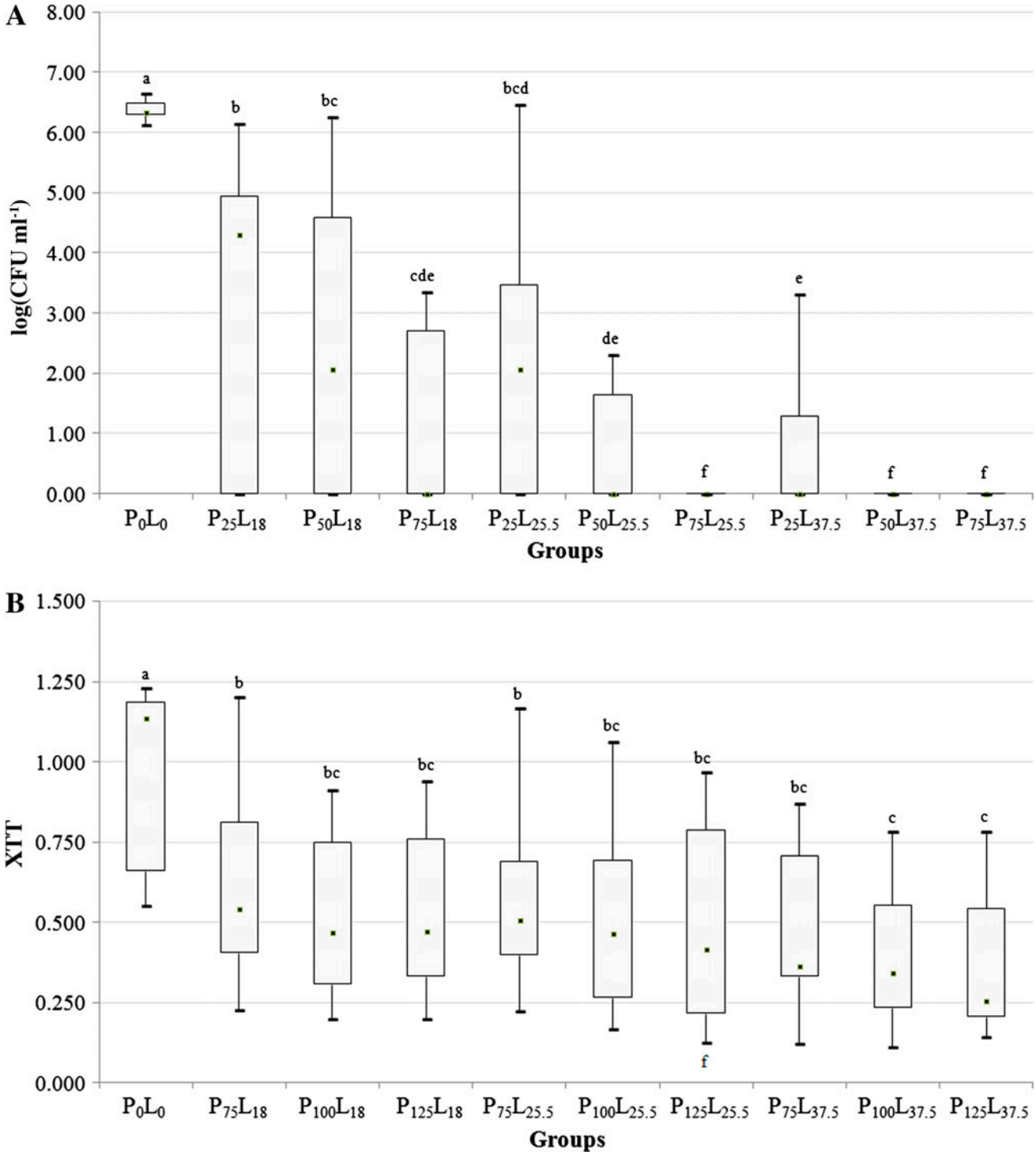

Figure 1. Summary of log CFU ml^{-1} (A) and XTT (B) values obtained after evaluation of the PDI groups of planktonic and biofilms cultures of *C. albicans*, respectively. The box plots use the median (bold square), the first and third quartiles (box), and the lowest and highest values (dashes). The same lower case letter denotes non-significant differences among groups according to non-parametric *post hoc* test ($p > 0.05$).

et al. (2010) who evaluated the susceptibility to PDI of *C. albicans* and *C. tropicalis* in the planktonic phase by the association of Photogem®, an hematoporphyrin derivative, and LED light. The use of high fluences of light associated with low concentrations of PS was able to promote the complete inviability of the suspensions of

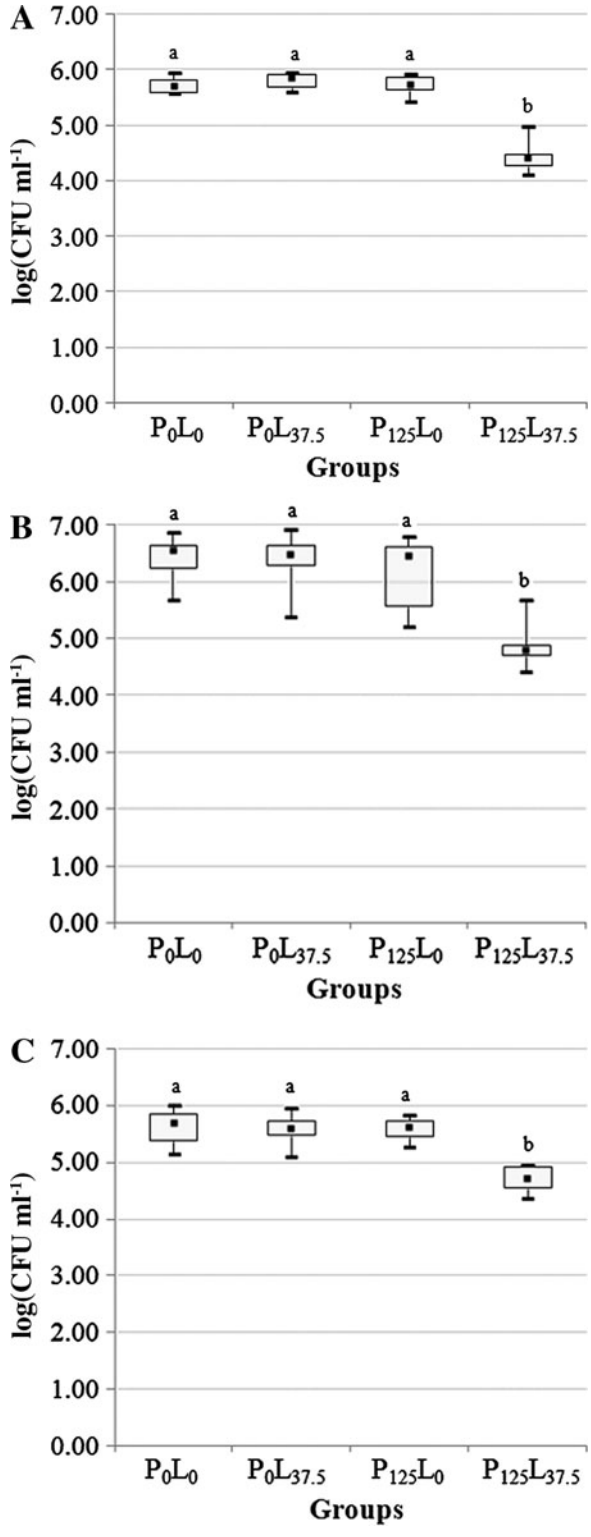

Figure 2. Summary of log CFU ml^{-1} values obtained after evaluation of biofilm cultures of *C. albicans* (A), *C. glabrata* (B), and *C. tropicalis* (C). The box plots use the median (bold square), the first and third quartiles (box), and the lowest and highest values (dashes). The same lower case letter denotes non-significant differences among groups according to non-parametric poshoc test ($p > 0.05$).

C. albicans and *C. tropicalis*. Another investigation (Dovigo, Pavarina, Mima, et al. 2011) demonstrated that PDI was effective in completely killing planktonic suspensions of *C. albicans* and *C. glabrata*, using Photogem® with LED light. In the present investigation, the eradication of *C. glabrata* and one strain of *C. tropicalis* were not observed. It can be explained by the fact that clinical isolates of *Candida* spp. from denture stomatitis patients were employed here, and in the investigations mentioned investigations, ATCC strains were used. It has been suggested that the response of strains to PDI is not homogenous among clinical isolates of the same species (Dovigo, Pavarina, Mima, et al. 2011).

The antimicrobial potential of PDI using chlorin e6 derivatives has been previously reported, especially against bacterial species (Demidova & Hamblin 2005; Hamblin et al. 2002; Park et al. 2010). However, the present authors were able to find only two investigations providing data on the use of PDZ and light to photoinactivate *Candida* species. In one investigation, the authors used different PDZ concentrations against planktonic suspensions of *C. guilhiermondii* and found <10% of the yeast cells survived when a concentration of 7 μM was used (which corresponds to $\sim 1.35\ mg\,l^{-1}$). However, the authors did not use the colony count method to determine yeast survival making it difficult to establish direct comparisons with the present results. The authors also compared the photosensitization efficiency of PDZ and other chlorin e6 derivatives, and concluded that PDZ was 25% more efficient (Strackhovskaya, Belenikina, Ivanova, et al. 2002). Another investigation showed that 10 μM PDZ caused a significant decrease in the colony-forming capacity of reference strains of *C. albicans* and *C. guilliermondii* after these species had been exposed to 4.5 and 3.0 J cm^{-2} of light, respectively (Strakhovskaya, Zhukhovitskii, Mironov, et al. 2002). These authors also verified that washing the cells before illumination reduced the yeast photosensitivity of the yeast and that increasing the concentration of PDZ from 2 to 20 μM promoted an increase in the photosensitivity of these strains, with the *C. guilliermondii* being 1.6 to 1.7 times more photosensitive than *C albicans* (Strackhovskaya, Zhukhovitskii, Mironov, et al. 2002). Moreover, a similar trend in the PDZ-mediated photosensitivity of eight clinical isolates of *C. albicans* (from pharynx, sputum, feces and urine) was also observed (three strains had similar photosensitive properties to the reference strain, the other three were not more than 20% less photosensitive, and two were more photosensitive) (Strackhovskaya, Zhukhovitskii, Mironov, et al. 2002). Together, these results are consistent with the hypothesis that *Candida* species can be efficiently killed by associating chlorin e6 derivatives and light.

Biofilms are the most common mode of fungal growth in clinical infections and are considered highly

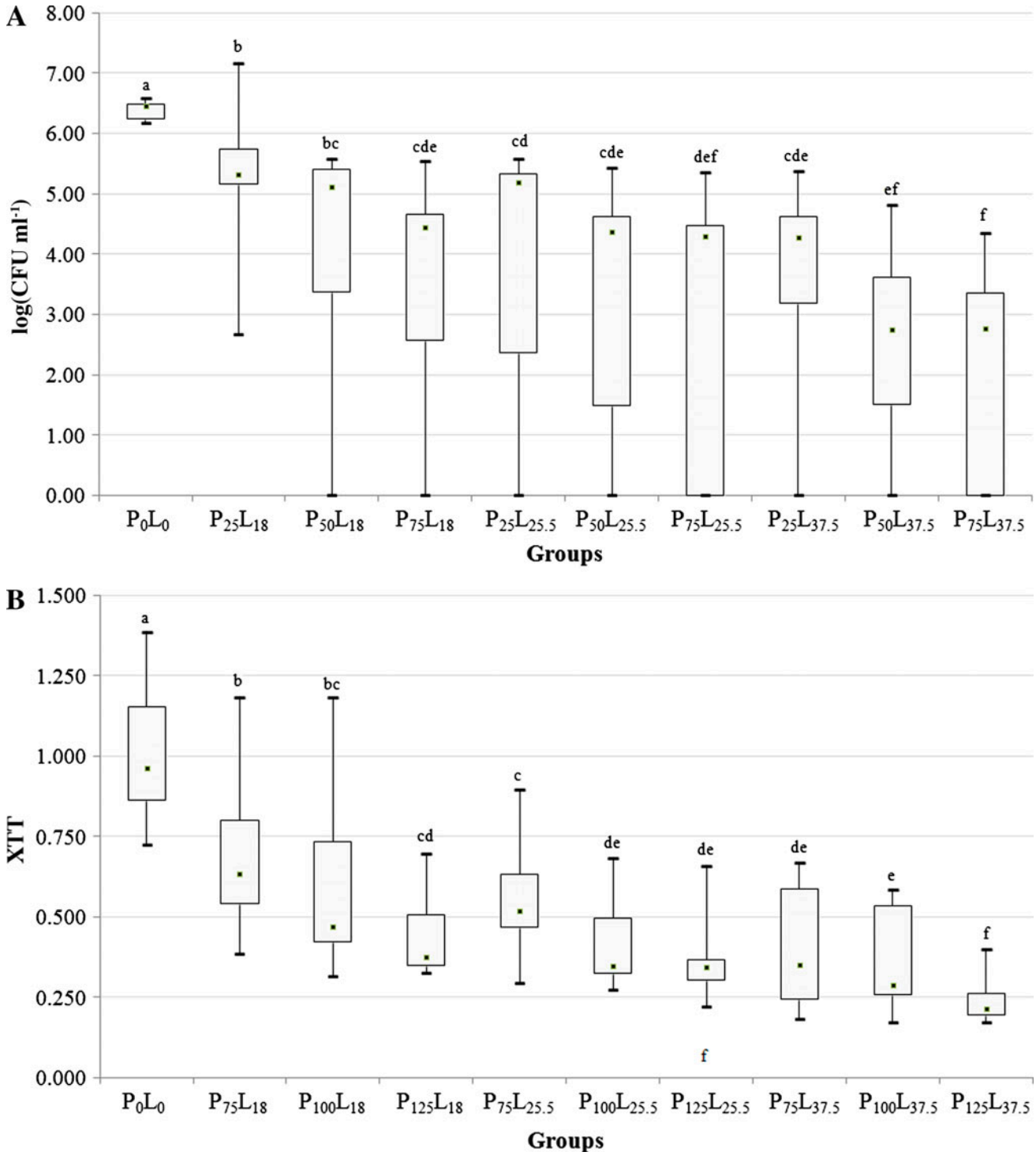

Figure 3. Summary of log CFU ml^{-1} (A) and XTT (B) values obtained after evaluation for the PDI groups of planktonic and biofilms cultures of *C. glabrata*, respectively. The box plots use the median (bold square), the first and third quartiles (box), and the lowest and highest values (dashes). The same lower case letter denotes non-significant differences among groups according to non-parametric *post hoc* test ($p > 0.05$).

resistant to antimicrobial agents (Costerton et al. 1999; Chandra et al. 2001). To the authors' knowledge, there is no other report describing the photoinactivation of the biofilm of *Candida* spp. mediated by PDZ. Nevertheless, Hope and Wilson (2006) showed the effectiveness of another commercial chlorin e6 derivative (SnCe6;

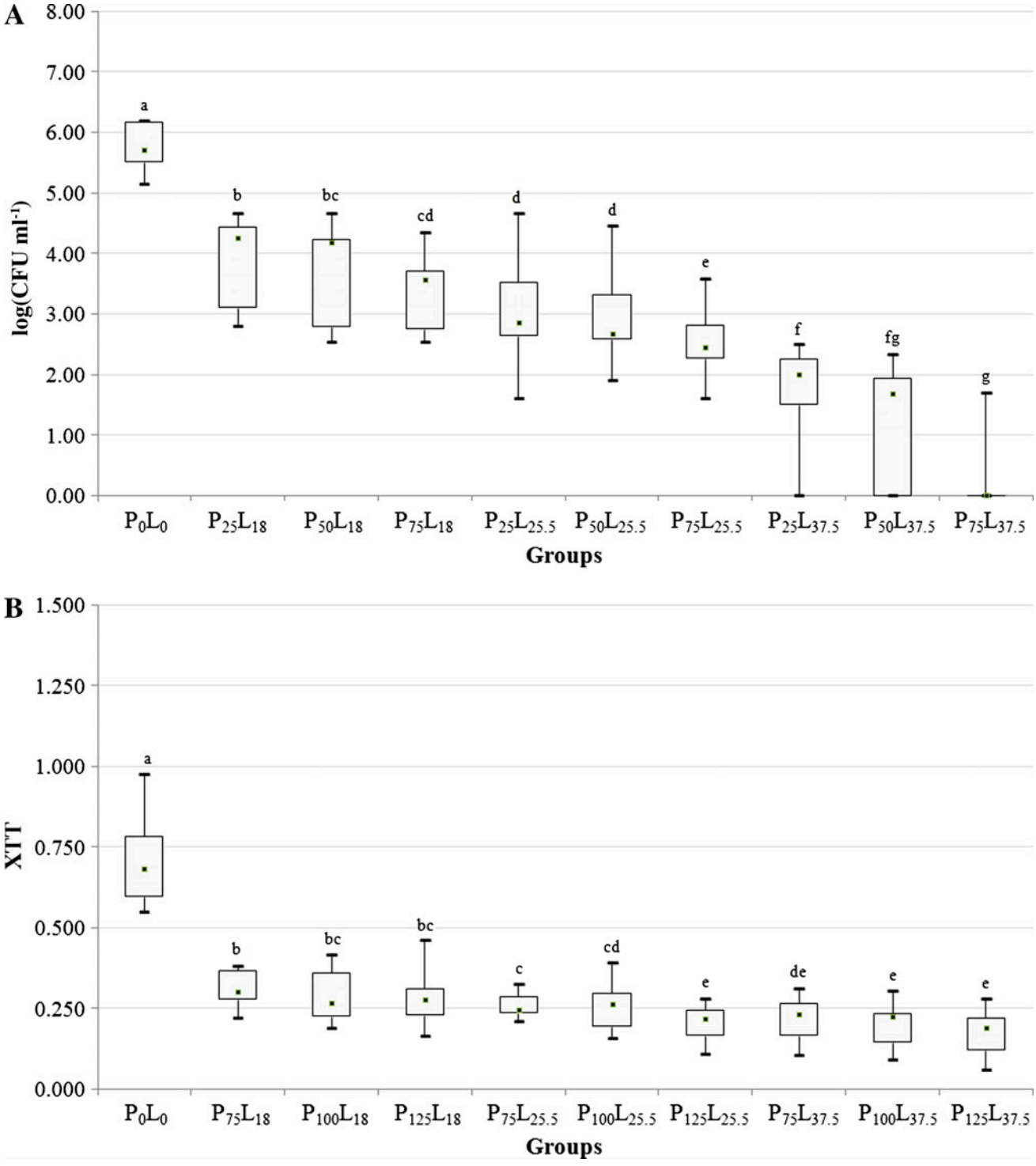

Figure 4. Summary of log CFU ml^{-1} (A) and XTT (B) values obtained after evaluation for the PDI groups of planktonic and biofilms cultures of *C. tropicalis*, respectively. The box plots use the median (bold square), the first and third quartiles (box), and the lowest and highest values (dashes). The same lower case letter denotes non-significant differences among groups according to non-parametric *post hoc* test ($p > 0.05$).

50 mg l^{-1}) against *Streptococcus pyogenes* biofilms. In the study presented in this paper, a significant reduction in the metabolic activity of *Candida* biofilm was observed after PDI treatment, compared with the positive control group P0L0. The highest percentage of reduction in biofilm metabolism was observed with the use of

125mgl^{-1} of PDZ and 37.5 J cm^{-2} of light. For *C. albicans*, there was a 62.1% reduction in the metabolic activity of biofilms, while reductions of 76.9% and 76.0% were observed for *C. glabrata* and *C. tropicalis*, respectively. All *Candida* spp. evaluated in the present investigation showed reduced viability after exposure to PDI when compared to the control groups by ~0.9, 1.4, and 1.5log (CFU ml^{-1}) for *C. albicans*, *C. tropicalis*, and *C. glabrata*, respectively. Another study evaluated the effectiveness of Photogem-mediated PDI against biofilms of *C. albicans* and non-*albicans* species. The authors verified that the PS promoted a reduction in the viable counts of <1 log10$_{10}$-unit in all species (Dovigo, Pavarina, Mima, et al. 2011). It is important to state that PDZ was more effective in reducing the cell viability of non-*albicans Candida* species, in comparison with the PS mentioned above. The higher susceptibility of *C. glabrata* and *C. tropicalis* biofilms, in comparison with *C. albicans*, may be associated with structural characteristics of *in vitro* single biofilms. It has been suggested that *C. albicans* biofilm is thicker and more organized than *C. glabrata* and *C. tropicalis* biofilms (Parahitiyawa et al. 2006; Seneviratne et al. 2009). In addition, some investigations have demonstrated that biofilms of non-*albicans* species do not form hyphae and these biofilms are exclusively composed of blastopores (Seneviratne et al. 2009). Although filamentous morphology (hyphae and pseudohyphae) is not necessary for biofilm formation, this morphology contributes to the survival of the fungus and it provides the development of a three-dimensional and arranged community (Nikawa, Nishimura, et al. 1998; Seidler et al. 2006; Thein et al. 2007). Thus, it is possible that the biofilms formed by *C. glabrata* and *C. tropicalis* were disrupted more easily with the application of PDI, resulting in a higher percentage reduction in XTT and log (CFU ml^{-1}), when compared with *C. albicans* biofilms. The observed susceptibility of *C. glabrata* biofilms to PDI may have important relevance since this species exhibits innate and acquired resistance to antifungal medicaments and is frequently associated with persistent infections (Vanden Bossche et al. 1992; Li et al. 2006). The mechanism of chlorin-phototoxicity towards bacterial systems differs completely from that of antifungal agents. The excited states of PDZ and their subsequent reactions with oxygen promote the formation of reactive oxygen species (ROS) that induce photo-damage to cell organelles and cell death (Strackhovskaya, Belenikina, Ivanova, et al. 2002). Studies on photoinactivation of cancer cells mediated by PDZ suggest that singlet oxygen ($^1O_2$) is a primary cytotoxic product of PDI that damages cellular structures in close proximity to the photoexcited PS molecules (Uzdensky et al. 2004).

In general a difference in susceptibility between planktonic and biofilm cells is expected (Donnelly et al. 2007; Dovigo, Pavarina, Mima, et al. 2011). For this reason, higher PDZ concentrations were tested against *Candida* biofilms, when compared with the protocols for planktonic suspensions. However, the use of high concentrations of PS was not enough to inactivate biofilms completely. The failure of conventional antifungal treatments is frequently related to the unsuccessful penetration of drugs through the extracellular polysaccharide matrix of biofilms (Hawser et al. 1998). Consequently, it is likely that the efficiency of photodynamic inactivation depends on the degree to which the PS penetrates into the biofilms. It is known that the structural complexity of biofilms is supported by exopolymeric substances in order to develop a three-dimensional community (Seneviratne et al. 2009). This complex structure can be divided in three different layers: the basal layer, the middle layer, and the superficial layer. Studies have shown that cells of *Candida* biofilms have distinct metabolic activity in the different layers (La Fleur et al. 2006; Uppuluri et al. 2006). Thus, this variety of physiological states of biofilm cells can influence the penetration of PS and promote poor cell sensitization. This fact could be responsible for biofilm resistance to PDI when compared with their planktonic counterparts. In an attempt to overcome this problem, investigations into the use of chlorin e6 for antibacterial PDI have frequently used chlorin conjugates to increase phototoxicity towards biofilms (Soukos et al. 2003; Garcez et al. 2007; Suci et al. 2010). The difficulty of PS in penetration into the inner layers of biofilms may also be explained by the possibility of aggregate formations at high concentrations of the PS. As seen with different types of PS in water solutions, the formation of dimmers and higher aggregates is a common problem in the photodynamic field. Aggregation has a direct influence on photophysical behavior, rendering normally active photosensitizers inactive through self-quenching (Bonnett & Martínez 2001; Macdonald & Dougherty 2001; Derosa & Crutchley 2002).

In the present investigation, the effect of PDI against different clinical isolates of *Candida* from denture stomatitis patients was evaluated. It was observed that the response of the strains to PDI was not homogenous within the same species. This result agrees with those of Strakhovskaya, Zhukhovitskii, Mironov, et al. (2002), Lambrechts et al. (2005) and Dovigo, Pavarina, Mima, et al. (2011), who reported variations in the sensitivity to PDI of different strains of *C. albicans*. The differences in susceptibility among isolates can be explained by the distinct characteristics that each strain may exhibit. A recent study showed that genetically heterogeneous yeasts can be isolated from denture stomatitis (Song et al. 2006). Although reference strains, such as ATCC, are commonly used for *in vitro* evaluations, the variation among clinical isolates of the same species in the manner in which they respond to PDI may have important

clinical relevance. It indicates the importance of investigating more than one isolate belonging to a single species before drawing conclusions with regard to the susceptibility to PDI. In addition, preliminary *in vitro* investigations have critical importance for new antifungal therapies, such as PDI, prior to clinical application. The results of the present study demonstrated that PDZ-mediated PDI promoted significant reduction in the viability of *Candida* biofilms. However, the effectiveness of this therapy may be improved with the use of PDZ conjugates. Further investigations are necessary to evaluate this hypothesis as well as the *in vivo* efficacy of this therapy against *Candida* infections.

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