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ORIGINAL ARTICLE

Action of antimicrobial photodynamic therapy on heterotypic biofilm: *Candida albicans* and *Bacillus atrophaeus*

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Abstract The increase in survival and resistance of microorganisms organized in biofilms demonstrates the need for new studies to develop therapies able to break this barrier, such as photodynamic therapy, which is characterized as an alternative, effective, and non-invasive treatment. The objective was to evaluate in vitro the effect of antimicrobial photodynamic therapy on heterotypic biofilms of *Candida albicans* and *Bacillus atrophaeus* using rose bengal (12.5 μM) and light-emitting diode (LED) (532 nm and 16.2 J). We used standard strains of *B. atrophaeus* (ATCC 9372) and *C. albicans* (ATCC 18804). The biofilm was formed in the bottom of the plate for 48 h. For the photodynamic therapy (PDT) experimental groups, we added 100 μL of rose bengal with LED (P+L+), 100 μL of rose bengal without LED (P+L−), 100 μL of NaCl 0.9 % solution with LED (P−L+), and a control group without photosensitizer or LED (P−L−). The plates remained in agitation for 5 min (pre-irradiation) and were irradiated with LED for 3 min, and the biofilm was detached using an ultrasonic homogenizer for 30 s. Serial dilutions were plated in BHI agar and HiChrom agar and incubated at 37 °C/48 h. There was a reduction of 33.92 and 29.31 % of colony-forming units per milliliter (CFU/mL) for *C. albicans* and *B. atrophaeus*, respectively, from the control group to the group subjected to PDT. However, statistically significant differences were not observed among the P+L+, P+L−, P−L+, and P−L− groups. These results suggest that antimicrobial photodynamic therapy using rose bengal (12.5 μM) with a pre-irradiation period of 5 min and LED for 3 min was not enough to cause a significant reduction in the heterotypic biofilms of *C. albicans* and *B. atrophaeus*.

Keywords Photodynamic therapy · *Bacillus atrophaeus* · *Candida albicans* · Biofilm

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Introduction

Due to increasing fungal and bacterial resistance to antimicrobials, many researchers have investigated non-conventional therapies for the treatment of infections. Photodynamic therapy (PDT) involves the application of a photoactive substance called a photosensitizer and a light source with a wavelength suitable to activate the photosensitizer molecules in the presence of oxygen. After activation, it generates an excited state of the photosensitizer molecules [1]. These procedures result in a cytotoxic photodynamic reaction involving the production of reactive oxygen species and sequential oxidative reactions, leading to cell death [2].

Bacillus atrophaeus is a Gram-positive bacteria used in studies as a classical non-pathogenic surrogate of *Bacillus*

anthracis [3]. It is easy to culture, has identified resistance characteristics, and is used in a variety of applications [4]. Despite being classified for many years solely as soil microorganisms, *Bacillus* are also commensal microorganisms in the human gut due to their great adaptability to the intestinal environment, representing a part of the natural life cycle [5].

Candida albicans is a commensal yeast and important human pathogen that presents as a major virulence factor with biofilm formation and is responsible for most cases of oral and systemic candidiasis [6, 7].

Biofilm formation is a survival mechanism that allows the on-site permanence of microorganisms. In the structure of the biofilm, bacteria and fungi are typically encapsulated in a matrix of glycoprotein and polysaccharide produced by microbial components, which often remain in a state of reduced metabolic activity [8, 9]. The microbial species of the biofilm are highly interactive and use intercellular signaling systems or quorum sensing. This phenomenon promotes collective behavior in the microbial population by improving access to nutrients and niches, as well as promoting a collective defense against other competitive organisms [10].

According to Martin et al. [11], the interactions between bacteria and fungi influence their survival and proliferation and may be beneficial for the microorganisms, increasing their resistance to antimicrobials or antagonists causing the death of even one component [12], as well as interfering with the survival of the host [13].

This study evaluated, in vitro, the effect of photodynamic therapy on a heterotypic biofilm formed by the association of *B. atrophaeus* and *C. albicans* using the photosensitizer rose bengal and a green light-emitting diode (LED).

Methodology

Microorganisms and growth conditions

We used standard strains of *B. atrophaeus* (ATCC 9372) and *C. albicans* (ATCC 18804) plated on brain heart infusion agar (Difco, Detroit, USA) and Sabouraud dextrose agar (Difco, Detroit, USA), respectively. After growth, the colonies of microorganisms were suspended in phosphate-buffered saline (PBS) and centrifuged for 10 min at 1300×*g* (MPW 350, Biosystems, Curitiba, Brazil). The cell pellet was then resuspended in 1 mL PBS. The number of viable cells in suspension was counted using a spectrophotometer (B582, Micronal, São Paulo, Brazil), and a standard suspension of the strain was prepared in PBS at a concentration of 10^7 cells/mL. The parameters of optical density and wavelength used were, respectively, 0.381 and 530 nm for *C. albicans* and 0.269 and 307 nm for *B. atrophaeus*.

Biofilm formation

The biofilm formation was based on the methodology described by Seneviratne et al. [14] and Bridier et al. [15] with modifications. Heterotypic biofilms were obtained by the association of *C. albicans* and *B. atrophaeus* and formed in the bottom of a 96-well microtiter plate (Costar Corning, New York, USA) by pipetting in 100 μL of a standardized suspension of 10^7 cells/mL of each microorganism. The plate was incubated under shaking at 75 rpm rotation (Quimis Diadema, England) for 90 min at 37 °C. The wells with formed biofilms were washed with PBS to remove the non-adhered cells and incubated for 24 h. After this period, the wells were washed again, and the broths were changed. The plates were incubated for 48 h.

In vitro photosensitization

After 48-h incubation, the wells were washed with PBS for the application of PDT. We used the photosensitizer rose bengal (Sigma, São Paulo, Brazil) at a 12.5-μM concentration and the incubation time of 5 min. A green LED (MMOptics, São Carlos, Brazil) with a wavelength of 532 ± 10 nm, 90 mW output power, and 16.2 J energy was used as the light source.

The experimental groups are shown in Table 1.

The plates containing the biofilm remained in pre-irradiation time for 5 min in an orbital shaker (Solab, Piracicaba, Brazil) to increase the penetration of the photosensitizer into the microorganisms. The cells were not washed after the pre-irradiation. Each experimental group was repeated 10 times (number of tests equal to 10), with a total of 40 trials for *C. albicans* biofilm and 40 trials for *B. atrophaeus*, resulting in 80 trials for the complete experiment.

After irradiation, excess photosensitizer was removed with PBS, and the wells were homogenized for 30 s in an ultrasonic homogenizer (Sonics Vibra Cell, Connecticut, USA) with 25 % amplification to detach the biofilm. Serial dilutions of suspensions were performed, and a 100-μL aliquot of each dilution was plated on brain heart infusion agar for *B. atrophaeus* and HiCrome *Candida* differential agar (Himedia, Mumbai, India) for *C. albicans* and incubated at 37 °C for 24 and 48 h, respectively. After the incubation period, colonies were counted to calculate the colony-forming units per milliliter (CFU/mL). The results were analyzed statistically by analysis of variance (ANOVA) with a 5 % significance level.

Results

Firstly, the results from this experiment were calculated as percent reduction in CFU/mL (Fig. 1). There was a reduction of CFU/mL for *C. albicans* (33.92 %) and *B. atrophaeus*

Table 1 Experimental conditions of groups subjected to PDT with the pre-irradiation time, irradiation time, photosensitizer, and light source

Irradiation		Experimental groups	
Pre-irradiation (with shaking) 5 min	Irradiation 3 min		
X	X	P+L+	Heterotypic biofilm + 100 μL rose bengal + LED
X		P+L−	Heterotypic biofilm + 100 μL rose bengal
	X	P−L+	Heterotypic biofilm + 100 μL solution NaCl 0.9 % + LED
Control Group		P−L−	Heterotypic biofilm + 100 μL solution NaCl 0.9 %

P+L+ photosensitizer and light, *P−L−* control group, *P−L+* photosensitizer, *P+L−* light

(29.31 %) when P+L+ groups were compared to the control groups (P−L−). The biofilms treated with rose bengal (P+L−) and laser (P−L+) alone demonstrated a number of CFU/mL more similar to the control group.

For statistical analysis, the data of CFU/mL were converted to logarithmic form ($\log_{10}$) and presented in Fig. 2. In this figure, it can be observed that *B. atrophaeus* showed a higher number of CFU/mL than *C. albicans* within heterotypic biofilms. For each microorganism, the P−L−, P+L+, P+L−, and P−L+ groups were compared by ANOVA test and statistically significant differences were not found among the groups.

Discussion

The susceptibility of bacterial and fungal species to the lethal photodynamic effects of various photosensitizing triggers has led to assorted studies seeking to better understand the mechanism of action of antimicrobial photodynamic therapy [16]. Most microorganisms, both in the environment and during the course of infections, form biofilms [17, 18]. The conditions selected for PDT application in this study, including the type and concentration of photosensitizer, light source, and pre-irradiation time, beyond the described methodology were based on the photoreduction results described in the literature [19].

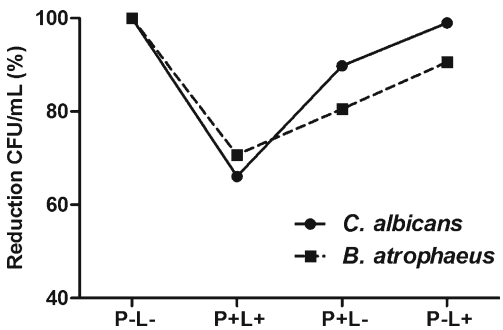

Fig. 1 Percent reduction in CFU/mL of *Candida albicans* and *Bacillus atrophaeus*. *P−L−* control group, *P+L+* photosensitizer and light, *P+L−* light, *P−L+* photosensitizer

In general, there are two basic mechanisms associated with photodynamic inactivation proposed to explain the lethal damage caused by PDT to microorganisms: changes in DNA and in the cytoplasmic membrane, allowing extravasation of the cellular contents or the inactivation of membrane transport systems and enzymes [20]. The photodynamic treatment also influences the biofilm structure, causing decreased layer thickness and biomass loss [21].

Regarding the fungal cell wall, there is a relatively thick layer of chitin and beta-glucan that produces a permeability barrier intermediate between Gram-positive and Gram-negative bacteria [22, 23]. The photodynamic mechanism damages fungal cells when reactive oxygen species penetrate the cell walls and membranes, thus allowing displacement of the photosensitizer into the cell. Then, oxidizing species generated by the excitation of light induce the photodestruction of internal cellular organelles, leading to cell death. Thus, the singlet oxygen generated by the excitation of the photosensitizer is a non-specific oxidizing agent against which there is no cellular defense [18, 24]. Antioxidant enzymes such as catalase and peroxidase protect against some reactive oxygen species but not against singlet oxygen, which inactivates some antioxidant enzymes such as catalase [18].

It has been suggested that the combination of the LED and rose bengal exerts a photodynamic effect on *C. albicans* biofilm. Costa et al. [25] used rose bengal and blue light LED for the photodynamic inactivation of planktonic cultures and

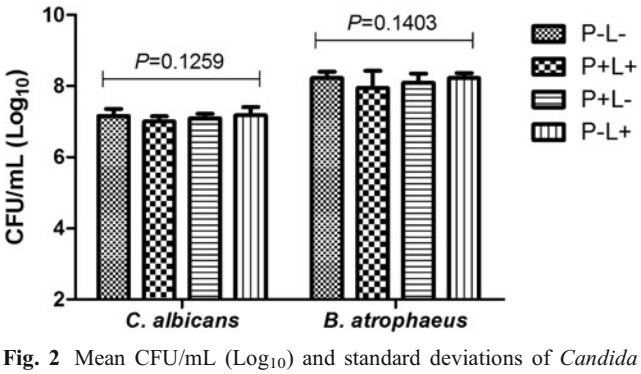

Fig. 2 Mean CFU/mL (Log_{10}) and standard deviations of *Candida albicans* and *Bacillus atrophaeus* for the following groups: *P−L−* control group, *P+L+* photosensitizer and light, *P+L−* light, *P−L+* photosensitizer (ANOVA, $p < 0.05$)

biofilms of *C. albicans* and observed a reduction of 1.97 and 0.12 $\log_{10}$, respectively. Freire et al. [19] compared the effects of rose bengal and eosin Y photosensitizers in PDT using a green light LED in planktonic cells and the biofilm of *C. albicans*. The concentrations of the photosensitizers used varied from 0.78 to 400 μM for planktonic cultures and 200 μM for biofilms. In the biofilms, rose bengal and eosin Y showed microbial reductions of 0.22 and 0.45, respectively. The result obtained by Costa et al. [25] showed a lower reduction of biofilm formed by *C. albicans* compared to the work performed by Freire et al. [19], suggesting that the concentration of the photosensitizer directly influences the action of photodynamic therapy.

The exposure time of the photosensitizer during pre-irradiation can also contribute to its access to different locations in the cell. Ito [26] and Usacheva et al. [27] showed that the inactivation mechanism of a cell depends mainly on the time of pre-irradiation to which the photosensitizer is subjected. The difference in the effectiveness of the photosensitizer due to exposure time in the pre-irradiation period may indicate the presence of cellular diffusion barriers that prevent the photosensitizer from reaching an optimal location inside the bacterial cell [27, 28].

Therefore, based on the experimental conditions carried out in this study, there was a slight reduction of *C. albicans* microbial biofilms (33.92 %) and statistically significant difference between the P+L+ and P−L− groups was not observed. The low susceptibility presented by *C. albicans* biofilm can be explained by the structural differences between bacteria and yeast biofilm or the limited penetration of light into the thick biofilms formed by *Candida* [29]. The non-significant reduction may also be related to the set of factors necessary to establish an appropriate relationship for an effective photodynamic effect: microorganism, photosensitizer (concentration and location in the target cell), type of light source, and pre-irradiation period.

Rossetti and colleagues [30] studied the effects of antimicrobial photodynamic therapy using toluidine blue as a photosensitizing agent in the production of reactive oxygen species, cell damage, and the ability of *C. albicans* to form biofilms. The authors found a significant decrease in cell growth and increased production of reactive oxygen species and cell permeability after treatment application, with the results dependent on and proportional to the concentration of the photosensitizer and the incubation time (1, 2, and 3 h) after PDT. After 3 h of incubation, the authors obtained better microbial reduction: 82 %.

Regarding the effect of photodynamic therapy in *B. atrophaeus*, the results of this study showed a decrease of only 29.31 % in the CFU/mL count, but statistically significant difference was not found between PDT and control groups. The ability of *Bacillus* spp. cells to differentiate into subtypes with specialized attributes in response to different environmental cues [31, 32] suggested that this response to adverse environmental conditions occurs by inducing the expression of adaptive genes. Stochasticity allows bacteria to deploy specialized cells in anticipation of possible adverse changes in the environment [32, 33].

Bacteria in biofilms are less accessible to antibiotics due to protection within the polymer matrix and bacterial adhesion to the surface [34]. Susceptibility testing evaluation studies have been performed, in vitro, in biofilm models and demonstrated significant survival of microorganisms after treatment with antibiotics [35]. Photosensitizer absorption into the matrix is prevented in the same way as antibiotic absorption [21]. Ergaieg and Seux [36] suggested that there are three basic types of photosensitizers: ones that bind tightly to the microorganism and thus penetrate the cell, ones that bind weakly, and ones that do not contact the cell. Thus, there can be variations in the absorption of the photosensitizer by microorganisms.

Studies have demonstrated the successful use of PDT on viruses, Gram-positive, Gram-negative and drug-resistant bacteria, and fungi and yeasts [37, 38]. Rose bengal is the most common photosensitizing agent used in PDT on these microorganisms [25, 39–41]. Brovko et al. [42] evaluated the application of photodynamic therapy with malachite green and rose bengal at concentrations of 5 to 500 μg/mL in the sanitizing methods of the food industry under the action of white light. Using planktonic cultures of Gram-positive (*Bacillus* spp. and *Listeria monocytogenes*) and Gram-negative bacteria (*Escherichia coli* and *Salmonella typhimurium*) and fungi (*Saccharomyces cerevisiae*), it was found that malachite green and rose bengal eliminated Gram-positive bacteria but were not effective against Gram-negative bacteria or fungi. For *Bacillus* spp., reductions were obtained with malachite green at a concentration of 500 μg/mL and rose bengal at all concentrations. This result suggests the intrinsic relationship between photosensitizer concentration and photodynamic therapy effect.

Thus, the photosensitive inactivation of microorganisms is a complex phenomenon and dependent on various parameters, including the probability of photosensitizer absorption in the cell outer membrane, concentration and location in the target cell [43], microbial species, type of photosensitizer, type of light source, and pre-irradiation time [23].

Conclusions

The development of mechanisms to promote the inactivation or death of microorganisms, as studied in this experiment, contributes notably to scientific knowledge because *B. atrophaeus* is considered a classic non-pathogenic *B. anthracis* substitute that is, in certain cases, more resistant; and *C. albicans* is an opportunistic pathogen causing many severe and recurrent infections, including fatal invasive

infections in immunodeficient patients. Thus, photodynamic therapy may be applied as an alternative or adjuvant treatment, acting against various diseases and microorganisms without developing resistance, which is an important and difficult problem that is becoming a key factor in the development of new research. Although the results of this study did not demonstrate significant microbial reduction, they raise the possibility of new experiments important for a wide variety of practical purposes, including the development of practices, strategies, and new formulations with different concentrations, photosensitizers, and light sources for more effective photodynamic action.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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