Photodiagnosis and Photodynamic Therapy 27 (2019) 124–131

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

DNase increases the efficacy of antimicrobial photodynamic therapy on *Candida albicans* biofilms

Beatriz H.D. Panariello[1], Marlise I. Klein, Fernanda Alves, Ana Cláudia Pavarina*

Department of Dental Materials and Prosthodontics, São Paulo State University (Unesp), School of Dentistry, Araraquara, São Paulo, Brazil

ARTICLE INFO

Keywords:
Candida albicans
Biofilm
Extracellular matrix
Hydrolytic enzymes
aPDT
Antimicrobial photodynamic therapy

ABSTRACT

Antimicrobial Photodynamic Therapy (aPDT) has been proposed as a means to treat *Candida* infections. However, microorganisms in biofilms are less susceptible to aPDT than planktonic cultures, possibly because the matrix limits the penetration of the photosensitizer. Therefore, the goals here were: (1) to target biofilm matrix components of a fluconazole-susceptible (S) and a fluconazole-resistant (R) *C. albicans* (Ca) strains using the hydrolytic enzymes β-glucanase and DNase individually or in combination; (2) to apply the best enzyme protocol in association with aPDT mediated by Photodithazine® (PDZ); (3) to verify under confocal microscope the penetration of PDZ in biofilms pre-treated or not with DNase at different periods of incubation. CaS and CaR 48h-old biofilms were incubated with the hydrolytic enzymes (5 min) and evaluated by cell viability, biomass, and matrix components. DNase showed the best outcomes by significantly reducing extracellular DNA (eDNA) and soluble proteins from the matrix of both strains; and water-soluble polysaccharides from CaR matrix. Subsequently, 48h-old biofilms were incubated with DNase for 5 min, followed by incubation with PDZ for 20 min and exposure to LED light (660 nm, 50 J/cm^2). Controls were biofilms treated only with aPDT without DNase, PDZ only, PDZ + DNase, light only, light + DNase, and biofilm without treatment. Pre-treatment with DNase allowed PDZ penetration into deeper biofilm layers, and the aPDT effect was enhanced, showing a significant reduction of the cell viability ($p = 0.000$) and eDNA amounts ($p \leq 0.047$). DNase affected the matrix composition improving the penetration of the photosensitizer, thereby, improving the effectiveness of subsequent aPDT.

1. Introduction

Candida albicans asymptomatically colonizes the oral cavity, gastrointestinal and reproductive tracts, and the skin of most humans [1]. Nevertheless, changes in the host microbiota, variations in the host immune response or alterations in the local milieu may promote the overgrowth of *C. albicans* and cause infections [1,2]. Most of *Candida* infections are associated with biofilm formation [3]. The biofilm development exhibits the following stages: adherence of planktonic cells to a surface, cell proliferation, hyphal development, production of extracellular matrix (ECM) and, then, biofilm maturation [4]. The ECM is considered essential for the existence of the cells and protects them from the attack by the immune system and antifungals [3,5]. Biochemical analyses have revealed the general composition of *C. albicans* biofilm ECM, which includes polysaccharides, proteins, lipids, and nucleic acids [6]. The ECM mediates adhesive and cohesive interactions, providing mechanical stability to the biofilms [6] and acting as a digestive system that provides a nutrient source for the conglomerate of cells [7].

The resistance of *Candida* biofilms to conventional antifungal drugs is considered multifactorial and is associated to the physiological state of the cells, the activation of drug efflux pumps and the protective effect of the ECM performed by β-glucans [8,9]. Hence, *Candida* biofilms are a reason for concern, since their cells may have reduced vulnerability to azoles and polyenes [10–12]. It has been shown that β-1,6 glucan and α-mannan in the ECM of *C. albicans* biofilms interact to form a mannan-glucan complex (MGCx) [7,13]. This interaction was found to be the key to protect biofilm from drug treatment with fluconazole and amphotericin-B [8,9,13]. Besides that, the extracellular DNA (eDNA) also contributes to the structural integrity of *C. albicans* biofilm and resistance against antifungals [6,14,15].

The antimicrobial Photodynamic Therapy (aPDT) has been studied

* Corresponding Author at: Rua Humaitá, 1680, 14801-903, Araraquara, São Paulo, Brazil.
E-mail address: ana.pavarina@unesp.br (A.C. Pavarina).
[1] Current address: Department of Cariology, Operative Dentistry and Dental Public Health, Indiana University– Purdue University Indianapolis, School of Dentistry, Indianapolis, IN, USA.

https://doi.org/10.1016/j.pdpdt.2019.05.038
Received 13 March 2019; Received in revised form 29 April 2019; Accepted 28 May 2019
Available online 29 May 2019
1572-1000/

as an alternative method to treat *Candida* infections. This treatment is based on the topical application of a photosensitizer (PS) followed by the irradiation with a light source in an appropriated wavelength. The light activates the PS and, in the presence of the oxygen, free radicals and singlet oxygen that are lethal to microorganisms are generated [16].

Photodithazine® (PDZ) is a chlorine e6 derived PS activated by a light in the red region of the spectrum (660 nm) [17]. Chlorine e6 products penetrate deep into tissues/biofilms, and they can produce a high amount of oxygen singlet [17]. The use of PDZ to mediate aPDT eradicated planktonic cultures of *C. albicans* [18]. In contrast, the application of aPDT mediated by PDZ against biofilms has not yet achieved complete eradication of *C. albicans* [18,19]. In vivo studies using PDZ-mediated aPDT to treat mice with induced oral candidiasis demonstrated that one or five consecutive sessions of aPDT significantly reduced *Candida* infection, but the eradication of the microorganism was not observed [20,21]. Therefore, when microorganisms are organized in biofilms, they are less susceptible to aPDT in comparison with their planktonic counterparts, probably because the ECM acts as a physical barrier [6] that limits PS penetration.

As the ECM is recognized as a critical characteristic related to biofilm protection, the use of enzymes that are capable of hydrolyzing polysaccharides and nucleic acid from its structure has been investigated as an alternative way to make *C. albicans* biofilms more susceptible to the available antifungals. Mannan accumulation was blocked with α-mannosidase—an enzyme that catalyzes the hydrolysis of terminal mannosides—enhancing the activity of fluconazole against susceptible *C. albicans* biofilms [13]. It has been demonstrated that 24 -hs exposure to the enzyme β-1, 3 glucanase at 2.5 units/mL eliminated the biofilm process in a rat catheter model [8]. Furthermore, 24 h-old biofilms challenged with RPMI 1640 containing different concentrations of antifungals alone or in combination with DNase showed that the addition of DNase enhanced the susceptibility of *C. albicans* cells to amphotericin B [14]. Moreover, it has been shown that the combination of biofilms with DNase plus amphotericin B and caspofungin significantly improved antifungal susceptibility in *Aspergillus fumigatus* biofilm [15].

Thus, the hypothesis studied here was that the hydrolytic enzymes potential to disturb the ECM could improve the weak PS penetration through biofilms, enhancing aPDT efficacy against *C. albicans* biofilms. Therefore, the goals of the present study were (1) to target ECM components of a fluconazole-susceptible and a fluconazole-resistant *C. albicans* strains using the hydrolytic enzymes β-glucanase and DNase individually or combined; (2) to select the best enzyme protocol to be associated with aPDT mediated by PDZ against biofilms of the fluconazole-susceptible *C. albicans* strain; (3) to verify under confocal microscope the penetration of PDZ in biofilms pre-treated or not with DNase at different periods of incubation.

2. Materials and methods

2.1. Biofilm formation

The microorganisms used for this study were *C. albicans* ATCC 90,028 (fluconazole-susceptible; CaS) and *C. albicans* ATCC 96,901 (fluconazole-resistant; CaR). The microorganisms stored at −80 °C were seeded onto Petri dishes with SDA (Sabouraud dextrose agar) culture medium supplemented with chloramphenicol (50 mg/L) and incubated at 37 °C for 48 h. Next, starter cultures containing about five colonies were grown using YNB medium (Yeast Nitrogen Base- DIFCO, Detroit, Michigan, USA) supplemented with 100 mM of glucose, and incubated at 37 °C. After 16 h of incubation, the starter cultures were diluted with fresh YNB medium supplemented with 100 mM glucose (1:20 dilution). These inoculum cultures were incubated at 37 °C until the two strains reached the mid-log growth phase [22]. Then, the OD_{540nm} of the inoculums was adjusted to reach 10^7 CFU/mL. Next, 1 mL of the inoculum of each strain was individually added to the wells of a 24-well polystyrene plate (Techno Plastic Products- TPP, Trasadingen, Switzerland). The culture plate was incubated at 37 °C under stirring (75 rpm) for cell adhesion to the substrate, which was the bottom of the 24-well polystyrene plates. After 90 min, the wells were washed twice with sterile 0.89% NaCl solution to remove non-adhered cells. Subsequently, one mL of Roswell Park Memorial Institute medium (RPMI 1640) buffered with morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich, St. Louis, Missouri, USA) at pH 7 was added to each well. After 24 h of biofilm formation, the culture medium was removed by aspiration and fresh RPMI 1640 buffered with MOPS (1 mL, pH 7.0) was added to each well, followed by another incubation during 24 h. After 48 h of biofilm formation, the wells were washed twice with 0.89% sterile NaCl solution, and the incubation of enzymes in the biofilms was performed.

2.2. Enzymes application in the biofilms

Stock solutions of β-(1→3)-D-Glucanase from *Helix pomatia* (67138, Sigma-Aldrich, St. Louis, MO, USA) and DNase I from bovine pancreas (AMPD1, Sigma-Aldrich, St. Louis, MO, USA) were freshly prepared before each experiment in 0.1 M sodium acetate buffer (pH 5.5). To disorganize the ECM, but not to destroy it, the concentrations of the enzymes were chosen based on previously determined alkali soluble polysaccharide (ASP), water-soluble polysaccharides (WSP), and eDNA content [22]. Additionally, preliminary tests were performed using the concentrations cited ahead and distinct incubation times (data not shown). The shortest time that indicated hydrolysis of ECM components was 5 min; hence, it was chosen for the study (data not shown). Thus, the final concentrations of the working solutions were 2 units/mL of β-glucanase and 20 μnits/mL of DNase. The enzymes combination (β + DNase) were used in the same final concentrations mentioned above for each enzyme. The control solutions were 0.89% NaCl and 0.1 M sodium acetate buffer (pH 5.5).

After 48 h of biofilm formation, the culture medium was removed; the biofilms were washed twice with 1 mL of 0.89% NaCl solution and 250 μL of enzyme solutions and control solutions were added to the biofilms. The volume of 250 μL is sufficient to cover the biofilm formed in the bottom of 24-well plates and was determined during pilot studies performed before starting the present study (data not shown). Plates were incubated at 37 °C for 5 min. Later, for the groups that DNase was applied, 20 units/mL of stop solution from the DNase I kit (AMPD1, Sigma-Aldrich) were applied to the biofilms. All biofilms, including those of the control groups, were washed twice with 0.89% NaCl solution. Then, the biofilms were removed by scraping each well with a pipette tip and 2 mL of sterile 0.89% NaCl. At this point, biofilms were vortexed and processed according to a previous study [23]. From the biofilm suspension, an aliquot of 0.1 mL was used for 10-fold serial dilution for CFU determination, and an aliquot of 0.1 mL was used for the total biomass determination (total dry-weight) [24], which comprehends cells and all ECM components. The remaining volume (1.8 mL) was centrifuged at 5500 xg for 10 min (4 °C). The supernatant containing the soluble part of the ECM was stored in another tube, and the precipitate with the cells and the insoluble components of the ECM was washed twice with 1.8 mL of sterile milli-Q water (5500xg /10 min/ 4 °C). The detailed methodology for the characterization of *C. albicans* ECM was described before [23]. Briefly, the supernatant (1.8 mL) was separated for quantifying WSP [25], eDNA [26] and proteins [27]. The pellet was resuspended in 1.8 mL of water, of which 0.8 mL was used to calculate the insoluble dry-weight (which includes cells and insoluble ECM components that were not removed during biofilm suspension washes), 0.05 mL for the quantification of total proteins [27] and 0.95 mL for the determination of ASP [25]. All experiments were repeated on three distinct occasions in triplicate (n = 9).

Table 1
Name of the groups and description of the treatments for aPDT treatment in association with DNase.

Name of the groups	Description of the treatments
P + L +	Biofilms incubated with PDZ (200 mg/L for 20 min in the dark), then, illuminated with red LED light (28 min, 50 J/cm^2)
P + L + DNase	Biofilms incubated with DNase I (20 units/mL) for 5 min. DNase was removed and the stop solution (AMPD1, Sigma-Aldrich) was added (20 units/mL), washed twice with saline solution and aPDT treatment was applied
P + L-	Biofilms were incubated only with PDZ (200 mg/L, 20 min)
P + L- DNase	Samples treated with DNase I (20 units/mL) for 5 min and then incubated with PDZ (200 mg/L, 20 min)
P-L +	Biofilms solely illuminated with red LED light (28 min, 50 J/cm^2)
P-L + DNase	Biofilms treated with DNase I for 5 min and illuminated with red LED light (28 min, 50 J/cm2)
P-L-DNase	Biofilms treated with DNase I (20 units/mL) for 5 min
P-L-	Samples that did not receive an application of DNase, PDZ neither illuminated with LED light (control group).

2.3. aPDT treatment in association with DNase

2.3.1. Photosensitizer and light source

Photosensitization of biofilms cells was performed with Photodithazine (PDZ, produced by VETA-GRAND Co., Russia). PDZ has a characteristic absorption peak in 660 nm and was supplied by the Biophotonics Laboratory of the São Carlos Institute of Physics (IFSC), University of São Paulo (USP). PDZ was applied at a concentration of 200 mg/L [19]. Thus, before each experiment, PDZ stock solution (5000 mg/L) was diluted in 0.89% NaCl solution to obtain the final concentrations of 200 mg/L. PDZ was irradiated with a red light emitting diode (LED) device (660 nm) at the dose of 50 J/cm^2. This LED apparatus is composed of red LEDs (LXHL-PR09, Luxeon® III Emitter, Lumileds Lighting, San Jose, California, USA) homogeneously distributed, with a constant power output of 30 mW/cm. Table 1 contains information about irradiation and pre-irradiation time for each experimental group.

2.3.2. Experimental groups

As a proof of concept that the use of DNase may allow the PS to reach the deeper layers of biofilms, the strain *C. albicans* ATCC 90,028 (CaS; fluconazole-susceptible) was selected as a standard for establishing parameters. DNase was applied as described on item 2.2. For this purpose, biofilms were grown at the same conditions as described on item 2.1. After 48 h of biofilm formation, the culture medium was removed and biofilms were washed twice with 0.89% NaCl solution. Then, biofilms were divided into the study groups (n = 9) described in Table 1.

2.3.3. Evaluation of the treatments

After the application of treatments, ECM was characterized as previously described in the literature [22,23], the viability of *Candida* cells was quantified (CFU/mL), as well as the dry-weight (mg) of the biofilms. Moreover, PDZ penetration was observed under a confocal laser scanning microscope (CLSM).

To verify the penetration of PDZ in biofilms pre-treated or not with DNase (20 units/mL) for 5 min, images were obtained under CLSM at the incubation periods of 10, 20 and 30 min with PDZ (200 mg/L). After each incubation time, biofilms were washed twice with 0.89% NaCl and incubated for 30 min at 37 °C with 100 μg/mL of conjugated fluorophore Concanavalin Alexa Fluor 488 (CON-A) prepared in PBS. CON-A has an affinity for the polysaccharide cell wall and shows the contour of the fungal cell wall in green color. In contrast, PDZ *per se* emits red fluorescence. After 30 min incubation, the fluorophore was removed, and biofilms were washed twice with 0.89% NaCl. As controls, *C. albicans* ATCC 90,028 biofilms images without incubation with PDZ were obtained with and without the pre-treatment with DNase. Biofilms formed in the bottom of 24-well polystyrene plates were then examined under a CLSM inverted microscope (Zeiss LSM 800 Confocal, Zeiss) using a 553 nm–700 nm filter for CON-A fluorophore and 483 nm–560 nm filter for PDZ detection [17].

Three-dimensional images were acquired using Zeiss software with 20x magnification at regular optical intervals across a depth of biofilms to create z-stacks. The evaluated area was 98,463,817 μm^2. Different fluorophores can be detected at different channels in CLSM. Con-A was detected in channel 1, and PDZ was detected in channel 2. For each group, the percentage of PDZ fluorescence was calculated based on the values obtained from channels 1 and 2. Two biofilms of each experimental group were evaluated, and one image of each was obtained.

2.4. Statistical analyses

Normality and homogeneity of variances were verified by Shapiro-Wilk and Levene's test, respectively. Data that met the assumption of normality were analyzed by one-way ANOVA with Tukey post-hoc test for homoscedastic data and Games-Howell post-hoc test for heteroscedastic data ($\alpha = 0.05$). Data that did not meet the assumption of normality were rank-transformed and analyzed by the Kruskal-Wallis test ($\alpha = 0.05$). If significant differences ($p < 0.05$) were found, pairwise multiple comparison procedures (Dunn test) were performed ($\alpha = 0.05$). Analyzes were performed using SPSS software (IBM® SPSS® Statistics, version 20, Chicago, IL, USA).

3. Results

3.1. Enzymes screening

Fig. 1 shows WSP (μg) data for ***CaR***. It was observed that the ***CaR*** has a significantly higher quantity of WSPs than ***CaR*** + ***DNase*** ($p = 0.010$) and ***CaR*** + ***β*** + ***DNase*** ($p = 0.023$). The same was observed for ***CaR*** + ***Buffer***, which has statistically higher WSP than, ***CaR*** + ***DNase*** ($p = 0.002$) and ***CaR*** + ***β*** + ***DNase*** ($p = 0.004$). Fig. 2 represents the eDNA (μg) for ***CaS*** and ***CaR***. It was observed that eDNA content was significantly reduced by DNase (***CaS*** + ***DNase***) when compared to the control groups ***CaS*** ($p = 0.020$) and ***CaS*** + ***Buffer*** ($p = 0.015$). In ***CaR***, statistical analysis showed that the presence of the enzyme DNase (***CaR*** + ***DNase***) significantly reduced the amount of eDNA in comparison to the control ***CaR*** ($p = 0.002$). In addition, ***CaR*** + ***β*** + ***DNase*** ($p = 0.001$) also showed significantly lower amounts of eDNA compared to the control ***CaR***. Besides, the enzyme DNase (***CaR*** + ***DNase***) caused a significant reduction of eDNA in comparison to ***CaR*** + ***Buffer*** ($p = 0.044$). Furthermore, the group ***CaR*** + ***β*** + ***DNase*** ($p = 0.045$) also presented significantly lower amounts of eDNA than the group ***CaR*** + ***Buffer***.

Soluble proteins (μg) data for ***CaS*** and ***CaR*** are represented in Fig. 3. It was observed that all enzymes studied, individually or in combination, significantly reduced the amount of soluble proteins compared to the control group ***CaS*** ($p \leq 0.004$). Likewise, statistical analysis of ***CaR*** soluble proteins data showed that there was a significant reduction in the amount of soluble proteins in all groups treated with enzymes (individually or in combinations) when compared to the control groups ***CaR*** ($p \leq 0.002$) and ***CaR*** + ***Buffer*** ($p \leq 0.004$).

No statistical differences were observed for fluconazole-susceptible *C. albicans* strain in Log_{10} (CFU/mL), biomass (mg), insoluble biomass

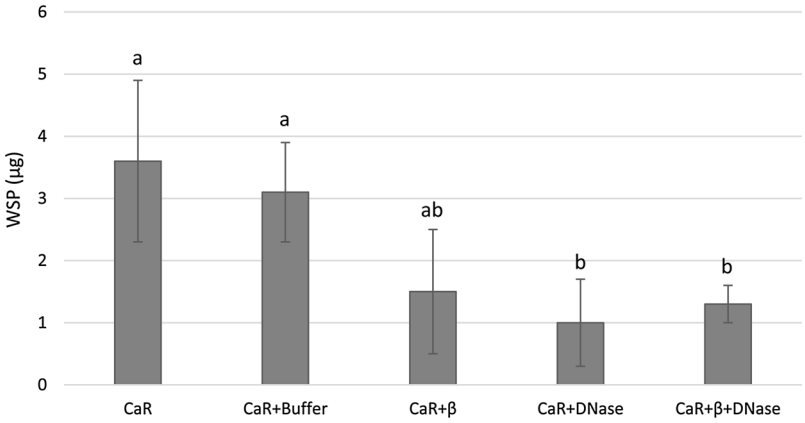

Fig. 1. Means and standard deviations of WSP (µg) of CaR after exposure to β-glucanase (β), DNase and the combination of these enzymes. 48 h-old biofilms were incubated at 37 °C for 5 min with 2 units/mL of β-glucanase, 20 units/mL of DNase and with the combination of these enzymes (β + DNase) following the same individual concentrations of each enzyme. Equal letters mean statistical correspondence ($p > 0.05$).

(mg), proteins (µg), ASP (µg) and WSP (µg) values between the groups treated or not with enzymes individually or combined ($p > 0.05$). For the fluconazole-resistant strain, no statistical differences were observed for Log_{10} (CFU/mL), biomass (mg), insoluble biomass, proteins (µg) and ASP (µg) between the groups ($p > 0.05$). Supplementary material on Tables S1 and S2 show the aforementioned data.

3.2. aPDT treatment in association with DNase

Regarding CaS viability after treatments (Log_{10} CFU/mL), it was observed that ***P-L-*** and ***P-L-DNase*** groups were statistically similar ($p = 0.159$) (Fig. 4 A). The group ***P + L + DNase*** presented the highest Log reduction in comparison to all the other groups ($p = 0.000$). Compared to ***P-L-***, this reduction was equivalent to 35.1%, corresponding to 2.16 Log_{10}; and compared ***P + L+***, this reduction was 13.7%, corresponding to 0.84 Log_{10}. When comparing the group submitted to aPDT (***P + L+***) with the respective control (***P-L-***), it was observed a significant reduction in the number of viable cells ($p = 0.000$), which was 21.5%, equivalent to 1.32 Log_{10} reduction. ***P + L-DNase*** showed a significant decrease in viability (0.43 Log_{10}) compared to ***P-L-*** ($p = 0.003$). However, ***P + L-DNase*** exhibited a higher number of viable cells than ***P + L + DNase*** ($p < 0.001$) and ***P + L+*** ($p < 0.001$). In contrast, the number of viable cells of ***P + L-*** is statistically comparable to ***P-L-*** group ($p = 0.712$). ***P-L + DNase*** also showed a significant reduction in cell viability compared to ***P-L-*** ($p < 0.001$). Finally, ***P-L+*** ($p = 0.598$) and ***P + L-*** groups ($p = 1.000$) were statistically similar to ***P-L-***.

Concerning total biomass (Fig. 4 B), the group with the greatest reduction compared to all the others was ***P-L + DNase*** ($p \leq 0.039$), and it was statistically comparable to ***P-L+*** ($p = 0.057$). In contrast, there were no statistically significant differences among the groups regarding the insoluble biomass ($p > 0.05$) (Fig. 4 C). Regarding proteins from the matrix, the group ***P + L-DNase*** showed higher amounts of this component compared to ***P-L-***, ***P + L+***, ***P + L + DNase*** ($p < 0.03$) (Fig. 4 D).

ASP amount in ***P + L-*** was significantly higher than the other groups ($p < 0.017$), however, it is statistically comparable to ***P + L-DNase*** ($p = 0.162$) (Fig. 5 A). ASP amount of the other groups was statistically similar ($p > 0.218$). Regarding WSP amount (Fig. 5 B), the group ***P-L + DNase*** showed the most significant reduction of this component compared to ***P-L-*** and ***P-L-DNase*** ($p = 0.000$), followed by ***P-L + ($p < 0.002$)***, and ***P + L-*** ($p < 0.032$). However, all groups showed a significant reduction compared to ***P-L-DNase*** and ***P-L-*** ($p \leq 0.032$).

Regarding eDNA (Fig. 5 C), the highest reduction was observed in the ***P + L + DNase*** group, being this reduction statistically significant in comparison to all the other groups ($p \leq 0.047$). Moreover, the presence of DNase significantly reduced eDNA amounts in the group ***P-L-DNase*** compared to ***P-L-*** ($p = 0.018$).

As shown in Fig. 5 D the soluble proteins amount from ***P-L-DNase*** group was significantly smaller than the other studied groups ($p \leq 0.020$). In contrast, regarding insoluble protein amounts (Fig. 4 D), the group ***P + L-DNase*** showed substantially higher amounts of this component than ***P-L-*** ($p = 0.003$), ***P + L + DNase*** ($p < 0.001$) and ***P + L+*** ($p = 0.003$). The other groups presented a similar amount of insoluble protein ($p > 0.05$).

Fig. 6 shows biofilm images obtained under CLSM. At 10 min of incubation with PDZ without pre-treatment with DNase, the mean fluorescence intensity value emitted by CON-A was 40.932 and the mean fluorescence intensity value emitted by PDZ was 13.316. When comparing with the control group, it represents 32.5% of PDZ detection. In contrast, when pre-treatment with DNase was applied, the mean fluorescence intensity value emitted by CON-A was 43.876 and the emission by PDZ was 29.740, which is equivalent to 67.8% of PDZ detection. Thus, when *C. albicans* cells were incubated with PDZ for 10 min, the pre-treatment with DNase increased the PDZ detection by 35.2% in comparison to the non-pretreated biofilm.

After 20 min of incubation with PDZ, the group not treated with DNase presented fluorescence mean intensity value emitted by CON-A of 43,006 and the mean PDZ intensity was 12,122. This is equivalent to 28.2% of PDZ detection when compared to the control group. In contrast, the group pre-treated with DNase presented mean of fluorescence intensity value emitted by CON-A of 38.021 and mean PDZ intensity of 24,436. It represents PDZ detection of 64.3% when the pre-treatment with DNase was applied. Thus, the presence of DNase increased the PDZ

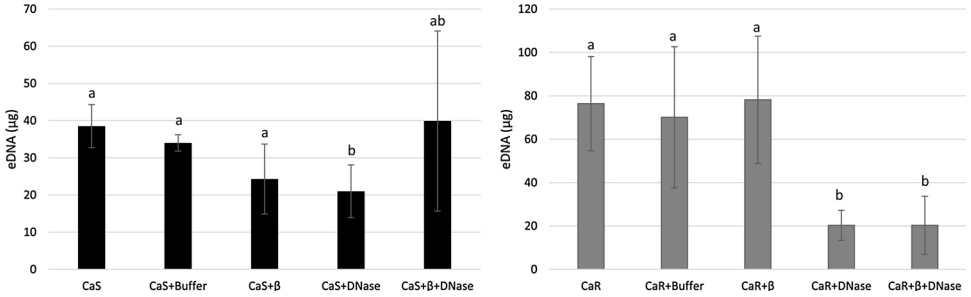

Fig. 2. Means and standard deviations of eDNA (µg) of CaS and CaR after exposure to β-glucanase (β), DNase and the combination of these enzymes. 48 h-old biofilms were incubated at 37 °C for 5 min with 2 units/mL of β-glucanase, 20 units/mL of DNase and with the combination of these enzymes (β + DNase) following the same individual concentrations of each enzyme. Equal letters mean statistical correspondence ($p > 0.05$).

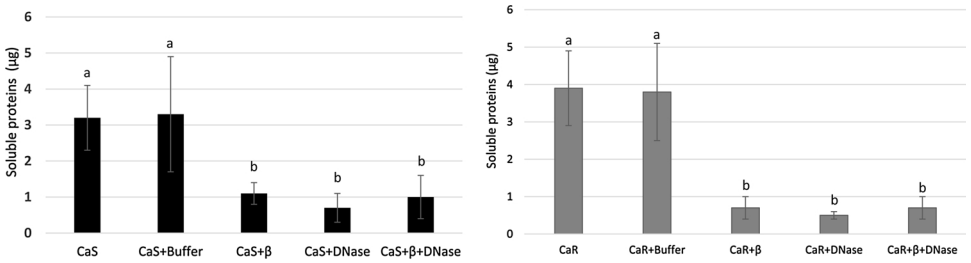

Fig. 3. Means and standard deviations of soluble proteins (µg) of CaS and CaR after exposure to β-glucanase (β), DNase and the combination of these enzymes. 48 h-old biofilms were incubated at 37 °C for 5 min with 2 units/mL of β-glucanase, 20 units/mL of DNase and with the combination of these enzymes (β + DNase) following the same individual concentrations of each enzyme. Equal letters mean statistical correspondence ($p > 0.05$.

detection is 36.1%, after 20 min of PDZ incubation.

After 30 min of incubation with PDZ without DNase pre-treatment, the mean value of fluorescence intensity emitted by CON-A was 47.046 and the mean PDZ intensity was 13.594. This outcome represents 28.9% of PDZ detection. In contrast, the pre-treatment with DNase resulted in a mean of fluorescence intensity value emitted by CON-A of 46,092 and a mean of PDZ intensity of 36,358. This represents 78.9% of PDZ detection on the biofilm. Therefore, after 30 min of incubation with PDZ, the pre-treatment with DNase increased the detection of PDZ by 50% compared to the biofilms that were not pre-treated.

4. Discussion

Based on the importance of the ECM for the protection of *Candida* cells in the biofilm [6], the present study focused on finding alternatives to open pathways through the ECM that may improve the action of antifungal therapies. The intention was to disorganize the ECM using hydrolytic enzymes. Overall, the quantities of ECM components are similar between the fluconazole-susceptible and -resistant strains studied, as shown previously [22]. However, the response to short time exposure to distinct hydrolytic enzymes and combinations of these enzymes yielded distinct patterns of ECM components amounts, indicating possible structural differences in the linkages of ECM building blocks that may contribute to *C. albicans* strains innate resistance to conventional antifungal drugs.

DNase was chosen for hydrolysis of the eDNA content from the ECMs of both strains, and β-glucanase was chosen for the hydrolysis of β-1,6-glucan. However, the presence of DNase alone and in combination with β-glucanase had an effect of reducing WSP in CaR. DNase also reduced soluble proteins from CaS and CaR matrices. Therefore, it is possible that polysaccharides and proteins are tangled with eDNA in the ECM of these biofilms, and when the enzyme acts on eDNA, it can also happen a reduction of the components intertwined to it. The reduction of soluble protein and WSP content by DNase indicates that eDNA might be linked to and/or enclosed by soluble and insoluble polysaccharides in this strain. It has been described that the secretion of β-1,6-glucan may be only possible because of its tight physical interaction with water-soluble mannans [7]. Moreover, the polysaccharides in *C. albicans* ECM are physically associated in the mannan-glucan complex (MGCx) [7,13]. Thus, it is hypothesized that DNase hydrolysis of eDNA breaks down the organization of complexes of eDNA-polysaccharides, consequently decreasing the amount of WSP in which eDNA molecules are attached to. However, none of the enzymes or combinations of them altered WSP content in the ECM of CaS or ASP in both strains. Apparently, the physical interaction between polysaccharides in the MGCx complex is different in each strain.

Considering the importance of eDNA to the ECM of *C. albicans*, contributing to biofilm structural integrity and resistance against antifungals [13–15], targeting eDNA via DNase demonstrated a positive result in the present study, as this enzyme significantly reduced the eDNA amounts in biofilms of both fluconazole-susceptible and -resistant strains. Moreover, the combination of DNase with β-glucanase was also effective in reducing the eDNA amounts in CaR. This result reinforces the possibility that eDNA is linked to and/or surrounded by the components of the MGCx complex in the ECM of *C. albicans* strains.

Proteins in the ECM may operate as an outer digestive structure that disrupts extracellular biopolymers as an energy source [7]. In the present study, the soluble protein (from the ECM) content was significantly reduced by the treatment with the enzymes alone and with the combinations of them, both in *C. albicans* strains used. In contrast, none of the enzymes or combinations of them reduced the total protein amount in fluconazole-susceptible and -resistant *C. albicans* biofilms. The total protein content includes proteins from the ECM that were not removed during the biofilm washes and proteins from the cells per se. Thus, as

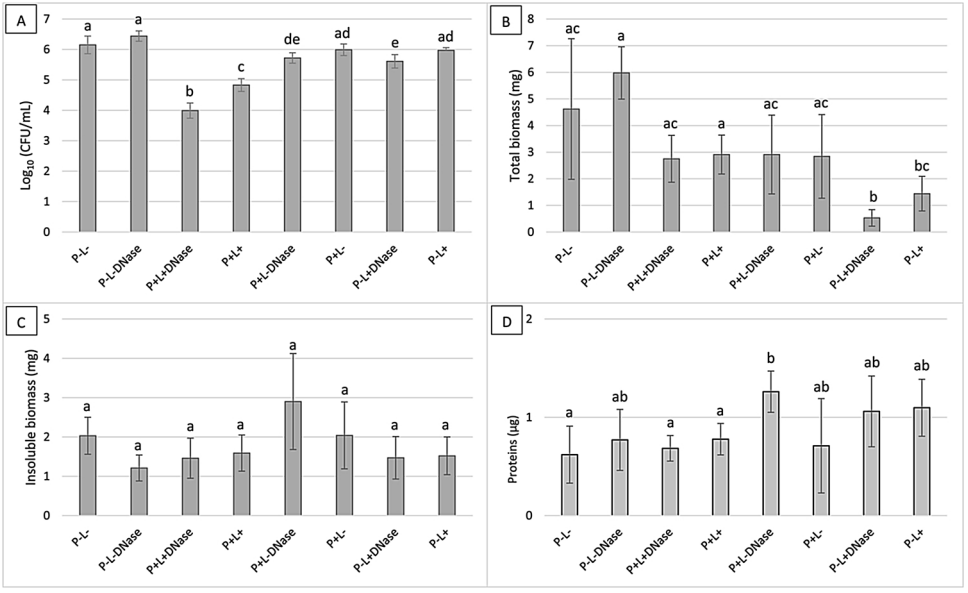

Fig. 4. Data are means and standard deviations of A) Log10 (CFU/mL), B) total biomass (mg), C) insoluble biomass (mg) and D) proteins from the insoluble part (µg) from fluconazole-susceptible *C. albicans* biofilms after the following treatments: ***P* + *L*+:** biofilms incubated with PDZ (200 mg/L, 20 min) and illuminated with red LED light (28 min, 50 J/cm2); ***P* + *L* + *DNase***: biofilms incubated with DNase (20 units/mL, 5 min) and treated with aPDT (as described in ***P* + *L*+**); ***P* + *L-*:** biofilms incubated only with PDZ; ***P* + *L- DNase***: biofilms treated with DNase and incubated with PDZ; ***P-L*+**, biofilms solely illuminated with red LED light; ***P-L* + *DNase***: biofilms treated with DNase and illuminated with red LED light; ***P-L-DNase***: biofilms treated solely with DNase; ***P-L-***: samples that did not receive any treatment (control group). Equal letters denote statistical similarity between the factors evaluated ($p > 0.05$).

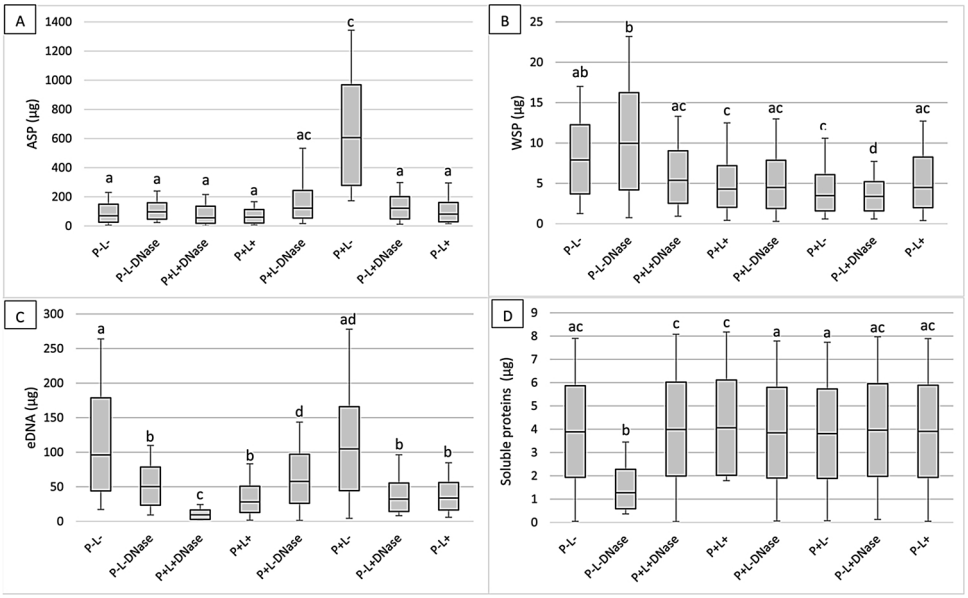

Fig. 5. Box-plot of A) ASP (µg), B) WSP (µg), C) eDNA (µg) and D) soluble proteins (µg) from fluconazole-susceptible *C. albicans* biofilms' ECMs after the following treatments: ***P + L+:*** biofilms incubated with PDZ (200 mg/L, 20 min) and illuminated with red LED light (28 min, 50 J/cm2); ***P + L+DNase***: biofilms incubated with DNase (20 units/mL, 5 min) and treated with aPDT (as described in ***P + L +***); ***P + L-:*** biofilms incubated only with PDZ; ***P + L-DNase***: biofilms treated with DNase and incubated with PDZ; ***P-L+***, biofilms solely illuminated with red LED light; ***P-L + DNase***: biofilms treated with DNase and illuminated with red LED light; ***P-L-DNase***: biofilms treated solely with DNase; ***P-L-***: samples that did not receive any treatment (control group). Equal letters denote a statistical similarity between the factors evaluated ($p > 0.05$).

none of the enzymes or combinations of them acted on the population of fluconazole-susceptible and -resistant strains, the unaltered quantity of total protein might be related to the unchanged number of cells. It has been demonstrated that proteins adhere to polysaccharides in the ECM [29,30]. Whereas proteases would certainly affect those proteins, which interact with polysaccharides within biofilms, it has been demonstrated that polysaccharases and polysaccharide lyases have a much greater effect [28,29,31]. The results of the present study support these previous observations, as β-glucanase and DNase reduced soluble proteins. Moreover, the present study demonstrates that the ECM components (polysaccharides, eDNA, and proteins) are interconnected in the ECM of fluconazole-susceptible and -resistant strains.

Overall, DNase treatment showed satisfactory outcomes decreasing eDNA and soluble proteins in both fluconazole-susceptible and -resistant *C. albicans* biofilms and reducing WSP in fluconazole-resistant strain. Moreover, DNase is cheaper, and its application is more practical than β-glucanase. Thus, DNase treatment might be an adjuvant to antibiofilm therapies, since it reduces most of ECM components that can hinder antifungal drug penetration into biofilms without interfering in the cell viability.

Based on the conclusions above, DNase was selected to test the hypothesis that degrading ECM components may improve the efficacy of aPDT mediated by Photodithazine® in fluconazole-susceptible *C. albicans* biofilms. Confirming this hypothesis, the group ***P + L + DNase*** presented greater Log_{10} reduction than the other groups ($p = 0.000$), corresponding to 2.16 Log_{10} (35.1%) when compared to the control group ***P-L-***. In contrast, group ***P + L+*** presented a significant decrease in cell viability compared to the control group, corresponding to 1.32 Log_{10}. It has been reported that PDZ-mediated aPDT promotes a significant reduction in the viability of *Candida* strains [18,21,32].

A reduction equivalent to 0.9 Log_{10} in clinical isolates of *C. albicans* was observed when biofilms were treated with 125 mg/L of PDZ and irradiated with LED light (37.5 J/cm^2) [18]. Later, it was verified that the use of PDZ at 100 mg/L and 37.5 J/cm^2 of LED light promoted a reduction of 1.01 Log_{10} in *C. albicans* ATCC 90,028 [32], the same strain evaluated here. Finally, PDZ at the concentration of 150 mg/L and a light dose of 37.5 J/cm^2 caused a reduction of 1.2 log_{10} in the cell viability of *C. albicans* ATCC 90,028 [21], a result very similar to this study. For this reason, the greater reduction observed in the group ***P + L + DNase*** in the present investigation may be because DNase would favor the penetration of PDZ and light through the biofilms' ECM leading to higher efficacy of the treatment. Indeed, the presence of

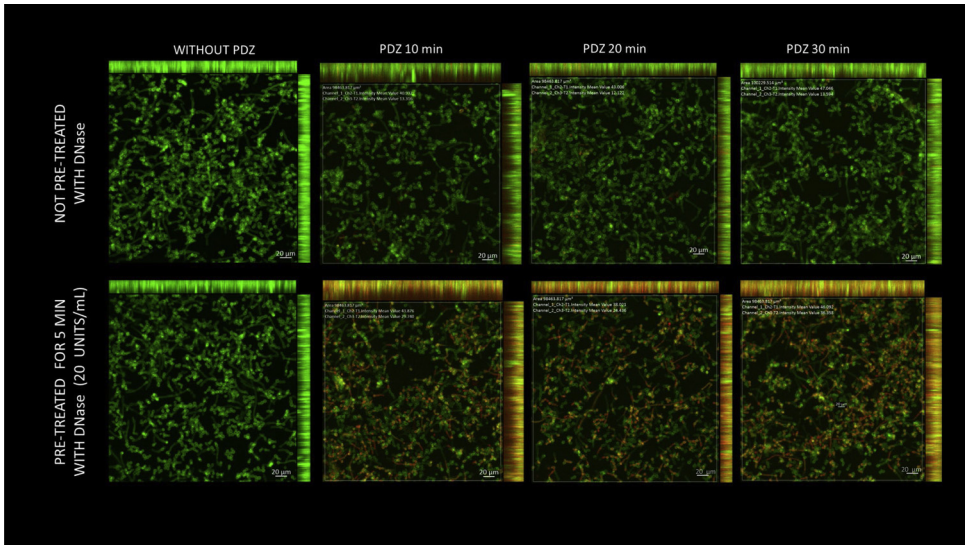

Fig. 6. Images of fluconazole-susceptible *C. albicans* biofilms obtained by CLMS. Upper images show biofilms that were not pre-treated with DNase and bottom images show biofilms that were pre-treated with DNase I (20 units/mL) for 5 min. Biofilms were incubated for 10, 20 and 30 min with PDZ (200 mg/L), except for the controls, and examined under a CLSM microscope (Zeiss LSM 800 Confocal, Zeiss) using a 553 nm–700 nm filter for CON-A fluorophore and 483 nm–560 nm filter for PDZ detection. CON-A has an affinity for polysaccharide and shows the contour of the fungal cell wall in green color (Channel 1). PDZ emits red color (Channel 2). Multidimensional imaging of the cells can be observed at different depths of the biofilms (z-stacks). The calculation of the percentage of PDZ fluorescence was based on the values obtained from channel 1 and channel 2.

DNase increased the penetration of PDZ into *C. albicans* biofilms, as shown in CLSM images (Fig. 7).

The separate application of PDZ (***P + L-***) or light (***P-L+***) did not show to reduce the number of viable colonies compared to the control ***P-L-***. Inversely, when DNase was associated with PDZ or light (***P + L-DNase*** and ***P-L+DNAse*** groups, respectively), a significant reduction in the cell viability was observed in comparison to ***P-L-***. A previous study demonstrated that the isolated application of PDZ or light did not reduce cell viability of planktonic *C. albicans* cells [33]. In contrast, the application of red light for at least 1 min reduced the CFU of *C. albicans* biofilms [34].

Pre-treatment with DNase increased PDZ penetration into the biofilm in all evaluated periods. PS uptake by microorganism cells is crucial for the effectiveness of aPDT since singlet oxygen and hydroxyl radicals have a short half-life, and only molecules and structures that are proximal to the area of their production (*i.e.*, areas of PS localization) are directly affected by aPDT and, subsequently, destroyed [35]. To determine if disruption of *C. albicans* biofilms was solely due to photoinactivation of biofilm-associated cells or also involved the extracellular matrix of the biofilm, the effect of aPDT on the components of the ECM was measured, associated or not with DNase.

It was observed that the total biomass of ***P-L+DNase*** and ***P-L+*** groups were lower than those obtained in the other groups, which were statistically similar among them, and that eDNA and WSP amounts in ***P-L+DNase*** and ***P-L+*** are significantly lower than the controls ***P-L-*** and/or ***P-L-DNase***. Thus, the presence of light reduced the soluble components (WSP, eDNA) of the ECM, leading to a significant decrease of the total biomass in the ***P-L+DNase*** and ***P-L+*** groups. Previous studies reported that light could reduce biofilm biomasses by reducing ECM components [34,36]. The effects of twice-daily blue light treatment (72 J/cm^2) on the dry-weight and soluble and insoluble exopolysaccharides of *Streptococcus mutans* UA159 biofilms have been evaluated, and it was found that insoluble exopolysaccharides and dry-weight were significantly reduced after light application [36]. Moreover, red and blue light treatment without a photosensitizer prevented *C. albicans* biofilm development by significantly reducing viable colony count and biomasses, and there was a slight tendency of reduction of soluble and insoluble exopolysaccharides by the lights [34]. Therefore, our results corroborate with previous studies that showed that the lights by itself influence biofilms and their ECMs.

There was no alteration in the ASP amounts, except for the ***P + L-*** group, which showed higher amounts of ASP compared to the other groups. It was also observed higher amounts of soluble proteins in ***P +L-DNase*** group. Since there was no light activation of the photosensitizer for these groups, one possible explanation for these results is that the photosensitizer could have penetrated the ECM and had its ASP quantified by the phenol-sulfuric acid test, and also its proteins might have been quantified in the Bradford test. A noteworthy finding of this study is that the pre-treatment with DNase for 5 min followed by aPDT (***P+L+DNase*** group) significantly reduced the eDNA content of CaS biofilms in comparison to all the other evaluated groups. This result is important since eDNA is related to the physical integrity and resistance to antifungals in *C. albicans* biofilms [14–16]. Previous studies have evaluated the effects of aPDT on the ECM components on different biofilms. The eDNA of *Pseudomonas aeruginosa* PAO1 biofilms after aPDT mediated by a cationic porphyrin was quantified [37]. The authors observed a decrease in biofilm eDNA of samples treated with aPDT and concluded that the disruption of *P. aeruginosa* PAO1 biofilms by aPDT was partially due to its effect on the eDNA matrix and not just by the photoinactivation of the cells within the matrix [37].

Enzymes can disturb the ECM arrangement enabling the detachment of biofilm, but an additional method is necessary to target the detached cells [38]. The aPDT parameters used in this study have been successfully applied clinically [39], which means that this amount of treatment time is clinically acceptable. However, within the limitations of this in vitro study, here we provide a method to improve aPDT results and reduce the treatment time for further clinical application. Enzymes can be applied as a synergistic assistant to aPDT to improve the photosensitizer penetration, consequently improving aPDT outcomes and reducing its time of application.

In conclusion, the reduction of biofilm ECM components is relevant, since it can open pathways in that could facilitate the diffusion of antifungal agents into the biofilm, overcoming the resistance derived from ECM components. Moreover, the reduction of extracellular polysaccharides and protein content by DNase indicate that these ECM components are intertwined with eDNA. Therefore, the exposure of 48h-old biofilms for 5 min to DNase reduces eDNA, polysaccharides, and soluble proteins from the ECMs of fluconazole-susceptible and -resistant *C. albicans*, being a promising adjuvant to anti-biofilm therapies. The association of enzymes to enhance aPDT effectiveness is a promising strategy to be further investigated.

Acknowledgments

This work was supported by the São Paulo Research Foundation (FAPESP, grant # 2014/18804-1 and # 2013/07276-1]; and by the National Institute in Basic Optics and Applied to Life Sciences (FAPESP grant # 2014/50857-8 and National Counsel of Technological and Scientific Development – CNPq grant # 465360/2014-9).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pdpdt.2019.05.038.

References

[1] C.J. Nobile, A.D. Johnson, Annu. Rev. Microbiol. 69 (2015) 71.
[2] B.J. Kullberg, A.M. Oude Lashof, Eur. J. Med. Res. 7 (2002) 183.
[3] C.G. Pierce, T. Vila, J.A. Romo, D. Montelongo-Jauregui, G. Wall, A. Ramasubramanian, J.L. Lopez-Ribot, J. Fungi (Basel) 3 (2017) 14.
[4] J.R. Blankenship, A.P. Mitchell, Curr. Opin. Microbiol. 9 (2006) 588.
[5] S.S. Branda, S. Vik, L. Friedman, R. Kolter, Trends Microbiol. 13 (2005) 20.
[6] K.F. Mitchell, R. Zarnowski, D.R. Andes, PLoS Pathog. 12 (2016) e1005828.
[7] R. Zarnowski, W.M. Westler, G.A. Lacmbouh, J.M. Marita, J.R. Bothe, J. Bernhardt, A. Lounes Hadj Sahraoui, J. Fontaine, H. Sanchez, R.D. Hatfield, J.M. Ntambi, J.E. Nett, A.P. Mitchell, D.R. Andes, MBio 5 (2014) e01333–14.
[8] J. Nett, L. Lincoln, K. Marchillo, R. Massey, K. Holoyda, B. Hoff, M. Van Handel, D. Andes, Antimicrob. Agents Chemother. 51 (2007) 510.
[9] G. Vediyappan, T. Rossignol, C. d'Enfert, Antimicrob. Agents Chemother. 54 (2010) 2096.
[10] M.A. Pfaller, D.J. Diekema, Clin. Microbiol. Rev. 20 (2007) 133.
[11] H.F. Tsai, L.R. Sammons, X. Zhang, Antimicrob. Agents Chemother. 54 (2010) 3308.
[12] R.S. Shapiro, N. Robbins, L.E. Cowen, Microbiol. Mol. Biol. Rev. 75 (2011) 213.
[13] K.F. Mitchell, R. Zarnowski, H. Sanchez, J.A. Edward, E.L. Reinicke, J.E. Nett, A.P. Mitchell, D.R. Andes, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) 4092.
[14] M. Martins, P. Uppuluri, D.P. Thomas, I.A. Cleary, M. Henriques, J.L. Lopez- Ribot, R. Oliveira, Mycopathologia 169 (2012) 323.
[15] R. Rajendran, L. Sherry, D.F. Lappin, C.J. Nile, K. Smith, C. Williams, C.A. Munro, G. Ramage, BMC Microbiol. 14 (2014) 303.
[16] M.R. Hamblin, Curr. Opin. Microbiol. 33 (2016) 67.
[17] J. Ferreira, P.F.C. Menezes, C. Kurachi, C. Sibata, R.R. Allison, V.S. Bagnato, Laser Phys. Lett. 5 (2008) 156.
[18] L.N. Dovigo, J.C. Carmello, M.T. Carvalho, E.G. Mima, C.E. Vergani, V.S. Bagnato, A.C. Pavarina, Biofouling 29 (2013) 1057.
[19] C.C. Quishida, E.G.O. Mima, L.N. Dovigo, J.H. Jorge, V.S. Bagnato, A.C. Pavarina, Lasers Med. Sci. 30 (2015) 2303.
[20] J.C. Carmello, F. Alves, F. Basso, C.A. de Souza Costa, V.S. Bagnato, E.G.O. Mima, A.C. Pavarina, PLoS One 11 (6) (2016) e0156947.
[21] J.C. Carmello, L.N. Dovigo, E.G.O. Mima, J.H. Jorge, C.A. de Souza Costa, V.S. Bagnato, A.C. Pavarina, Photochem. Photobiol. Sci. 14 (2015) 1319.
[22] B.H.D. Panariello, M.I. Klein, E.G.O. Mima, A.C. Pavarina, J. Oral Microbiol. 10 (2018) 1476644.
[23] B.H.D. Panariello, M.I. Klein, A.C. Pavarina, S. Duarte, J. Oral Microbiol. 9 (2017) 1385372.
[24] H. Koo, M.F. Hayacibara, B.D. Schobel, J.A. Cury, P.L. Rosalen, A.M. Park, Vacca-Smith, W.M. Bowen, J. Antimicrob. Chemother. 52 (2003) 782.
[25] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Anal. Chem. 28 (1956) 350.
[26] K.C. Rice, E.E. Mann, J.L. Endres, E.C. Weiss, J.E. Cassat, M.S. Smeltzer, K.W. Bayles, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 8113.

[27] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[28] I.W. Sutherland, Carbohydr. Polym. 38 (1999) 319.
[29] I.W. Sutherland, Microbiology 147 (2001) 3.
[30] P.M. Bales, E.M. Renke, S.L. May, Y. Shen, D.C. Nelson, PLoS One 8 (2013) e67950.
[31] K.A. Hughes, I.W. Sutherland, J. Clark, M.V. Jones, J. Appl. Microbiol. 85 (1998) 583.
[32] F. Alves, E.G.O. Mima, R.C.P. Passador, V.S. Bagnato, J.H. Jorge, A.C. Pavarina, Lasers Med. Sci. 32 (2017) 815.
[33] F. Alves, A.C. Pavarina, E.G.O. Mima, A.P. McHale, J.F. Callan, Biofouling 34 (2018) 357.
[34] P.V. da Silveira, B.H.D. Panariello, S.M. C.A.G de Araújo Costa, S.M. Maule, M.N. Maule, I.C.J. Zanin Janal, S. Duarte, Lasers Med. Sci. (2018), https://doi.org/10.1007/s10103-018-2610-x.
[35] C.A. Robertson, D.H. Evans, H. Abrahamse, J. Photochem. Photobiol. B 96 (2009) 1.
[36] D.L. de Sousa, R.A. Lima, I.C. Zanin, M.I. Klein, M.N. Janal, S. Duarte, PLoS One 10 (2015) e013194139.
[37] T.L. Collins, E.A. Markus, D.J. Hassett, J.B. Robinson, Curr. Microbiol. 61 (41) (2010).
[38] H. Koo, R.N. Allan, R.P. Howlin, P. Stoodley, L. Hall-Stoodley, Nat. Rev. Microbiol. (2017) 740–755.
[39] F. Alves, G.C. Alonso, J.C. Carmello, E.G.O. Mima, V.S. Bagnato, A.C. Pavarina, Photodiagnosis Photodyn. Ther. (2018) 168–171.