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The importance of combining methods to assess Candida albicans biofilms following photodynamic inactivation

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ABSTRACT

Background: Methylene blue (MB)-mediated photodynamic inactivation (PDI) has shown good results in killing *Candida spp*. Although MB solutions are commonly used, new formulations have been designed to improve PDI. However, chemical substances in the formulation may interfere with the PDI outcome. In this sense, different methodologies should be used to evaluate PDI *in vitro*. Herein, we report different methodologies to evaluate the effects of PDI with an oral formulation (OF) containing 0.005% MB on *Candida albicans* biofilm. Methods: Biofilms were treated using the MB-OF, with 5 min pre-irradiation time and exposure to a 640 nm LED device (4.7 J/cm^2). PDI was evaluated by the XTT reduction test, counting the colony forming units (CFU), a filamentation assay, crystal violet (CV) staining, and scanning electronic microscopy (SEM). Results: PDI was able to reduce around 1.5 $\log_{10}$ CFU/mL, even though no significant differences were noted in metabolic activity in comparison to the control immediately after PDI. A significant decrease in yeast to hyphae transition was observed after PDI, while the biofilm exhibited flattened cells and a reduced number of yeasts in SEM. The CV assay showed increased biomass. Conclusion: MB-OF-mediated PDI was effective in *C. albicans* biofilms, as it significantly reduced the CFU/mL and the virulence of surviving cells. The CV data were inconclusive, since the OF components interacted with the CV, making the data useless. Taken together, our data suggest that the association of different methods allows complementary responses to assess how PDI mediated by a formulation impacts biofilms.

1. Introduction

Candidiasis is an opportunistic fungal infection that is considered to be the most common in humans. Recently, *Candida ssp* infections have grown worldwide due to the increased misuse of antifungals, associated with the higher use of medical devices, such as heart valves, vascular bypass, dental implants, and catheters, where biofilms can establish [1]. The organized biofilm structure facilitates the persistence of colonization, dissemination, and invasion of the yeast. Indeed, the biofilm also protects the pathogen from the immune system of the host and enables the yeast to tolerate a high concentration of antimicrobial agents [1,2]. The treatment of *Candida* infections is a significant unsolved clinical problem worldwide, firstly due to the antifungal toxicity, especially to patients presenting other conditions (such as HIV) and also due to the emergency of drug-resistant strains, such as *Candida auris* [2,3]. New antifungal therapies are required to meet the great clinical challenge posed by Candida diseases [4–8].

Photodynamic inactivation (PDI) has been reported as an innovative approach for the reduction of *Candida* yeasts [9–15]. It involves the use of a light source, a photosensitive drug, and oxygen to generate reactive oxygen species and kill microbial cells by oxidative stress [16]. A pivotal difference between PDI and most antifungal agents is that the development of resistance to PDI by microorganisms is considered an unlikely event, since PDI is considered a typical multi-target process. Different photosensitizers, light dosimetry, and photosensitizer formulations have been widely investigated [12,13,17–20]. Drug delivery systems such as nanoparticles, liposomes, penetration enhancers, and other formulations have gained attention in recent years [21–23].

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In this regard, the literature presents different methodologies to evaluate the antimicrobial effects on *Candida* biofilms *in vitro*, which are based upon optical density assays, such as XTT (2-methoxy-4-nitro-5-sulfophenyl-5-phenylalanine-carbonyl-2H-tetrazolium hydroxide) [24,25], dry weight by crystal violet (CV) staining for biomass [25], counting the number of colony forming units (CFU) per mL, filamentation assay [26], microscopies such as fluorescence microscopy [27], and scanning electron microscopy (SEM) [25,28]. Each method provides specific information and carries its own strengths and weaknesses.

Nonetheless, singly these methods may provide incomplete information, depending upon the time point evaluated after PDI, as well as the possibility of interaction of the photosensitizer or formulation components to the assay. Indeed, it is not clear whether using drug delivery systems/formulations may affect the results obtained by the classical methods. In this work, we used different assays to evaluate the PDI action of an oral formulation containing methylene blue (MB-OF) on biofilms of *Candida albicans* [13]. We aimed to show how the data obtained by different methods complements each other and highlight some drawbacks of the methods that may confuse the interpretation of the PDI outcome.

2. Material and methods

2.1. Suspension preparation

Candida albicans yeast (ATCC 10,231) was grown for 48 h at 37 °C on Sabouraud dextrose agar (Kasvi, Curitiba, Brazil). The yeast was collected and transferred to a test tube containing Milli-Q water. After the preparation of the water suspension, the number of cells were counted using a Neubauer chamber and the suspension diluted to reach 3.0×10^7 cells/mL of *C. albicans* [13].

2.2. Biofilm growth

Forty-eight-well plates (Sarstedt, Nümbrecht, Germany) were treated with 200 μL/well of fetal bovine serum (FBS) (Vitrocel, São Paulo, Brazil) for 24 h at room temperature. The FBS was removed, and 100 μL of the yeast suspension (3.0×10^7 cells/mL), prepared as described previously, were added to the wells, along with 300 μL of the Sabouraud dextrose broth medium (Kasvi, Curitiba, Brazil). The plates were incubated for 48 h at 37 °C for biofilm formation. Then, the supernatant was discarded, and the biofilm was washed with phosphate buffer saline (PBS) at pH 7.2 (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4; the salts were purchased from Synth (Diadema, Brazil)) [13]. The biofilm was then submitted to PDI, according to the parameters described below, followed by the different methods of analysis.

2.3. Experimental conditions

After biofilm formation, samples were exposed to PDI using methylene blue (MB) 0.005% delivered in an oral formulation (MB-OF). This formulation, which is under patent (BR1020170253902), was developed to control MB aggregation [12,13] due to the presence of sodium dodecyl sulfate (SDS) 0.25% and has been shown to improve PDI efficacy [12,13]. The MB-OF was prepared by diluting a 1 mg/mL water MB (Sigma-Aldrich, Saint Louis, USA) stock solution in OF to reach the desired concentration (0.005%). A physiological solution of NaCl 0.9% was used as a control (CT), and an OF without MB was also used for comparison.

The biofilms were exposed to the conditions presented above. A pre-irradiation time of 5 min was set, to allow MB uptake by the biofilm, followed by a red light-emitting diode (LED) irradiation of 30 min (Condulai, São Paulo, Brazil) with emission at 640 ± 12.5 nm and 2.6 mW/cm^2, positioned exactly above the plate (radiant exposure of 4.7 J/cm^2). This protocol was based upon a previous study of the group [13]. After irradiation, the following assays were performed, as described below: XTT reduction test, CFU/mL, filamentation assay, CV staining, and SEM. All the experiments were performed both in the dark and irradiated.

2.4. Analysis of biofilm by XTT

The biofilms were subjected to XTT assay. A 1 mg/mL XTT solution (Sigma-Aldrich, Saint Louis, USA) was prepared in PBS, filtered with a 0.2 μm membrane, and stored at −70 °C. A 200 mM glucose (Sigma-Aldrich, Saint Louis, USA) solution was prepared in PBS. A water menadione solution (0.4 mM; Sigma-Aldrich, Saint Louis, USA) was prepared immediately before the experiment. The XTT solution was thawed on ice before the experiment. Then, a mixture of these solutions was prepared at a proportion of 79 glucose: 20 XTT: 1 menadione v/v.

The biofilms were washed immediately after MB-OF mediated PDI. The control biofilms (CT and OF) were equally managed. One plate received Sabouraud dextrose broth medium and was incubated for 24 h at 37 °C before submission to the XTT assay. The other plate was immediately evaluated by XTT assay. The wells received the glucose/XTT/menadione solution, and the plates were incubated in the dark for 3 h at 37 °C. After incubation, 150 μL of the solution was transferred to a 96-well plate, and the optical density of the solution was measured using a microplate reader (SpectraMax – Molecular Devices, San José, USA) at 492 nm [24]. Three independent experiments were performed, each of which was carried out in triplicate.

2.5. Analysis of biofilm by counting CFU/mL

Immediately after the end of the treatment, the wells were washed with PBS, receiving 0.2 mL of PBS. The biofilm was disrupted by multiple pipetting and vortexing for 1 min. Then, an aliquot of 20 μL of each sample was diluted (10^{-1} to 10^{-5}). An aliquot of 20 μL of each dilution was seeded in lines into Petri dishes containing Sabouraud dextrose agar, which were incubated at 37 °C for 24 h [13,29]. After incubation, the CFU/mL values were determined. For statistical analysis and comparison between groups, the CFU/mL data were converted to the linear scale. Three independents experiments were performed, each of which was carried out in triplicate.

2.6. Analysis of biofilm by filamentation assay

The filamentation assay was performed following a methodology described previously [26]. After treatment and CFU/mL count, colonies of *C. albicans* were incubated at 37 °C in Falcon tubes with 10 mL of Sabouraud dextrose broth medium for 24 h. These tubes were then centrifuged (1300 x g 10 min) once, and the number of *C. albicans* was standardized in a Neubauer chamber.

The *C. albicans* suspension (10^7 cells/mL) was mixed with 1 mL of distilled water with 10% FBS in a 24-well plate (Sarstedt, Nümbrecht, Germany). The plate was incubated at 37 °C for filamentation. After 24 h of incubation, 50 μL from the inoculum of each well were transferred to glass slides previously demarcated in 10 similar areas on the back of the slide. In each area, a randomly chosen microscopic field was observed under a light microscope Nikon Eclipse E200 (Tokyo, Japan) at 40x magnification and analyzed regarding *C. albicans* hyphae morphology. The following scores were attributed to the number of hyphae present in each microscopic field: 0, no hyphae; 1, 1–10 hyphae; 2, 11–20 hyphae; 3, 21–30 hyphae; 4, 31–40 hyphae; and 5, more than 41 hyphae. Two independent experiments were performed. Considering all the incubations carried out during this method, the filamentation analysis was performed 72 h after PDI.

2.7. Analysis of biofilm by CV staining assay

Crystal violet (Synth, Diadema, Brazil) staining was used to evaluate the total biomass. Immediately after PDI, the biofilm was gently washed with PBS, fixed in methanol for 20 min, and dried in air for 30 min. The samples were then stained for 15 min with 0.05% CV, and the CV solution was then removed and the samples gently washed in water. Finally, ethanol was used to dissolve the bound CV for 30 min, and the absorbance was measured using a microplate reader (SpectraMax – Molecular Devices, San José, USA) using a 492 nm filter. The data are presented as a percentage of the control (% CT), calculated by $A_{sample}*100/A_{CT}$.

2.8. Analysis of biofilm by SEM

Based upon the methodologies previously applied, acrylic resin discs of 11 mm diameter with a pin (Clássico, São Paulo, Brazil) were used as substrates for growing the biofilm [30]. Each substrate received two layers of nail polish (Risqué, São Paulo, Brazil), in the pin and base. These discs were placed in 24-well plates for biofilm formation, as previously reported. Immediately after PDI, the discs were fixed with 1 mL of glutaraldehyde at 2.5% for 1 h and then dehydrated with several ethanol washes (10%, 25%, 50%, 75%, and 90% for 20 min, and 100% for 1 h). The samples were then incubated at 37 °C for 24 h to dry the discs. No further sample preparation was needed. The biofilms were examined and photographed by low-vacuum scanning electronic microscopy (SEM, TableTop Microscope, TM3000, Hitashi, Tokyo, Japan), operating at 15 kV, in increments of 1000 and 5000 times [31]. Experiments were performed in triplicate on two different days.

2.9. Statistical analysis

The statistical analysis was performed using Microcal Origin software, and the Kolmogorov-Smirnov test was employed to evaluate data normality. For log CFU/mL and the CV assay, the gaussian data were compared by One-Way ANOVA followed by the Tukey post-hoc test; for XTT analysis, the gaussian data were analyzed by Two-Way Repeated measures ANOVA, followed by the Tukey post-hoc test. In the case of the filamentation test, the Kruskal-Wallis test was used for comparison of the non-parametric data, followed by the Mann-Whitney test. Differences between groups with $p<0.05$ were considered statistically significant.

3. Results

The results showed that MB-OF mediated PDI on *C. albicans* biofilm was able to reduce around 1.5 $\log_{10}$ of CFU/mL, while no effect was observed in the OF group compared to the CT group (Fig. 1a). No effects were observed in the dark controls (data not shown).

Although PDI significantly reduced the mitochondrial metabolic

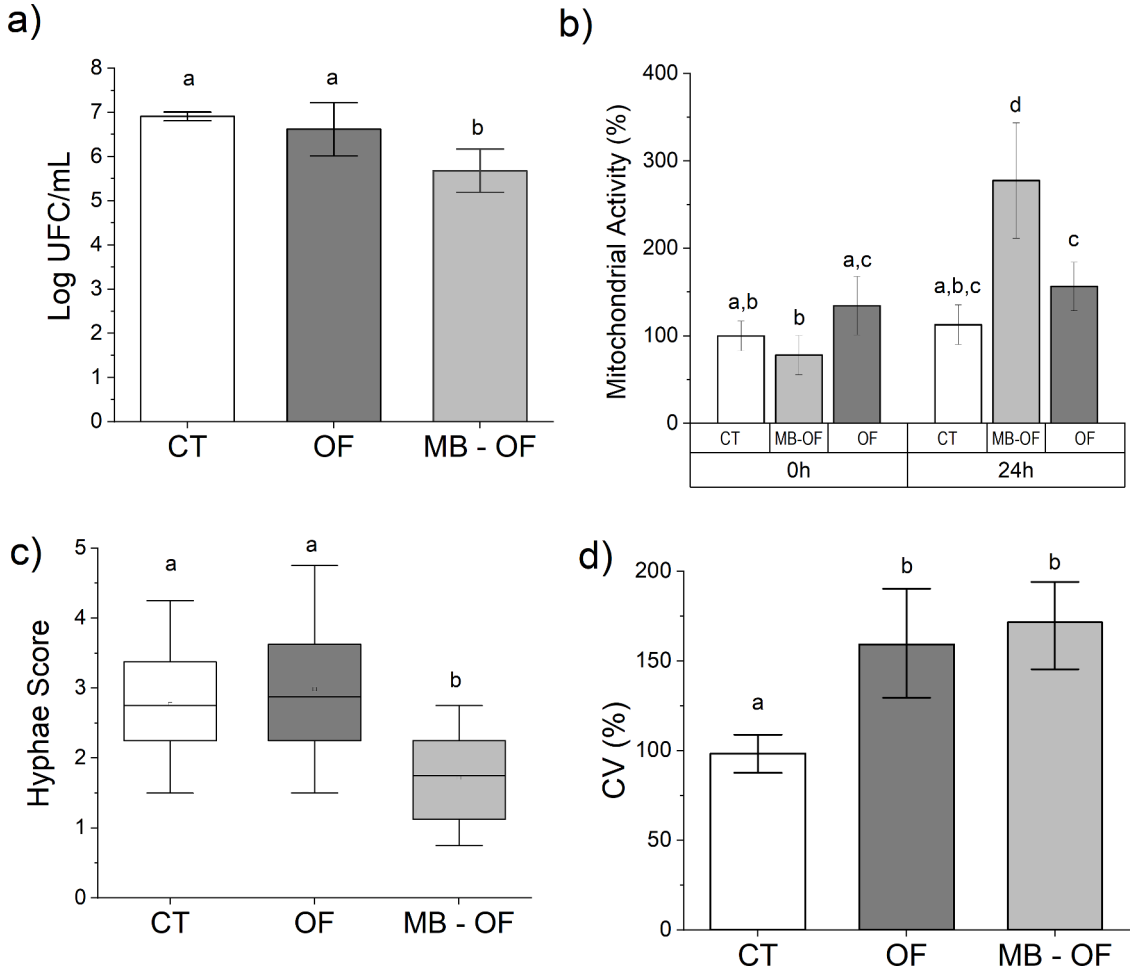

Fig. 1. Biofilm analysis after PDI treatment. Cells were treated with physiological solution (NaCl 0.9%) – CT (control) group; OF (oral formulation without MB); MB-OF (0.005% MB in oral formulation). a) CFU/mL count of *C. albicans* grown on Sabouraud agar. b) Evaluation of mitochondrial activity by XTT assay, immediately after PDI (0 h) and 24 h after PDI. c) Hyphae count in filamented samples 72 h post-PDI. d) Crystal violet staining for biomass analysis, immediately after PDI. Groups sharing the same letters present no statistically significant differences.

activity immediately after light exposure (0 h) compared to the OF group, no statistically significant differences were noted between the MB-OF and CT groups (Fig. 1b). However, 24 h after PDI, metabolic activity increased substantially in the MB-OF group, to around three times.

MB-OF-mediated PDI caused a significant reduction in yeast to hyphae transition, 72 h after irradiation (Fig. 1c). This finding suggests that PDI can prevent hyphae formation, thus reducing the virulence factors and infection ability.

The biofilms treated with OF and MB-OF immediately after PDI exhibited an increase in total biomass. This was a surprisingly result in comparison to others found here, since colony forming and hyphae numbers were reduced.

SEM was used to evaluate the biofilm architecture and cellular morphology, as displayed in Fig. 2. Our results showed that the CT group presented a pronounced number of hyphae, pseudohyphae, and yeasts with round morphology – the blastospores (Fig. 2A). In the OF (Fig. 2B) and MB-OF (Fig. 2C) groups, the biofilm exhibited flattened cells and a drastically reduced number of yeasts, which were smaller in the MB-OF group.

4. Discussion

Different methodologies can be designed to evaluate the antimicrobial action of pharmaceuticals on biofilms [8]. Thus, assessing a new photosensitizer, formulation, and/or drug delivery system for PDI may be a challenge. In this work, we used a range of assays to verify the PDI impact of an oral formulation containing MB. We noticed that methodologies may complement each other, and also that formulation substances may interfere with the results.

Here, we submitted *C. albicans* biofilm to MB-OF-mediated PDI. We used an OF with MB as the active compound, and SDS as a surfactant to reduce MB aggregation [12,13,32]. For this, the PDI group (0.005% MB-OF) was compared to either OF without photosensitizer or to CT with saline solution. The improvement of MB aggregation, promoted by its association to SDS in the OF, has been shown to be an effective alternative to potentiate PDI efficacy in vitro in planktonic *C. albicans* cultures [12]. However, the effects on biofilm needed further investigation.

The CFU/mL assay is the most widely used methodology to analyze the effect of antifungal strategies [25]. This technique allows the quantification of all viable cells, including the low metabolism ones, and excluding dead cells and debris [33]. However, CFU may have sub-counts in its quantification [34,35]. Indeed, the biofilm needs to be disorganized before dilutions, and the non-homogeneous disruption of biofilm, together with the serial dilutions, can bring strong variability to the data. Additionally, due to the method itself, sample collection occurs immediately after PDI, but the data may only be analyzed 24 h later, due to the time needed for colony growth. Using the CFU method, we could note that MB-OF was effective in reducing the viable cells of the biofilm immediately after PDI.

SEM was used to visualize the structure of the biofilm, to address possible morphological changes in the cells and the biofilm structure before and after treatment. However, this method mostly involves qualitative analysis. Indeed, we observed flattened and languished cells immediately following PDI, which corroborates the cell killing provided by PDI (around 95%). We also noticed surviving cells, which could regrow to keep the biolfim structure.

XTT data, collected at two different time points, confirmed this hypothesis. We observed no differences in cell metabolic activity immediately after PDI compared to the control, but increased metabolic activity was noted 24 h after PDI. These findings suggest that the surviving cells could be persister cells, whose behavior is not fully understood, even though their presence is associated with a biphasic killing of the yeast [36]. Indeed, yeast cells respond to stress, showing metabolic changes, and it is known that PDI action promotes pronounced oxidative stress.

Although the literature indicates that the XTT assay correlates with CFU/mL results [37,38], our study did not reproduce this finding. Our data matched that reported by Zago and collaborators, who suggested that CFU/mL and XTT analyses are complementary [33]. Furthermore, it is important to keep in mind the time point evaluated. Indeed, we observed the immediate effect of PDI by XTT, as well as the cell behavior after the strong oxidative stress promoted by PDI. Besides, both techniques, CFU/mL and XTT, could be used to evaluate the adherence inhibition, biofilm development inhibition, or biofilm disruption, depending on the PDI application during the time course of the biofilm formation (adherence – first 90 min, maturation – first 24 h, or mature biofilm – after 24 h) [39].

Bapat and colleagues suggested that the evaluation of XTT metabolic activity in PDI studies can show strong interference in optical density measurements since the photosensitizers absorb visible light [39]. This is completely relevant when evaluating PDI. However, in our study, the photosensitizer solution was removed before incubation in XTT; in this way, the effect was minimized. Besides, XTT absorption was measured at 492 nm, while MB absorbs light at higher wavelengths.

On the hand, data from the CV assay was strongly influenced by the OF. This assay is based on the detachment of adherent cells from the cell culture plates during cell death, with CV staining the remaining attached live cells. Our data showed an increased biomass immediately after PDI in both OF and MB-OF. This data was surprising, since other data showed a reduction in viable cells (CFU/mL) and also a reduced number of yeasts in the biofilm structure (SEM). Some hypotheses were raised regarding this result. First, the SDS contained at the OF interacted with CV, affecting the spectroscopic features. It has been shown previously that CV and SDS interact by forming ion-pairs, while CV is incorporated into the micellar surface at higher SDS concentrations [40]. These interactions affect the CV absorption spectra and molar absortivities, thus affecting the measurement. Second, since CV binds to negatively charged molecules, the SDS present at the OF could interact in some way, to the cell wall or extracellular matrix, and attract CV molecules to bind, increasing the total amount of bound CV, without a real increase in

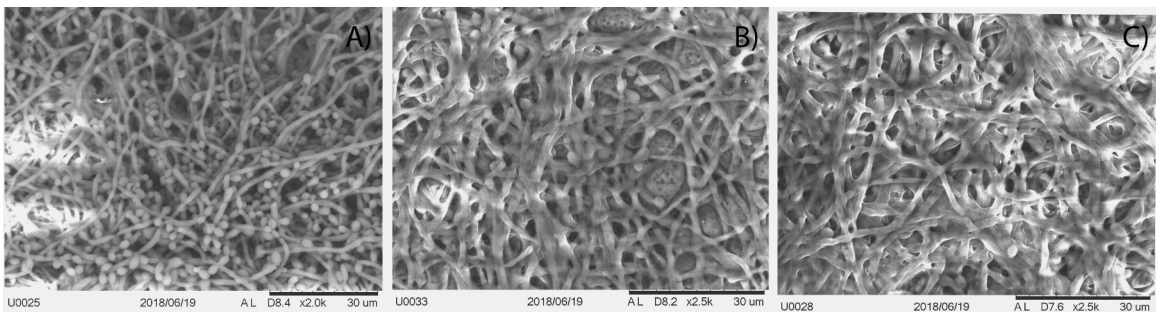

Fig. 2. Electron-micrographs of *C. albicans* biofilms treated by PDI. A) Control group (CT - physiological solution), B) oral formulation (OF), C) 0.005% methylene blue in oral formulation (MB–OF).

biomass. SDS is known to bind to proteins [41], thus reinforcing this hypothesis.

Filamentation is an important assay, since it allows identification of the ability to form hyphae, correlating it with virulence [33,42,43]. This is because the hyphae have a morphology that favors tissue penetration and the formation of a mature biofilm [26,44,45]. In addition, this morphology avoids the mechanism of death by host phagocytic cells. Phagocytosis induces the transition from yeast to hyphae, in which the cell can perforate the macrophage membrane. After lysis and death of the macrophages, *C. albicans* can evade the host's immune defenses [26, 44–46]. Our data showed that MB-OF mediated PDI was able to reduce virulence due to the smaller capacity of hyphae formation. This reduction was observed by filamentation assay but not in the SEM images. However, it is noteworthy that the hyphae were already present in the biofilm structure submitted to PDI in the SEM images, while yeasts were induced to hyphae transition in the filamentation assay.

This work highlights the importance of using different methodologies to assess the PDI. Furthermore, the use of formulations, nanoparticles, liposomes, and other drug delivery systems increases the complexity of the technique and can bring unexpected results. It is essential to gather data from various methodologies and also from different moments to obtain a complete understanding and to advance the use of PDI in mainstream medicine.

5. Conclusion

Understanding the therapeutic potential of new photosensitizers and/or formulations/drug delivery systems for PDI is challenging. Various methods are known; however, isolated assays may not bring conclusive information due to the possibility of an interaction between the photosensitizer or formulation components and the assay. The evaluated time point is also essential. In this study, we noticed that MB-OF-mediated PDI significantly reduced CFU/mL and decreased virulence, even though the surviving yeast cells presented higher mitochondrial metabolic activity after 24 h, in an attempt to regrow biofilm. This could be indicative of the need for extra and/or multiple applications of this therapy. In contrast, CV data were inconclusive, since the SDS present in the OF interacted with CV, making the data useless. Taken together, our data suggest that the association of different methods allows complementary responses to the assessment of the impact of PDI on biofilms.

Declarations of interest

None

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