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Susceptibility of *Candida albicans* and *Candida dubliniensis* to erythrosine- and LED-mediated photodynamic therapy

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ABSTRACT

The effect of erythrosine- and LED-mediated photodynamic therapy (PDT) on planktonic cultures and biofilms of *Candida albicans* and *Candida dubliniensis* was evaluated. Planktonic cultures of standardized suspensions (10^6 cells/mL) of *C. albicans* and *C. dubliniensis* were treated with erythrosine concentrations of 0.39–200 μM and LEDs in a 96-well microtiter plate. Biofilms formed by *C. albicans* and *C. dubliniensis* in the bottom of a 96-well microtiter plate were treated with 400 μM erythrosine and LEDs. After PDT, the biofilms were analysed by scanning electron microscopy (SEM). The antimicrobial effect of PDT against planktonic cultures and biofilms was verified by counting colony-forming units (CFU/mL), and the data were submitted to analysis of variance and the Tukey test ($P < 0.05$). *C. albicans* and *C. dubliniensis* were not detectable after PDT of planktonic cultures with erythrosine concentrations of 3.12 μM or higher. The CFU/mL values obtained from biofilms were reduced 0.74 $\log_{10}$ for *C. albicans* and 0.21 $\log_{10}$ for *C. dubliniensis*. SEM revealed a decrease in the quantity of yeasts and hyphae in the biofilm after PDT. In conclusion, *C. albicans* and *C. dubliniensis* were susceptible to erythrosine- and LED-mediated PDT, but the biofilms of both *Candida* species were more resistant than their planktonic counterparts.

1. Introduction

Candida albicans is a commensal yeast from the oral cavity and is the most virulent species of the genus. A pathogenic phase that produces superficial to systemic infections by disrupting the balance between microorganism and host can result from alterations in the host environment, such as the use of immunosuppressive drugs, antibiotics, estrogen or prostheses, xerostomia and inadequate oral hygiene.[1–3]

In immunosuppressed individuals, such as those with acquired immunodeficiency syndrome (AIDS), oral candidosis is the most common fungal manifestation; 84–100% of HIV-infected individuals develop at least one episode of colonization

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by *Candida* spp., and up to 90% develop pseudomembranous candidiasis.[4]

The treatment of oral candidosis in HIV-positive individuals is complicated by its recurrent nature; previous exposure reduces its susceptibility to conventional antifungals. *C. albicans* and other *Candida* species can develop resistance to antifungals used to treat oral candidosis, such as fluconazole.[5,6]

Colonization and infection by yeasts of the *Candida* genus are mediated by the formation of a biofilm, which is composed of a heterogeneous mixture of blastoconidia, pseudohyphae and hyphae embedded in extracellular polymeric substances that form channels and pores and exhibit different phenotypic characteristics than planktonic *Candida*.[7] The extracellular matrix is composed of polysaccharides, proteins, hexosamine, uronic acid and DNA to promote biofilm adhesion and formation, protect the cells from phagocytosis, maintain the integrity of the biofilm and limit the diffusion of substances.[7,8]

The biofilms formed by yeasts of the *Candida* genus are resistant to a range of chemicals and antifungal agents. Biofilms of *C. albicans* and *C. parapsilosis* are resistant to fluconazole, voriconazole, amphotericin B, nystatin, ravuconazole, terbinafine and chlorhexidine and are sensitive to caspofungin, micafungin, amphotericin B lipid complex and liposomal amphotericin B.[9]

C. dubliniensis, a species with phenotypic characteristics similar to those of *C. albicans*, is isolated predominantly from the oral cavities of patients with AIDS.[6,10] *C. dubliniensis* produces a complex mature biofilm composed of the same fungal morphologies expressed by *C. albicans*, forming a multilayer extracellular matrix that acts as a reservoir for the release of cells into the oral environment. *C. dubliniensis* seems to be well-adapted to colonization of the oral cavity, with important clinical repercussions.[11]

As fungal infections caused by *C. albicans* and their reduced susceptibility to conventional antifungals have increased, the antifungal potential of photodynamic therapy (PDT) has been evaluated.[12,13] PDT consists of irradiating an administered photosensitizer, a photosensitive and non-toxic agent, with a light source of a suitable wavelength. In the presence of oxygen, reactive oxygen species or free radicals are produced, causing cell damage by disrupting the cytoplasmic membrane; the increased permeability causes damage to intracellular targets and reduces the formation of germ tubes.[14–17]

The main photosensitizers used in antifungal PDT are phenothiazine dyes, phthalocyanines and porphyrins associated with lasers and other non-coherent light sources.[12,18–20] Erythrosine has attracted interest as a photosensitizer because it is not toxic to the host and has already been approved for use in dentistry.[21] Erythrosine is used to detect dental biofilms. This dye has shown potent photodynamic activity and can reduce 3.0–3.7 $\log_{10}$ of *Streptococcus mutans* biofilm.[21,22]

Light-emitting diodes (LEDs) have been suggested as alternative light sources to lasers due to their wider emission bands, smaller size, reduced weight and cost, greater flexibility in treatment irradiation time and easy operation.[23,24] LEDs are used in dentistry as bleaching tools that do not damage oral tissues. LEDs have shown potent activity in PDT and lack of absence of antimicrobial action alone.[19,25,26]

In PDT against *Candida* spp., red and blue LEDs were used with phenothiazines (methylene blue and toluidine blue) and Photogem photosensitizers, reducing planktonic cultures by 3.41 $\log_{10}$ and biofilms by less than 1 $\log_{10}$.[19,25,26] However, the effect of erythrosine dye and green LEDs against *Candida* spp. has not been described. The aim of this study was to evaluate the effect of PDT mediated by erythrosine dye and green LEDs on planktonic cultures and biofilms of *C. albicans* and *C. dubliniensis*.

2. Materials and methods

2.1. Photosensitizer and light source

Erythrosine (Aldrich Chemical Co., Milwaukee, WI, USA) was used for the sensitization of yeasts. Erythrosine solution was prepared by dissolving the powdered dye in phosphate-buffered saline (PBS, pH 7.4) and sterilized by filtration through 0.22-μm pore diameter membranes (MFS, Dublin, CA, EUA). After filtration, the dye solution was stored in the dark. The absorption spectrum (400–800 nm) of the erythrosine solution (1.0 μM in PBS) was verified in a spectrophotometer (Cary 50 Bio, Varian Inc., Palo Alto, CA, USA) coupled to a microcomputer.

A green light-emitting diode (LED) (MMOptics, São Carlos, SP, Brazil) was used as the light source with a wavelength of 532 ± 10 nm, an output power of 90 mW, an energy of 16.2 J, a time of 3 min, a fluence rate of 237 mW cm^{-2} and a fluence of 42.63 J cm^{-2}. The area irradiated in planktonic cultures and biofilms was 0.38 cm^2.

The temperature at the bottom of the 96-well microtiter plates (Costar Corning, New York, NY, USA) was monitored using an infrared thermometer (MX4, Raytek, Sorocaba, SP, Brazil); no increases in temperature were observed after irradiation with the LED.

2.2. Photodynamic therapy of planktonic cultures of *C. albicans* and *C. dubliniensis*

Reference strains of *C. albicans* (ATCC 18804) and *C. dubliniensis* (ATCC 7978) were seeded onto Sabouraud dextrose (Himedia, Mumbai, Maharashtra, India) agar and incubated at 37 °C for 24 h. The microorganisms were then harvested in Sabouraud dextrose (Himedia) broth and incubated at 37 °C for 16 h.

The microbial growth in the broths was centrifuged at 358 × *g* for 10 min and washed twice with PBS. The sediments were resuspended in PBS. Standardized suspensions of each strain were then prepared at a concentration of 10^6 cells/mL with an optical density (OD) of 0.284 in PBS using a spectrophotometer (B582, Micronal, São Paulo, SP, Brazil) set to 530 nm.

To establish the death curve for the planktonic cultures, 220 assays with the standardized suspensions of each strain were performed with erythrosine dye at concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 μM, with 10 assays for each concentration.

The assays were divided into four experimental groups for each *Candida* species: treatment with erythrosine at concentrations of 200–0.39 μM and LED irradiation (P+L+, $n = 100$);

treatment with erythrosine at concentrations of 200–0.39 μM only (P+L−, $n = 100$); LED irradiation (P−L+, $n = 10$); control group, treated with PBS only (P−L−, $n = 10$).

A 0.1 mL aliquot of the standardized suspension of each strain was added to each well of a 96-well flat-bottom microtiter plate (Costar Corning). The assay groups P+L+ and P+L− received 0.1 mL of each concentration of the erythrosine solution, whilst the assay groups P−L+ and P−L− received 0.1 mL of PBS. The plate was then shaken for 5 min (pre-irradiation) in an orbital shaker (Solab, Piracicaba, SP, Brazil). The wells containing the assay groups P+L+ and P−L+ were irradiated according to the protocol described.

After irradiation, serial dilutions were prepared, and aliquots of 0.1 mL were seeded in duplicate onto Sabouraud dextrose (Himedia) agar plates and incubated at 37 °C for 48 h. After incubation, the number of colony-forming units (CFU/mL) was determined.

2.3. Photodynamic therapy on biofilms of C. albicans and C. dubliniensis

The methodology described by Seneviratne et al.[27] was used for biofilm growth, with some modifications.

Cultures of *C. albicans* (ATCC 18804) and *C. dubliniensis* (ATCC 7978) that were grown on Sabouraud dextrose (Himedia) agar at 37 °C for 18 h were harvested in yeast nitrogen base (YNB, Himedia) supplemented with 50 mM glucose (Vetec, Duque de Caxias, RJ, Brazil). After an 18-h incubation at 37 °C, the yeasts were centrifuged at 358 × *g* for 10 min, washed twice with PBS, resuspended in YNB supplemented with 100 mM glucose (Vetec) and adjusted to an optical density of 0.381 at 530 nm (10^7 cells/mL) using a spectrophotometer (B582, Micronal).

A 250 μL aliquot of each suspension was pipetted into each well of a 96-well flat-bottom microtiter plate (Costar Corning). The plate was incubated for 1.5 h at 37 °C in a shaker at 75 rpm (Quimis, Diadema, SP, Brazil) for the initial adhesion phase. After this period, the wells were washed with 250 μL of PBS to remove loosely adhered cells. A 250 μL aliquot of YNB (Himedia) with 100 mM glucose was then pipetted into each of the washed wells, and the plates were incubated at 37 °C in a shaker at 75 rpm (Quimis) for 48 h. The broth was changed every 24 h. The plates with biofilms formed by *C. albicans* and *C. dubliniensis* were then washed with 250 μL of PBS to remove loosely adhered cells.

The biofilm formed by each strain was immersed in 250 μL of a solution of 400 μM erythrosine for 5 min (pre-irradiation time) in an orbital shaker (Solab). The photosensitizer concentration for biofilms was determined after results obtained for planktonic cultures and in a pilot study on biofilms. Subsequently, the suspended plates were irradiated according to the protocol described (P+L+, $n = 10$). The effects of the isolated erythrosine photosensitizer (P+L−, $n = 10$) and light source (P−L+, $n = 10$) and the control group, treated with PBS in the absence of light (P−L−), were evaluated as well.

After the treatments, the biofilm cells were scraped off the well wall using a sterile toothpick and transferred to Falcon tubes containing 10 mL of PBS. To disrupt the biofilms, the contents of the tubes were homogenized for 30 s using an ultrasonic homogenizer (Sonoplus HD 2200; Bandelin Electronic, Berlim, Brandemburgo, Germany) with an output power of 50 W. The solutions in the Falcon tubes were considered to be a dilution factor of 10^{-1}. Serial dilutions were then made using each original 10^{-1} dilution, and aliquots of 0.1 mL were seeded onto Sabouraud dextrose (Himedia) agar plates that were then incubated at 37 °C for 48 h. After the incubation period, the CFU/mL values of each plate were determined.

The irradiation of planktonic cultures and biofilms was performed under aseptic conditions in a laminar flow hood in the dark. During irradiation, the plates were covered with a black matte screen with an orifice the same size as the wells to prevent the spread of light to neighbouring wells.

2.4. Scanning electron microscopy (SEM)

Biofilms of *C. albicans* and *C. dubliniensis* from the groups P+L+ ($n = 2$) and P−L− ($n = 2$) were submitted to SEM analysis.

The biofilms were formed as described above and treated according to the experimental groups P+L+ and P−L−, but the biofilms were formed on polystyrene discs approximately 8 mm in diameter that had been previously sterilized in a 20-kGy gamma radiation chamber (cobalt 60) for 6 h (Embrarad, São Paulo, SP, Brazil). The discs were placed into 24-well plates (Costar Corning) in which the volume of suspension, PBS, broth culture and photosensitizer solution was 1 mL. After biofilm formation, the discs were transferred to 24-well plates (Costar Corning), fixed in 2.5% glutaraldehyde for 1 h and dehydrated in several ethanol washes (10, 25, 50, 75, and 90% for 20 min and 100% for 1 h). The plates were then incubated at 37 °C for 24 h to dry the discs.

The discs were transferred to aluminium stubs and covered with gold for 120 s at 40 mA (BAL-TEC 50D 050 Sputter Coater, Liechtenstein). After metallization, the biofilms were examined and photographed by SEM (Jeol JSM5600, Tokyo, Japan), operating at 15 kV in increments of 1000 and 5000 times.

2.5. Statistical analysis

The data for CFU/mL were converted to logarithmic form and submitted to analysis of variance and the Tukey test. A P value < 0.05 was statistically significant.

The percentage of CFU/mL reduction for *C. albicans* and *C. dubliniensis* biofilms were calculated, considering the groups P+L−, P−L+ and P+L+ in relation to the control group (P−L−).

3. Results

The chemical structure and absorption spectrum of the erythrosine dye are shown in Fig. 1. Erythrosine absorbs between 460 and 560 nm with an absorbance maximum at approximately 530 nm.

The death curves obtained for the planktonic cultures of *C. albicans* and *C. dubliniensis* are shown in Fig. 2. The antimicrobial activity of PDT was photosensitizer concentration-dependent for planktonic cultures of *C. albicans* and *C. dubliniensis*.

For *C. albicans*, an erythrosine concentration of at least 0.39 μM was required for a statistically significant reduction in CFU/mL in the P+L+ group relative to the control group (P−L−).

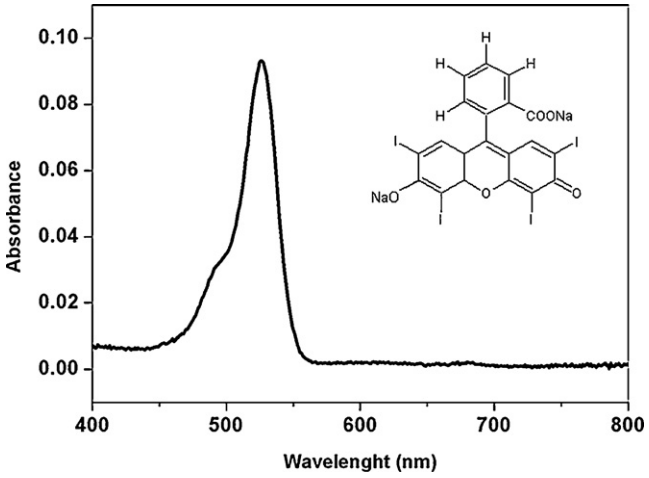

Fig. 1 – Chemical structure and absorption spectrum of the erythrosine photosensitizer (1.0 μM in PBS).

For *C. dubliniensis*, erythrosine concentrations of 1.56 μM or higher resulted in a statistically significant reduction in CFU/mL in the P+L+ group relative to the control group (P−L−).

For both species, PDT eliminated microbial growth when erythrosine was used at concentrations of 3.12 μM or higher.

PDT mediated by 400 μM erythrosine of biofilms resulted in 0.74 $\log_{10}$ and 0.21 $\log_{10}$ reductions of *C. albicans* and *C. dubliniensis*, respectively (Fig. 3). The differences for the P+L+ groups of both species were statistically significant relative to the remaining groups (P−L−, P−L+ and P+L−), with *P* values relative to the control group of 0.001 for *C. albicans* and 0.015 for *C. dubliniensis*.

SEM revealed that the biofilm of the *C. albicans* control group (P−L−) was composed of blastoconidia, pseudohyphae and hyphae. The characteristics of the biofilm formed by *C. dubliniensis* were similar to those of the *C. albicans* biofilm, but the *C. dubliniensis* biofilm exhibited a greater amount of filamentous forms (Fig. 4-1A–1D). The biofilms exposed to PDT (P+L+) showed a decrease in fungal structures, and *C. dubliniensis* primarily demonstrated a reduction in filamentous forms (Fig. 4-2A–2D).

4. Discussion

The production of reactive oxygen species by PDT depends on the interaction the photosensitizer with photons of visible light of suitable wavelength. For this interaction to occur, the laser or LED must emit light at a wavelength that the photosensitizer is able to absorb.[28] In the present work, an LED with an emission of 532 ± 10 nm was chosen for the photodynamic reaction so that the emission of the light source coincided with the absorption maximum (530 nm) of the erythrosine photosensitizer.

PDT mediated by erythrosine and LED-irradiation significantly reduced planktonic cultures and biofilms of *C. albicans* and *C. dubliniensis*. These results are the first report of antimicrobial PDT of *Candida* spp. mediated by erythrosine and green LEDs.

PDT of *C. albicans* planktonic cultures reduced cell viability in a statistically significant manner at the lowest erythrosine concentration used (0.39 μM), whilst the lowest suitable concentration for reduction of *C. dubliniensis* was 1.56 μM. Both strains were reduced completely at concentrations of erythrosine 3.12 μM and higher with LED irradiation of 3 min and a fluence of 42.63 J cm^{-2}.

Candida were previously shown to be completely inactivated when a blue LED (37.5 J cm^{-2}) was used in association with Photogem (25 mg/mL) on planktonic cultures of reference and fluconazole-resistant strains of *C. albicans* and *C. glabrata*.[19]

In contrast, the present study resulted in a greater microbial reduction at lower concentrations of photosensitizer than that reported by Peloi et al.[25] These authors assessed the photodynamic action of a methylene blue photosensitizer at a concentration of 35.2 μM irradiated by a red LED (2–12 J cm^{-2}) for 10–60 min against planktonic cultures of *Staphylococcus aureus*, *Escherichia coli* and *C. albicans*, obtaining reductions of 2.34–3.71, 1.61–3.41 and 2.77–3.87 $\log_{10}$, respectively. However, the fluence of the LED used by Peloi et al.[25] was approximately 3.5 times smaller than the fluence of the LED used here.

We demonstrated greater microbial reductions with a smaller fluence of LED, irradiation time and dye concentration

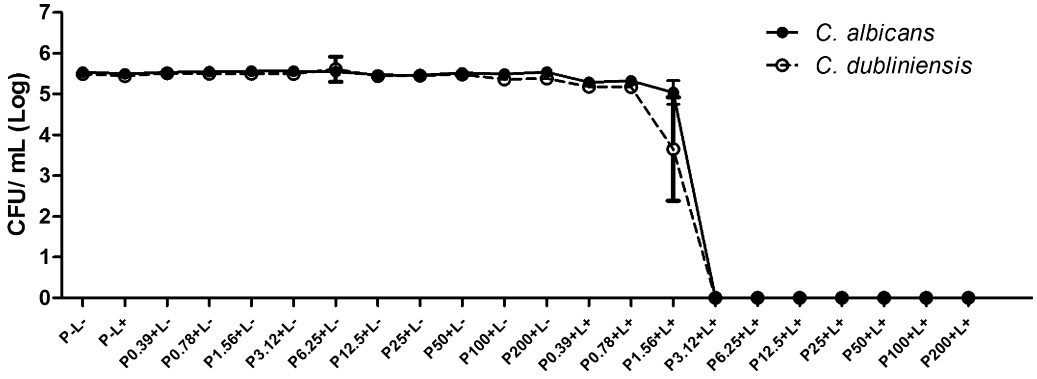

Fig. 2 – Means and standard deviations of the CFU/mL (Log) values from the death curves of the planktonic cultures of *C. albicans* and *C. dubliniensis* exposed to the following treatments: PBS control (P−L−), PBS with LED (P−L+), erythrosine only (P+L−) and erythrosine with LED (P+L+).

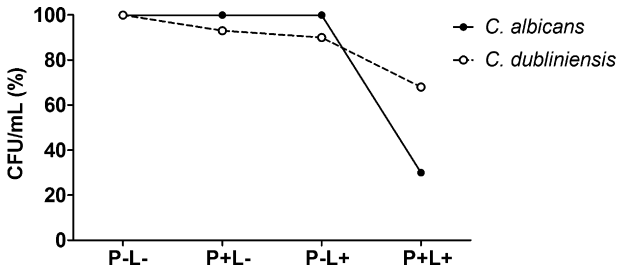

Fig. 3 – Percentage of reduction, expressed as mean values (CFU/mL), in the viability of *C. albicans* and *C. dubliniensis* exposed to photosensitizer (P+L−), LED (P−L+) or both photosensitizer and LED (P+L+) relative to the control group (P−L−).

than that reported by Soares et al.,[26] who used a red LED with a fluence of 180 J cm^{-2} and an irradiation time of 15 min in association with 25 μM toluidine blue to achieve a 3.41 log_{10} reduction in fluconazole-resistant and -sensitive *Candida* strains. These authors also demonstrated that PDT inhibited 55% of the adhesion of the *Candida* strains to buccal epithelial cells, highlighting the important impact of LED in association with toluidine blue on the inhibition of growth and virulence factors of the fluconazole-resistant and -sensitive *Candida* strains.

The biofilms of *C. albicans* and *C. dubliniensis* exposed to PDT mediated by 400 μM erythrosine and a green LED exhibited statistically significant reductions in CFU/mL of 0.74 log_{10} and 0.21 log_{10}, respectively. The result obtained for the *C. dubliniensis* biofilms corroborates those described by Dovigo et al.[19] for the PDT of biofilms of *C. albicans* and *C. glabrata*, which were reduced 0.24 log and 0.16 log respectively.

The biofilms of *C. albicans* and *C. dubliniensis* were less susceptible to PDT than their planktonic counterparts, which could be due to the heterogeneity of the biofilm population, the restriction of antimicrobial penetration by the extracellular matrix material, the slower growth rate of the cells in the biofilms and differences in gene expression levels.[11,29]

Chabrier-Roselló et al.[30] evaluated the effects of Photofrin- and Hg arc lamp-mediated PDT on biofilms and germ tubes of *C. albicans*. The metabolisms of biofilms treated with PDT were significantly reduced relative to biofilms treated with the antifungal amphotericin B. The treated germ tubes displayed a loss of membrane integrity and cell death. The authors highlighted the potential of PDT as an adjuvant or alternative treatment against cutaneous and mucocutaneous infections caused by *C. albicans*.

SEM of the biofilms of the control group showed a complex structure formed by blastoconidia, pseudohyphae and hyphae, but the extracellular polysaccharide matrix was not apparent. The absence of the extracellular polysaccharide matrix is likely due to the fixation process required for SEM. Fixation can remove the extracellular polysaccharide matrix and prevent its visualization by microscopy.[7,11]

The biofilms of the group P+L+, which were exposed to PDT, displayed a decrease in fungal structures, in agreement with previous work by Pereira et al.[31] They evaluated the effects of methylene blue (312.6 μM) and an indium–gallium–aluminium–phosphide (InGaAlP) laser on single- and multi-species biofilms formed by *C. albicans*, *S. aureus* and *S. mutans*. A decrease in cell aggregates was observed in the outer layers of both biofilms. The multi-species biofilms were more resistant to PDT, suggesting that biofilm complexity increases resistance to PDT.

SEM revealed a reduction of blastoconidia, pseudohyphae and hyphae in the *C. albicans* biofilms submitted to PDT and an important reduction of hyphae in the *C. dubliniensis* biofilms. According to Bliss et al.,[32] the filamentous forms of *Candida* uptake more photosensitizer and are therefore more sensitive to Photofrin-mediated PDT than the blastoconidia.

The green LED and the erythrosine photosensitizer used in the present work did not exhibit cytotoxic effects when used alone against either planktonic cultures or biofilms of both species, as shown previously for red and blue LEDs used in association with erythrosine against microbial cells and fibroblasts.[19,25,26,33,34]

C. dubliniensis may be less sensitive to PDT than *C. albicans* because this species required higher concentrations of erythrosine than *C. albicans* to achieve the same microbial reduction. The CFU/mL (Log) of *C. dubliniensis* biofilms were also reduced less than those of *C. albicans* biofilms. According to Paugam et al.,[35] *C. dubliniensis* acquires secondary resistance to fluconazole more quickly than *C. albicans*. de Souza et al.[36]

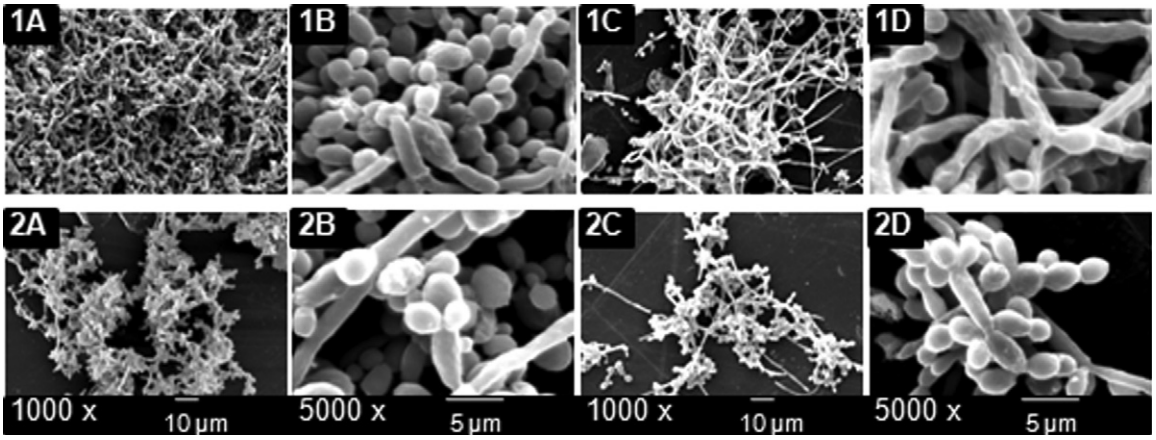

Fig. 4 – Scanning electron microscopy. *C. albicans* biofilms: PBS control (P−L−) (1A and 1B) and 400 μM erythrosine with LED (P+L+) (2A and 2B); *C. dubliniensis* biofilms: P−L− (1C and 1D) and P+L+ (2C and 2D).

have also identified different responses to PDT amongst different species of *Candida*, highlighting the need for studies of the effects of photosensitizers on specific *Candida* species.

C. albicans and *C. dubliniensis* were both susceptible to erythrosine- and LED-mediated PDT. However, biofilm structures were more resistant to PDT than planktonic cultures for both species of *Candida*.

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