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Photoinactivation of single and mixed biofilms of *Candida albicans* and non-*albicans Candida* species using Photodithazine®

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ABSTRACT

This study evaluated the effectiveness of antimicrobial photodynamic therapy (aPDT) mediated by Photodithazine® (PDZ) formulated in hydrogel, in the inactivation of mono and duo-species biofilms of *Candida albicans, Candida glabrata* and *Candida tropicalis*. Standardized suspensions of each strain were prepared and after biofilm formation, mono-species were treated with 150 and 175 mg/L of PDZ for 20 min (pre-irradiation time), and exposed to LED light at a dose of 37.5 J/cm^2 (660 nm). The duo-species biofilms (*C. albicans + C. glabrata and C. albicans + C. tropicalis*) were treated with 150 mg/L of PDZ and light. Additional samples were treated with PDZ or light only, and the control did not receive any treatment. Next, microbiological evaluation was performed by spreading the cells on Sabouraud Dextrose Agar and CHROMagar *Candida* for colony forming units (CFU/mL). Moreover, the total biomass of biofilm was verified using the crystal violet staining assay (CV). The data were submitted to ANOVA and Tukey post-hoc ($\alpha = 0.05$). The use of PDZ 150 mg/L promoted a reduction of 1.0, 1.2, 1.5 $\log_{10}$ in the viability of *C. glabrata, C. albicans* and *C. tropicalis*, respectively. The same concentration reduced in 1.0 $\log_{10}$ the viability of each species grown as duo-species biofilms. The crystal violet assay showed that the use of 150 mg/L reduced 24.4%, 39.2% and 43.7% of the total biomass of *C. albicans, C. tropicalis* and *C. glabrata*, respectively. aPDT did not reduce the total biomass to the duo-species biofilms. Thus, PDZ-mediated aPDT was more effective in the inactivation of mono-species biofilms of *Candida* spp. compared with duo-species biofilm.

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1. Introduction

The colonization of the oral mucosa by *Candida* spp. has been identified as being responsible for the development of oropharyngeal candidiasis (OPC) [1]. Although *Candida albicans* has been considered the predominant causative agent of many forms of candidiasis, the non-*albicans Candida* species (NAC) such as *Candida glabrata* and *Candida tropicalis* have often been isolated [2,3] in nosocomial invasive candidaemias, surpassing *C. albicans* [4,5]. *Candida* species virulence is attributed to the ability to modify its morphology in yeast, pseudohyphae or true hyphae [6]. Hyphae development is a prerequisite for strong biofilm formation, as well as the interactions between the cells, and extracellular matrix production, which are primordial steps in biofilm formation [7].

Biofilms consist of structured communities of microorganisms surrounded by an extracellular polymeric matrix attached to living or inert surfaces [8,9]. This complex structure promotes fungus survival even in unfavorable conditions, increasing resistance to antifungal drugs [9]. In addition, polymicrobial or multi-species biofilms assist the microorganisms by promoting mutually beneficial interactions among them [10]. Pereira-Cenci et al. [11] investigated the interactions between *C. albicans* and either *Candida glabrata* or *Streptococcus mutans* in biofilms grown on various surfaces, with or without saliva. The authors verified that dual *Candida* species biofilms did not show competitive interaction between the two species. Thus, the presence of polymicrobial biofilms has been considered an important factor for increasing microorganism resistance, contributing to the failure of treatment against OPC [12].

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Treatments against infections caused by *Candida* spp. are usually based on the use of medication, which may be topical or systemic [13]. However, the use of antifungal therapy may be limited due to its toxicity, low efficacy, and microorganism resistance after long drug exposure time [14]. In fact, the resistance of non-*albicans* species to antifungal agents is a major challenge for the development of therapeutic and prophylactic strategies [15]. One of these strategies, the antimicrobial photodynamic therapy (aPDT), has been suggested as a promising alternative for the treatment of superficial fungal infections [16,17].

Antimicrobial photodynamic therapy consists of the administration of a photosensitizing agent (PS) of high specific light absorption, known as photosensitizing process. When this PS is subjected to irradiation with visible light in the presence of oxygen it produces reactive oxygen species (ROS) capable of inducing cell inactivation [16,17]. It has been suggested that the mechanism involves the absorption of photons from the light source by the PS, promoting the electrons to an excited state. In the presence of oxygen, the PS excited by light may react with neighboring molecules transferring electrons or hydrogen (type I reaction) and/or energy to oxygen (type II reaction), producing ROS [18]. Both methods can lead to cell death. These reactive species have no specific reactivity with organic molecules, which means that any cellular macromolecule may be a potential target for aPDT [18].

In addition, different photosensitizing agents have been assessed for aPDT, including phenothiazines [19,20], porphyrins [21] and phthalocyanines [22]. Studies have shown that aPDT presents fungicidal activity against planktonic suspensions of *C. albicans*, including resistant strains [21]. However, complete inactivation of these microorganisms, when organized in biofilms, has not been observed [23,24]. Second-generation photosensitizers have been considered an advantage as they present short periods of photosensitization, absorb longer wavelengths, and produce high levels of singlet oxygen. Among these compounds are the chlorins, reduced hydrophilic porphyrins that exhibit strong absorption band in the red region of the electromagnetic spectrum. Photodithazine® (PDZ) is a glucosamine salt of chlorin e_6, soluble in water and an amphiphilic molecule obtained from the cyanobacterium *Spirulina platensis* [25]. These molecules are capable of penetrating into the biological membranes, which generally promotes greater efficacy of aPDT [26]. Previous studies have shown that aPDT mediated by PDZ and other chlorin e_6 derivatives associated with red LED inactivated planktonic suspensions of *C. albicans* and *Candida guilliermondii* [27–29]. PDZ diluted in saline was effective in the inactivation of planktonic suspensions and it was able to reduce the metabolic activity of single biofilms in clinical isolates of *C. albicans*, *C. glabrata* and *C. tropicalis* [30]. Quishida et al. [31] reported that the PDZ diluted in saline, at a concentration of 175 and 200 mg/L, was effective in reducing the cell viability of mixed biofilms formed by *C. albicans, C. glabrata* and *S. mutans*. Recently, Carmello et al. [32,33] reported the effectiveness of this PS, formulated in solution [32] and in hydrogel [33], in the photoinactivation of *C. albicans* present in the oral lesions of mice.

Although aPDT mediated by PDZ has been effective in the inactivation of *C. albicans in vitro* [30,31] and *in vivo* [32,33] demonstrating its potential as an antifungal method, there are no reports that tried to develop a new formulation to improve its topical application as a photosensitizing agent against *Candida* species. Considering the above mentioned aspects, the novelty of the study was to assess the effectiveness of aPDT mediated by PDZ formulated in hydrogel on the inactivation of single and mixed biofilms of *Candida* albicans and non-*albicans Candida* species. Additionally, it was evaluated the ability of the therapy in reducing the total biomass of *Candida* spp. biofilms.

2. Materials and method

2.1. Microorganisms and growth conditions

Candida species were maintained in Yeast-Peptone-Glucose medium and frozen at −70 °C. Before each experiment, the strains of *C. albicans* (ATCC 90028, Rockville, MD), *C. glabrata* (ATCC 2001) and *C. tropicalis* (ATCC 4563) were thawed and spread on Sabouraud Dextrose Agar – SDA plates (Acumedia Manufacturers, Inc., Baltimore, MD, USA) with 5 μg/ml of chloramphenicol and incubated at 37 °C for 48 h.

To obtain standardized suspensions of *Candida* spp., each strain was individually inoculated in 5 ml of RPMI 1640 medium (Brand). All microorganisms were incubated at 37 °C for 16 h. The cells were washed twice with PBS (pH 7.2), centrifuged at 5000 xg for 5 min and resuspended in PBS. Suspensions of *Candida* spp. were adjusted using a spectrophotometer at a concentration of 10^7 cells/ml [30].

Single-species biofilms of *Candida* spp. were formed with standard suspensions. For this purpose, aliquots of 100 μl of each standard cell suspension were inoculated on 96-well microtiter plates and incubated for 90 min at 37 °C on an orbital shaker at 75 rpm (adhesion phase) [30]. After this, the non-adherent cells were removed by washing them twice with 200 μl of PBS. Then, 200 μl of RPMI 1640 medium was added to each well and the plates were incubated for 48 h at 37 °C on an orbital shaker at 75 rpm, for the biofilm formation.

Two dual-species biofilms were formed: 1) biofilm constituted of *C. albicans* + *C. tropicalis*; 2) biofilm constituted of *C. albicans* + *C. glabrata.* For this, 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). After this, the non-adherent cells were removed by washing them twice with 200 μl of PBS. Then, 200 μl of RPMI 1640 medium was added to each well and the plates were incubated for 48 h at 37 °C on an orbital shaker at 75 rpm, for the biofilm formation [31]. All experiment was performed in duplicate on three different occasions.

2.2. Photosensitizer agent and parameters of irradiation

Photodithazine® (Moscow, Russia) formulated in Natrosol–based hydrogel (Pharmacy Santa Paula, Araraquara, SP, Brazil) at concentrations of 150 and 175 mg/L was used to sensitize the biofilms. The illumination of the samples was performed using a LED device (660 nm) at a light dose of 37.5 J/cm^2 and output power of 71.7 mW/cm^2 [30]. It is important to mention that the parameter of irradiation and the concentration of the PS used in the present investigation were determined previously, in a pilot study (data not shown).

2.3. Photodynamic therapy and microbiological evaluation

The susceptibility of single-species biofilms of *Candida* spp. to aPDT was assessed by exposing them to two concentrations of PDZ (150 and 175 mg/L) and light dose of 37.5 J/cm^2 (groups P+L+ using 150 mg/L or 175 mg/L, respectively). Additional samples were treated only with PDZ (P+L- with 150 mg/L or 175 mg/L) or LED (P-L+). Positive control consisted of samples that were not sensitized or illuminated (P-L-). Negative control group consisted of RPMI 1640 medium without microorganisms. After biofilm formation, the wells of the plates were washed twice with PBS and, according to the experimental groups described, 200 μl of PDZ was added to groups P+L+ and P+L- and aliquots of 200 μl of saline solution was added to groups P-L+ and P-L-. The plates were then incubated in the dark for 20 min (pre-irradiation time). After this period, the groups P+L+ and P-L+ were illuminated for

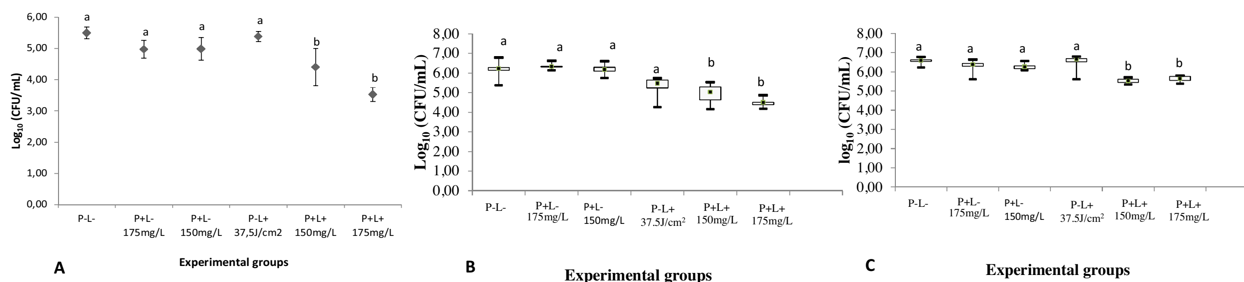

Fig. 1. A: Mean values and standard deviation [$\log_{10}$ (CFU/ml)] of the viability of mono-species biofilm of *C. albicans*. Different lower case letters denote significant differences among the groups in comparison with the control (P-L-) ($p < 0.05$); B: Log $_{10}$ (UFC/mL) values obtained in the experimental groups for the strains of *C. tropicalis*. The box plot shows the median (bold square), the 1st and 3rd quartile (box), and the smallest and highest values obtained (traces). Different lower case letters denote significant differences among the groups in comparison with the control (P-L-) ($p < 0.05$); C: Log $_{10}$ (UFC/mL) values obtained in the experimental groups for the strains of *C. glabrata*. The box plot shows the median (bold square), the 1 st and 3rd quartile (box), and the smallest and highest values obtained (traces). Different lower case letters denote significant differences among the groups in comparison with the control (P-L-) ($p < 0.05$). (Data were submitted to ANOVA and Tukey post-hoc for *C. albicans* and Kruskal-Wallis followed by Dunn post- hoc for *C. tropicalis and C. glabrata).*

9 min (37.5 J/cm^2). The most effective concentration of PDZ associated with LED (37.5 J/cm^2) was used to inactivate the dual-species biofilms of *Candida* spp.

Biofilm viability was assessed after completion of the treatments. The cells were detached from the plate orifices with the aid of sterile swabs (Johnson) and serial dilutions of 10^{-1} to 10^{-3} were obtained and spread on Petri plates containing the SDA culture medium (Acumedia Manufacturers, Inc., Baltimore, MD, USA) with 5 μg/ml of chloramphenicol and presumptive CHROMagar *Candida* medium (Difco, Laboratories, Detroit, MI, USA) for simple- and dual-species biofilms, respectively. The plates were incubated (37 °C/48 h) to determine the colony forming units per milliliter (CFU/ml) and the data were transformed into a logarithm ($\log_{10}$). The experiment was performed in duplicate on 3 different occasions.

2.4. Total biomass quantification

The test to quantify the total biomass of biofilms was performed by crystal violet staining (CV). Immediately after the completion of the treatments proposed, the biofilms were washed with PBS and the cells were fixed in 200 μl of methanol for 15 min. Methanol was removed and the plates were left at room temperature for 40 min. Next 200 μl of CV (1% v/v) was added into each well of the plate, which was 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 verified in a microplate reader at 570 nm [34].

2.5. Statistical analysis

For the data that met the assumptions of normality and homogeneity, the values were submitted to analysis of variance (ANOVA), followed by post-hoc Tukey's test ($\alpha = 0.05$). For the data that did not meet the assumptions of normality, they were analyzed by the Kruskal-Wallis test and post hoc Dunn's test ($\alpha = 0.05$).

3. Results

3.1. Photoinactivation of single-species biofilms of Candida spp.

The exposure of single-species biofilms of *Candida* spp. to aPDT promoted a significant reduction in viability irrespective of the PDZ concentration used for all yeasts evaluated ($p < 0.001$). There was no significant difference between the groups submitted to aPDT. Fig. 1 shows the behavior of each biofilm formed by three species of *Candida* in the different experimental groups. It was observed 1.0 and 1.2 $\log_{10}$ reduction of *C. albicans* after aPDT mediated by 150 mg/L and 175 mg/L of PDZ, respectively (Fig. 1A). Similarly, aPDT mediated by 150 mg/L of PDZ was able to promote 1.0 and 1.1 $\log_{10}$ of reduction of *C. tropicalis* (Fig. 1B) and *C. glabrata* (Fig. 1C), respectively. When 175 mg/L of PS was used, it was found 1.6 and 1.0 $\log_{10}$ of reduction respectively (Fig. 1B and C, respectively). Additionally, there was no statistical difference between the groups P+L-, P-L+ and P-L-, showing that the application of light or photosensitizer separately was not toxic to fungal species.

Table 1
Mean values of absorbance (optical density – OD) obtained after crystal violet assay to the mono-species biofilms of *Candida* spp. for all groups evaluated.

Groups	P-L-	P+L- 150	P+L- 175	P-L+	P+L+ 150	P+L+ 175
C. albicans	2401^{a}	2312^{a}	2371^{a}	2354^{a}	1813^{b}	2301^{a}
C. tropicalis	2288^{a}	2262^{a}	2263^{a}	2281^{a}	1390^{b}	2140^{a}
C. glabrata	2462^{a}	2333^{a}	2436^{a}	2257^{a}	1386^{b}	1824^{b}

Equal lowercase letters in the same column denote statistical similarity between the groups for each specie evaluated. (Data were submitted to ANOVA followed by Tukey post-hoc for *C. albicans*, *C. tropicalis*, and *C. glabrata* – $p > 0.05$).

3.1.1. Total biomass assay

The total biomass of biofilms of *C. albicans* and *C. tropicalis* was significantly lower in the group submitted to aPDT with 150 mg/L of PDZ ($p < 0.001$). aPDT significantly reduced the total biomass of biofilms of *C. glabrata* using the two concentrations of PDZ ($p < 0.001$). The highest percentage of biomass reduction was observed in the samples submitted to aPDT mediated by 150 mg/L of the PS associated with 37.5 J/cm^2 of light, which was equivalent to 24.4%, 39.2% and 43.7% in comparison with the P-L- group for *C. albicans, C. glabrata* and *C. tropicalis*, respectively (Table 1).

3.2. Photoinactivation of the dual-species biofilms of Candida spp.

3.2.1. Biofilm constituted of C. albicans + C. tropicalis

Mean values and standard deviation of $\log_{10}$ (CFU/ml) for biofilm constituted of *C. albicans* + *C. tropicalis* assessed for all experimental conditions are shown in Fig. 2. aPDT promoted significant reduction in viability for both species assessed in comparison with the positive control group (P-L-), which was equivalent to 1.04 $\log_{10}$ and 1.14 $\log_{10}$ for *C. albicans* (Fig. 2A) and *C. tropicalis* (Fig. 2B), respectively ($p < 0.001$). Additionally, there was no statistically significant difference between the groups P-L-, P+L- and P-L+ for each species assessed ($p > 0.05$).

3.2.2. Total biomass assay of C. albicans + C. tropicalis

The results obtained from the CV staining showed no significant differences among the groups ($p = 0.060$). Table 2 shows the mean

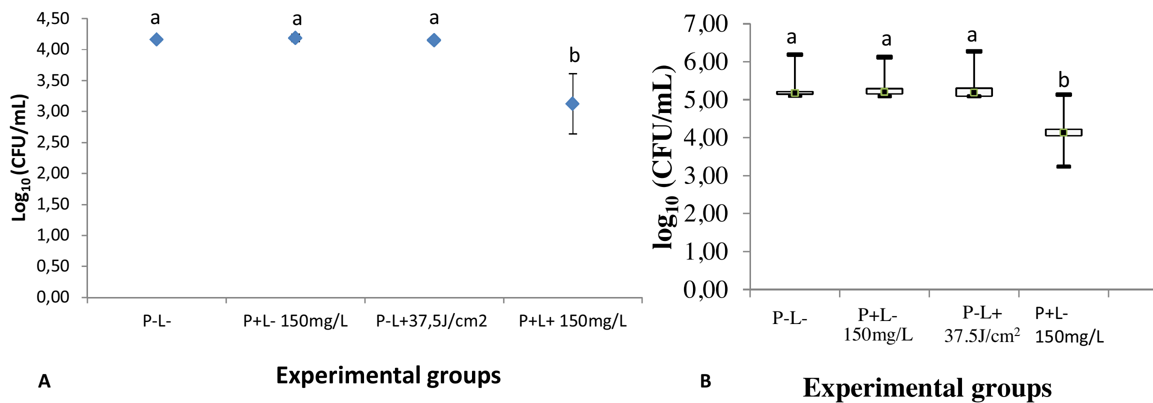

Fig. 2. Log $_{10}$ (UFC/mL) values of the viability of duo-species biofilms constituted of *C. albicans* + *C. tropicalis*. A: Mean values and standard deviation [$\log_{10}$ (CFU/ml)] of the viability of *C. albicans*. Different lower case letters denote significant differences among the groups in comparison with the control (P-L-) ($p < 0.05$). B: Log $_{10}$ (UFC/mL) values obtained in the experimental groups for the strains of *C. tropicalis*. The box plot shows the median (bold square), the 1 st and 3rd quartile (box), and the smallest and highest values obtained (traces). Different lower case letters denote significant differences among the groups in comparison with the control (P-L-) ($p < 0.05$). As the samples were spread on CHROMagar *Candida*, it was possible to identify presumptively each species present in the mixed biofilm for each experimental group. (Data were submitted to ANOVA and Tukey post-hoc for *C. albicans* and Kruskal-Wallis and Dunn post- hoc for *C. tropicalis).*

Table 2
Mean values of absorbance (optical density – OD) obtained after crystal violet assay to the duo-species biofilms of *C. albicans* + *C. tropicalis* for all groups evaluated.

Groups	P-L-	P+L- 150	P-L+	P+L+ 150
Mean values (OD)	2833[a]	2823 [a]	2758 [a]	2606 [a]
Standard Deviation (SD)	±0,21	±0,23	±0,07	±0,10

Equal lowercase letters denote statistical similarity between the groups. (Data were submitted to ANOVA followed by Tukey post-hoc for *C. albicans*, and *C.tropicalis* – $p > 0.05$).

values and standard deviation of the absorbance values obtained in the evaluation of total biofilm biomass (crystal violet assay).

3.2.3. *Biofilm constituted of* C. albicans + C. glabrata

With regard to the biofilm constituted of *C. albicans* + *C. glabrata*, aPDT mediated by 150 mg/L of PDZ associated with LED promoted significant reductions in viability equivalent to 1.04 and 1.27 $\log_{10}$ for *C. albicans* (Fig. 3A) and *C. glabrata* (Fig. 3B), respectively, in comparison with the P-L-group ($p < 0.001$). There was no statistically significant difference between the groups P+L-, P-L+ and P-L- ($p > 0.05$) (Fig. 3).

3.2.4. *Total biomass assay of* C. albicans + C. glabrata

The quantification of the total biofilm biomass formed by *C. albicans* + *C. glabrata* showed that aPDT did not cause significant reductions in total biomass and the mean absorbance values were statistically similar to the groups P-L-, P+L- and P-L+ ($p = 0.070$) (Table 3).

4. Discussion

According to the available literature, few studies have reported the effectiveness of aPDT mediated by PDZ in the inactivation of *Candida* species [27,28,30]. Dovigo et al. [30] assessed the effectiveness of aPDT mediated by PDZ on viability and on cell metabolism of suspensions and single-species biofilms of clinical isolates of *C. albicans, C. glabrata* and *C. tropicalis*. According to the authors, the use of 100 mg/L of PDZ associated with 37.5 J/cm^2 promoted reductions in viability equivalent to 0.9, 1.4 and 1.5 $\log_{10}$ and reductions in cellular metabolism of approximately 62%, 76% and 76.8% for *C. albicans, C. tropicalis* and *C. glabrata*, respectively. In the present study, a higher concentration of PDZ (150 mg/L) was used to promote reduction in fungal viability similar to those reported by Dovigo et al. [30], which were equivalent to 1.2, 1.2 and 0.9 $\log_{10}$ for *C. albicans, C tropicalis* and *C. glabrata*, respectively. These results can be explained by the incorporation of PDZ in hydrogel, which may have reduced its availability, thus reducing its effectiveness. Moreover, it has been reported that the clinical isolates of *Candida* spp. exhibits reduced susceptibility to aPDT when compared with standard strains [21]. In addition, Dovigo et al. [30] reported that the susceptibility to aPDT of 5 clinical isolates of the same species were not homogeneous among each other. It may be explained by the different characteristics of each strain. These findings reinforce the importance of investigating the reference strains, such as ATCC and non-*albicans Candida* species, to search for new antifungal therapies as these strains have a standard behavior. It is important to mention that only one previous investigation reported the effectiveness of aPDT mediated by PDZ diluted in hydrogel, *in vivo* [33]. In this study, mice with induced oral candidiasis were treated with PDZ-mediated aPDT for 5 consecutive days. In accordance with the results, PDZ was as effective as the antifungal Nystatin to inactivate *C. albicans* present in the oral lesions of mice. Moreover, macroscopic analysis showed that 24 h after completion of treatment, all animals submitted to aPDT presented total remission of oral lesions, which was not observed in the group treated with Nystatin. Thus, the effectiveness of PDZ entrapped in hydrogel against *C. albicans in vivo* lead us to evaluate this PS for the photoinactivation of non-*albicans Candida* species in the present investigation.

The results of viability showed that biofilms formed by *C. glabrata* were less susceptible to aPDT. The average number of viable colonies ($\log_{10}$ CFU/ml) for biofilms of *C. glabrata* submitted to aPDT was higher than those observed for other species. This finding corroborates with those observed in other studies, which also found reduced susceptibility of *C. glabrata* to photoinactivation [21,35]. It has been suggested that under unfavorable conditions, *C. glabrata* can develop an alternative respiration pathway due to damage in the nucleus or mitochondria [36]. This alternative

Table 3
Mean values of absorbance (optical density – OD) obtained after crystal violet assay to the duo-species biofilms of *C. albicans* + *C. glabrata* for all groups evaluated.

Groups	P-L-	P+L- 150	P-L+	P+L+ 150
Mean values (OD)	2814 [a]	2776 [a]	2916 [a]	2801 [a]
Standard Deviation (SD)	± 0,14	± 0,15	± 0,20	± 0,40

Equal lowercase letters denote statistical similarity between the groups. (Data were submitted to ANOVA followed by Tukey post-hoc for *C. albicans*, and *C.glabrata* – $p > 0.05$).

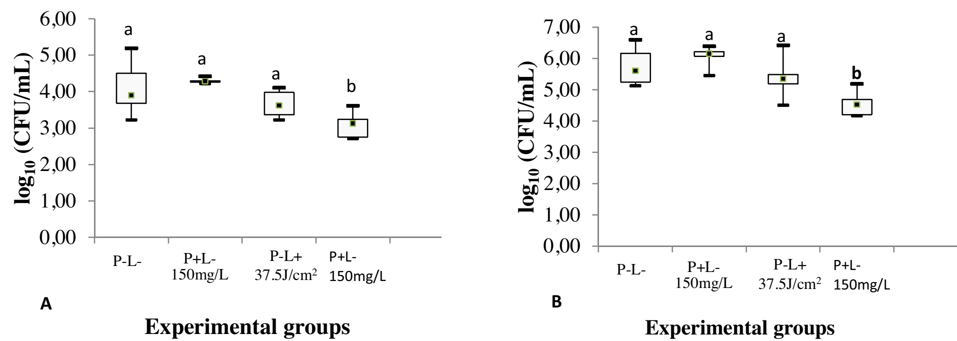

Fig. 3. Log $_{10}$ (UFC/mL) values of the viability of duo-species biofilms constituted of *C. albicans* + *C. tropicalis.* A: Mean values and standard deviation [$\log_{10}$ (CFU/ml)] of the viability of *C. albicans.* Different lower case letters denote significant differences among the groups in comparison with the control (P-L-) ($p < 0.05$). B: Log $_{10}$ (UFC/mL) values obtained in the experimental groups for the strains of *C. glabrata*. The box plot shows the median (bold square), the 1 st and 3rd quartile (box), and the smallest and highest values obtained (traces). Different lower case letters denote significant differences among the groups in comparison with the control (P-L-) ($p < 0.05$). As the samples were spread on CHROMagar *Candida*, it was possible to identify presumptively each species present in the mixed biofilm for each experimental group. (Data were submitted to Kruskal-Wallis followed by Dunn post- hoc for *C. albicans* and *C. glabrata).*

respiratory pathway reduces the fungal metabolism rate, making it less susceptible to antifungal agents in general and to reactive oxygen species (ROS) [36], which provides greater resistance to aPDT action.

The evaluation of total biomass of the single-species biofilms of *C. albicans, C. glabrata* and *C. tropicalis* was assessed by measuring the absorbance of the crystal violet stain. According to the results, the three species showed equivalent total biomass value in the control groups, suggesting that they have a similar capacity for biofilm formation. These results are not in agreement with those found by Dovigo et al. [24] in which the authors observed higher total biofilm biomass of clinical isolates of *C. albicans* in comparison with *C. tropicalis* and *C. glabrata*. The results also showed significant variation in the individual response to aPDT among each species. The combination of 150 mg/L of PDZ associated with LED light produced the greatest reductions in total biomass equivalent to 24.4%, 39.2% and 43.7% in comparison with the P-L- groups for *C. albicans, C. tropicalis* and *C. glabrata*, respectively. These findings suggest that aPDT caused the disruption of biofilms matrix. The highest disruption observed in the biofilm biomass of *C. glabrata* may be explained by the fact that this specie produces a biofilm exclusively composed of blastospores, without the presence of hyphae/pseudohyphae [9], that would act as a structure for the extracellular matrix, facilitating its disruption. Recently, another research showed that aPDT mediated by curcumin also promoted the disruption of biofilms of clinical isolates of *Candida* spp., including *C. glabrata* [24].

The results of the present study related to dual-species biofilms formed by the association of *C. albicans* + *C. tropicalis* and *C. albicans* + *C glabrata* showed that the use of 150 mg/L of PDZ in hydrogel associated with LED light promoted significant reductions in fungal viability equivalent to 1.04 and 1.14 $\log_{10}$ for *C. albicans* and *C. tropicalis* and 1.02 and 1.27 $\log_{10}$ for *C. albicans* and *C. glabrata*, respectively. In the study conducted by Quishida et al. [31], the aPDT mediated by PDZ diluted in saline at a concentration of 175 and 200 mg/L associated with 37.5 J/cm^2 was also effective for the inactivation of mixed biofilms formed by *C. albicans* + *C. glabrata* + *S. mutans*, promoting reductions in viability equivalent to 1.21, 1.19 and 2.39 $\log_{10}$, respectively. It is important to note that the concentration of PDZ required to achieve a significant reduction in viability in the present study (150 mg/L) was lower than that used by Quishida et al. [31] (175 and 200 mg/L). This may have occurred because the cited study assessed multi-species biofilms formed by standard strains of *C. albicans* + *C. glabrata* + *S. mutans* and the interaction among these species resulted in richer and more robust biofilm in the extracellular matrix hindering the action of aPDT.

In accordance with the mean values of $\log_{10}$(CFU/ml) of the control groups for each species, the species *C. tropicalis* and *C. glabrata* showed higher growth than *C. albicans*. These findings corroborates with those reported by Pereira-Cenci et al. [11]. The authors investigated the interactions between *C. albicans* and either *C. glabrata* or *S. mutans* in biofilms grown on various surfaces, with or without saliva. They verified that the values in the control group were higher for *C. glabrata* in comparison with *C. albicans* in biofilms formed by *C. albicans* and *C. glabrata*, which could indicate the occurrence of a synergistic relationship among the species. The synergistic relationship can be characterized as complex interaction of microorganisms, as they may coordinate the use of nutrients and resist to adverse conditions [37] promoting the adhesion of other species [38].

In the present study, aPDT did not cause any reduction in the total biomass of the dual-species biofilms even with the use of the new vehicle for PDZ, such as hydrogel. The penetration of the PS into biofilms seems to have been sufficient to cause the disruption of dual-species biofilms that may have different features than a mono-species biofilm. It is possible to suggest that extracellular matrix hindered the effect of aPDT on the structure of the biofilm. These findings are consistent with those found by Quishida et al. [31] who verified no significant reductions in total biomass of mixed biofilms formed by *C. albicans* + *C. glabrata* + *S. mutans* after application of aPDT mediated by high concentrations of PDZ (175 and 200 mg/L). A recent research conducted by Pathak et al. [39] reported that *C. albicans* is stimulated to produce extracellular matrix in the presence of non-*albicans* species, resulting in a higher amount of total biomass for biofilms formed by *C. albicans* + *C. tropicalis* and *C. albicans* + *C. glabrata*.

Only one clinical study reported the effectiveness of aPDT mediated by the photosensitizer Photogem diluted in solution or hydrogel as a disinfection method of complete dentures [40]. According to the results, the Photogem formulated in hydrogel at a concentration of 100 mg/L was more effective in reducing the biofilm attached to the inner surface of the prosthesis, providing a 60% of reduction of microorganisms when compared with the PS in solution [40]. The results obtained in the present investigation indicate that aPDT mediated by PDZ entrapped in hydrogel was effective in the inactivation of the biofilms evaluated. These findings reinforce that this formulation may facilitates the topical application of the photosensitizer agent, rendering it more clinically feasible.

In summary, given the incidence of Nosocomial candidemia by strains of *Candida* spp, and the results obtained in the present

investigation, it may be concluded that aPDT mediated by PDZ formulated in hydrogel can be effective for inactivating single- and dual-species biofilms of non-*albicans Candida* species, *in vitro*, and it was able to produce the greatest reductions in total biomass for all single-species biofilms tested. In addition, some aspects of the therapeutic approach need to be further investigated, as well as new drug delivery systems of the PS such as liposomes, nanoparticles or nanoemulsions. Likewise, the formulation of PDZ in hydrogel may be considered a good option for this PS to be used in a new animal model infected by non-*albicans Candida* species in order to improve the application of the therapy for clinical trials.

Conflict of interest

All authors declare no conflict of interest

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