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ORIGINAL ARTICLE

# Photodynamic inactivation of a multispecies biofilm using curcumin and LED light

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**Abstract** This study evaluated the potential of curcumin-mediated antimicrobial photodynamic inactivation (API) on multispecies biofilms of *Candida albicans*, *Candida glabrata*, and *Streptococcus mutans* of different ages. Acrylic samples ($n = 480$) were made with standardized rough surfaces and incubated with bacteria and yeast for 24 or 48 h. API was performed with curcumin (80, 100, 120 μM) and LED light. Additional acrylic samples were treated with curcumin or LED light only. Positive control samples received neither light nor curcumin. After API, colony counts were quantified (CFU/mL), cell metabolism was determined by means of XTT assay, and the total biofilm biomass was evaluated using Crystal Violet (CV) staining assay and images were obtained by confocal laser scanning microscopy (CLSM). The data were analyzed by nonparametric two-way ANOVA and post hoc Tukey tests ($\alpha < 0.05$). For 24-h biofilm, API resulted in statistically significant difference ($\rho < 0.001$) of viability of *C. albicans* compared with control (P−L−) for all Cur concentrations. For 48-h biofilm, API resulted in statistically significant difference ($\rho < 0.001$) compared with control only when Cur at 120 μM was used. API promoted statistically significant difference ($\rho \leq 0.001$) in the viability of *S. mutans* and *C. glabrata* for all Cur concentrations in the two biofilm ages. In addition, API produced a statistically significant difference ($\rho < 0.001$) of metabolic activity and of total biomass ($\rho < 0.001$) of multispecies biofilms compared with control for all Cur concentrations. It can be concluded that both 24- and 48-h biofilms were susceptible to API mediated by Cur; however, 24-h biofilm was more sensitive than the 48-h biofilm.

**Keywords** Photodynamic inactivation · Multispecies biofilm · Fungi · Bacteria

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## Introduction

The disease most commonly found in the oral mucosa of patients wearing removable dental prostheses is denominated denture stomatitis (DS) [1, 2]. The etiology of DS is considered multifactorial; however, infection by *Candida* spp., especially *Candida albicans*, is considered the main etiologic factor [3, 4]. The species of the genus *Candida* are capable of adhering directly to the denture surface, or by means of a bacterial layer primarily formed on this surface [1, 5]. The continuous denture wearing associated with inadequate cleaning promotes conditions favorable to the proliferation of microorganisms [6]. Baena-Monroy et al. [7] demonstrated high prevalence of the microorganisms *C. albicans*, *Streptococcus mutans*, and *Staphylococcus aureus* (66.7, 49.5, and 49.5.4 %, respectively) on surface of complete dentures. In a previous study, it was observed that *Candida* spp. were the most predominant microorganism (60.0 %) on dentures, followed by mutans Streptococci (53.3 %) and *Staphylococcus* spp. (48.3 %). Among the dentures positive for *Candida* spp., three species were identified: *C. albicans* was the

most frequent yeast, followed by *Candida glabrata* and *Candida tropicalis*, respectively [8].

The biofilm adhered to the surface of dentures is characterized as being a complex microbial community, surrounded by a polymeric matrix, favoring the proliferation and survival of these microorganisms [9–12]. Innumerable mechanisms contribute to these microorganisms organized in biofilm presenting greater resistance to antimicrobial agents and being protected from the action of the host immunologic system [13, 14]. Different treatments have been considered for DS, involving the prescription of topical and systemic antifungal medications and procedures for cleaning and disinfection of the dentures [15–17]. However, a high rate of recurrence of the infection has been observed [17–19]. The use of azole substances associated with failures in the therapeutic procedures may culminate in the resistance of microorganism to these drugs, which contributes to the failure of conventional therapies [20–22].

Due to the difficulties found in the treatment of oral infections using conventional treatments, antimicrobial photodynamic inactivation (API) has been used as an alternative for the inactivation of microorganisms. The photodynamic therapy has been used for more than 100 years for inactivating microorganisms, when Oscar Raab described for the first time the lethal effect of acridine hydrochloride and visible light on *Paramecia caudatum*. The photodynamic process requires the use of a chemical compound-denominated photosensitizer (PS), with the subsequent application of a light that corresponds to the absorption band of the PS in the presence of oxygen [23]. The interaction of the light with the PS results in oxygen reactive species capable of inducing inactivation of microbial cells [24–26]. In vitro studies have been demonstrated that photodynamic therapy is effective in killing *Candida* spp. in planktonic cultures [27–34] and reducing biofilm [35, 36] formation using methylene blue [29, 30, 32–34, 37, 38], toluidine blue O [32, 34, 36, 39], photofrin [35], tionin [34], porphyrins [28], phthalocyanine [31, 34], and malachite green [32]. In general, the studies that have evaluated API have related that their greatest efficacy occurs when the microorganism is presented in the planktonic form, promoting their complete photoinactivation [26, 40]. Nevertheless, when microorganisms are organized in biofilm, only reduction in the number of microorganisms has been observed and the complete elimination was not achieved [26, 40–43]. According to Ramage et al. [12], there is a linear relationship between the increase in cell density, resulting from the time for the development of the biofilm, and the increase in its metabolic activity. These authors have also observed an increase in the resistance of mature biofilms to conventional antifungal treatment when compared with planktonic cultures. However, studies evaluating the efficacy of API considering different times of biofilm formation were not found.

Another factor that may influence the efficacy of API is the interaction between the different species. Pereira et al. [44] observed that photoactivation mediated by methylene blue and laser light of biofilms formed by *C. albicans*, *S. aureus*, and *S. mutans* was greater in single-species biofilms (2.32 to 3.29 $\log_{10}$ of reduction) when compared with multispecies biofilms (1.00 to 2.44 $\log_{10}$ of reduction). Quishida et al. [43], using different concentrations of Photodithazine® associated with LED light, observed greater bacterial inactivation compared with the fungal species of multispecies biofilms of *S. mutans*, *C. albicans*, and *Candida glabrata*.

Different PS have been employed for API, among them curcumin (diferuloylmethane, Cur), an extract from the rhizome of the plant *Curcuma longa* L. [45]. Various studies have suggested that Cur has anti-inflammatory, anticarcinogenic, immunomodulatory, and antimicrobial effects [46–48], which may be exacerbated by means of illuminating it at an adequate wavelength [49–51]. Dovigo et al. [41] demonstrated that the association between Cur at the concentration of 20 μM and light dose of 5.28 J/cm$^2$ promoted complete inactivation of a planktonic suspension of *C. albicans*. However, for reducing the cell metabolism of the fungal biofilm, it was necessary to have a higher concentration of Cur (40 μM) with the same light dose, demonstrating lower susceptibility of the biofilm to API (the metabolic activity of *C. albicans*, *C. glabrata*, and *C. tropicalis* biofilms was reduced by 85, 85, and 73 %, respectively, at 18 J/cm$^{-2}$). Pileggi et al. [52] verified that the PSs eosin-Y, rose bengal, and Cur in the concentrations of 5, 1, and 5 μM, respectively, were capable of significantly reducing (Log CFU/mL) the planktonic culture of *Enterococcus faecalis* after the application of blue light (from 7.8 ± 0.70 for Controls—no light to 2.9 ± 0.30 for Eosin 5 M, 0.5 ± 0.30 for rose bengal 1 M and 0.15 ± 0.36 for Cur). For bacterial biofilm, the concentrations of 100, 10, and 10 μM of the PSs Eosin-Y, rose bengal, and Cur, respectively, resulted in complete suppression of the viability of *E. faecalis*. Araújo et al. [53] observed that the exposure of the biofilm to 0.75, 1.5, and 3.0 g/L of Cur and subsequent illumination with light resulted in 97.5, 95, and 99.9 % reductions in viable cells (respectively) in the multispecies biofilm of *Streptococcus mutans* and *Lactobacillus acidophilus* formed on dentin samples. When the Cur concentration was 4.0 and 5.0 g/L, a decrease of 100 % was obtained.

Despite this, the investigation of the photodynamic ability of Cur against multispecies biofilm composed by bacteria and yeast of different ages could demonstrate better its antimicrobial potential for a further clinical evaluation. Besides, this kind of multispecies biofilm on acrylic resin should be investigated in order to mimic a biofilm found on dentures, where bacteria and yeasts are found. Therefore, the aim of this study

was to evaluate the capacity of API mediated by the PS CUR for the inactivation of multispecies biofilm of *C. albicans*, *C. glabrata*, and *S. mutans*, formed on denture base acrylic resin for different periods of time.

## Material and methods

### Preparation of acrylic resin samples

The samples ($n = 480$) were fabricated from an acrylic denture base resin (Vipi Wave; VIPI Indústria e Comércio Exportação e Importação de Produtos Odontológicos Ltda, Pirassununga, SP, Brazil), in accordance with the manufacturer's specifications. The resin discs were fabricated using a metal mold with compartments (10 × 2 mm). This metal mold was invested in the flask in dental stone sandwiched between two glass slabs that were sandblasted with aluminum oxide to obtain rough specimens with a roughness of approximately 3 μm [54]. The acrylic resin polymerization cycle was performed in a 500-W domestic microwave oven (Brastemp, Manaus, AM, Brazil) at 20 % power for 20 min, and subsequently at 90 % power for 5 min. The flasks were allowed to bench cool at room temperature; the specimens were deflasked, and excess flash was removed with a bur (Maxi-Cut; Lesfils de August Malleifer S.A., Ballaigues, Switzerland).

The surface roughness of all specimens was measured with a profilometer (Mitutoyo SJ 400, Mitutoyo Corporation, Japan). Four measurements were made for each specimen and the average reading was designated as the Ra (μm) value of the specimen. The resolution was 0.01 μm, interval (cutoff length) 0.8 mm, transverse length 2.4 mm, stylus speed 1 mm/s, and the diamond stylus tip radius was 5 μm. Only specimens with surface roughness between 2.7 and 3.7 mm (Ra) were selected for the study, in order to simulate the internal surface of the denture [55]. After this, the samples were sterilized with ethylene oxide at the ACECIL (Central de Esterilização Com. Ind. Ltda, Campinas, SP) facilities.

### Biofilm production

In this study, the microorganisms used to produce multispecies biofilm were *C. albicans* (ATCC 90028), *Candida glabrata* (ATCC 2001), and *Streptococcus mutans* (ATCC 25175) obtained from the American Type Culture Collection strains (ATCC; Rockville, MD, USA). *C. albicans* and *C. glabrata* were seeded on Sabouraud Dextrose Agar (SDA, Acumedia Manufacturers Inc., Baltimore, MD, USA) with 5 μg/mL of chloramphenicol and *S. mutans* on Mitis-Salivarius Agar (Difco, Laboratories, Detroit, MI, USA) supplemented with 15 % sucrose and 0.2 IU/mL bacitracin (MSB plates), which were incubated at 37 °C for 48 h. To obtain the standardized suspension of each microorganism, the yeasts were individually inoculated in Yeast Nitrogen Base (YNB) medium supplemented with 100 mM glucose and the bacteria were inoculated in Brain Heart Infusion (BHI, Himedia Laboratories, Mumbai, India). All microorganisms were incubated at 37 °C overnight under appropriate conditions. In all experiments, *S. mutans* was incubated in candle jar. Microbial cells were harvested, washed twice with phosphate-buffered saline (PBS, pH 7.2), and resuspended in BHI as previously described in the study of Quishida et al. [43]. After this, the microbial suspensions were spectrophotometrically standardized at concentration of $10^7$ cells/mL for *Candida* species and $10^8$ cells/mL for *S. mutans*, using wavelengths of 520 and 600 nm, respectively [56]. Aliquots of 500 μL of each standardized cell suspension were added to each well of a 24-well microtitler plate containing an acrylic resin specimen, which were incubated at 37 °C in orbital shaker at 75 rpm (adhesion phase) for 90 min [57]. The nonadherent cells were removed by washing samples twice with PBS. For biofilm formation, 2 mL of BHI medium was added in each well and the plates were incubated at 37 °C in orbital shaker at 75 rpm for a period of 24 or 48 h. The negative control groups consisted of BHI medium without microorganisms. Experiments were repeated three times with individual samples in triplicate.

### API

The Cur used as photosensitizer (PS) to sensitize the microbial biofilm was obtained from Sigma-Aldrich (St. Louis, Missouri, USA). A stock solution of Cur (800 μM) was obtained by dissolving it in dimethylsulfoxide (DMSO) at 10 %. Immediately before the experiment, this solution was diluted in physiological solution (0.85 % NaCl) to the final concentrations of 80, 100, and 120 μM. The 10 % concentration of DMSO was maintained in the final solutions, and it was selected on the basis of a previous study that had shown that it had no effect on *Candida* viability and no influence on API results [41]. In addition, histological analysis also revealed that this solution had no adverse effects on the tissue of mice when it was applied topically [58]. Cur was exposed to light-emitting diode (LEDs, LXHL-PR09, Luxeon® III emitter, Lumileds Lighting, San Jose, CA) in the blue region, and with emission from 440 to 460 nm, the intensity of light emitted was 22 mW/cm$^2$ and fluence was 37.5 J/cm$^2$.

API was performed by the administration of Cur and exposure to 37.5 J/cm$^2$ of LED light (P+L+ group). After biofilm formation (24 or 48 h), the samples were washed twice with PBS, and according to the experimental groups described, 2 mL of Cur was added to groups P+L+ (24 h), P+L− (24 h), P+L+ (48 h), and P+L− (48 h), while 2 mL of physiological solution was added to groups P−L+ (24 h), P−L+ (48 h), P−L− (24 h), and P−L− (48 h). Next, the plates were incubated in the dark for 20 min (pre-irradiation time) [41]. After this period, the groups

P+L+ (24 h), P+L+ (48 h), P−L+ (24 h), and P−L+ (48 h) were exposed to LED light, illuminating samples for 29 min (37.5 J/cm$^2$). Additional samples were treated either with Cur (P+L−) or LED light (P−L+) only. Untreated control samples received neither light nor Cur (P−L−).

After the samples had been submitted to the experimental conditions, the following biofilm viability evaluations were performed:

### Quantification of colonies

Each specimen was individually placed in a centrifuge tube containing 4.5 mL of sterile physiological solution, and these tubes were vortexed for 1 min to detach the biofilms from the acrylic samples [59]. After this, aliquots of 25 μL of serial dilutions ($10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$) were seeded in duplicate on CHROMagar *Candida* (Difco, Laboratories, Detroit, MI, USA) and MSB (Mitis Salivarius Agar supplemented with 15 % of sucrose and 0.2 IU/mL bacitracin) for the identification of *Candida* spp. and *S. mutans*, respectively. Green and pink colonies grown on CHROMAgar *Candida* were presumptively identified as *C. albicans* and *C. glabrata*, respectively. After incubation at 37 °C for 48 h, the colony-forming unit per milliliter (CFU/mL) was determined and log-transformed ($\log_{10}$).

### Metabolic activity (XTT reduction assay)

The effect of API on the metabolic activity of the multi-species biofilms was measured by XTT reduction assay. After treatments, acrylic samples with biofilms were transferred to another plate with wells containing 2 mL of XTT solution {containing 1580 μL PBS with 200 mM glucose, 400 μL XTT 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide, and 20 μL menadione}. The plates were incubated in the dark at 37 °C for 3 h. After this, 200 μL of the solution was transferred to a 96-well microtitler plate and colorimetric measurement was read at 492 nm by means of a spectrophotometer (Thermo Plate—TP Reader) [60].

### Total biomass quantification

Crystal Violet (CV) staining was performed for the quantification of total biomass. Acrylic samples were washed with PBS and then fixed with 2 mL of methanol for 15 min. Acrylic samples with fixed biofilms were removed, transferred to another plate, and allowed to dry at room temperature. After drying, 2 mL of CV (1 %, *v*/*v*) was added to the wells and incubated for 5 min. Acrylic samples were washed with PBS and transferred to another plate with 2 mL of acetic acid (33 %, *v*/*v*) in each well in order to dissolve the stain. After this period, an aliquot of 200 μL was transferred to a 96-well microtitler plate and the absorbance of the final solution was read at 570 nm by means of a spectrophotometer (Thermo Plate—TP Reader) [61].

### CLSM

The confocal laser scanning microscopy (CLSM) images were obtained of the groups P+L+ (24 h) and P+L+ (48 h) with 120 μM of curcumin because they showed greater microbial inactivation, and these groups were compared with the positive control groups [P−L− (24 h) and P−L− (48 h)]. In addition, an image of the group P+L− with 120 μM of curcumin was obtained, because the presence of 120 μM Cur, in the dark, promoted microbial reduction. After treatment, acrylic samples with biofilm were washed twice with PBS and stained with the Live/Dead BacLight viability kit containing SYTO-9 and Propidium Iodide (PI) (Molecular Probes, Inc., Eugene, OR, USA). Biofilms were stained in the dark and incubated at room temperature for 15 min, according to the manufacturer's instructions. The maximum excitation/emission used for these stains was approximately 480/500 nm for SYTO-9 stain and 490/635 nm for PI [43]. Images were obtained with a Leica TCS SPE confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

### Statistical analysis

All experiments were conducted using three specimens on three independent occasions. CFU/mL values were log-transformed ($\log_{10}$). Since the assumptions of normality and homoscedasticity were not always met, a nonparametric two-way ANOVA (using the groups of treatment and biofilm age as independent factors) followed by the Tukey test for multiple comparisons was used to analyze the data [ranks of $\log_{10}$(CFU/mL) and absorbance values]. The significance level was 0.05, and SPSS software (IBM® SPSS® Statistics, version 20, Chicago, IL) was used.

## Results

### Quantification of colonies

For *C. albicans*, ANOVA on ranks showed that biofilm age was not a significant factor ($\rho = 0.472$), while groups of treatment and the interaction between biofilm age and groups of treatment were significant factors ($\rho < 0.001$). When biofilms were cultivated for 24 h, API (P+L+) resulted in significant difference ($\rho < 0.001$) compared with control (P−L−) for all

Cur concentrations, which showed no significant difference ($\rho \geq 0.998$) among them. The application of light only did not affect the viability of *C. albicans* ($\rho = 1.000$), but treatment with Cur alone at 100 and 120 μM demonstrated significant difference ($\rho \leq 0.001$) compared with control (P−L−). For 48-h biofilm, API (P+L+) resulted in significant difference ($\rho < 0.001$) compared with control (P−L−) only when Cur at 120 μM was used. Application of light alone (P−L+) and Cur alone (P+L−) at all concentrations evaluated, and API with Cur at 80 and 100 μM did not show significant difference ($\rho \geq 0.989$). API with Cur at 120 μM showed no significant difference ($P = 1.000$) between the two biofilm ages (24 and 48 h) (Fig. 1).

For *C. glabrata*, ANOVA on ranks demonstrated significant effects of biofilm age ($P < 0.001$), groups of treatment ($P < 0.001$), and the interaction between these factors ($\rho = 0.038$). API (P+L+) mediated by Cur at all concentrations evaluated resulted in significant difference ($\rho < 0.001$) compared with control (P−L−) for the two biofilm ages (24 and 48 h). Concentrations of Cur showed no significant difference ($\rho \geq 0.999$) among API (P+L+) groups for the two biofilm ages. When biofilms (24 and 48 h) were treated with light only (P−L+) or Cur only (P+L−), no significant difference ($\rho \geq 0.085$) was found compared with control (P−L−) and among them, except for Cur only at 120 μM, which showed significant difference compared with control ($\rho = 0.001$) and light only ($\rho = 0.004$) for 24-h biofilm. Controls (P−L−) of the two biofilm ages (24 and 48 h) showed no significant difference between them ($\rho = 0.999$), nor did the API groups (P+L+) of the two biofilm ages ($\rho \geq 0.128$) (Fig. 2).

For *S. mutans*, ANOVA on ranks also showed significant effect for biofilm age ($\rho = 0.001$), groups of treatment ($\rho < 0.001$), and the interaction between them ($\rho < 0.001$). API resulted in significant difference ($\rho \leq 0.001$) for all Cur concentrations in the two biofilm ages. No significant difference ($\rho \geq 0.055$) was observed for P−L− (controls), P−L+, and P+L− groups for all Cur concentrations in the two biofilm ages, except for P+L− group with Cur 120 μM, which showed significant difference compared with control (P−L−, $\rho = 0.010$) and P−L+ ($\rho = 0.002$) for 24-h biofilm. For the two biofilm ages (24 and 48 h), no significant difference ($\rho = 1.000$) was verified among API (P+L+) groups when Cur concentrations were compared. Control (P−L−) groups of the two biofilm ages showed no significant ($\rho = 1.000$) difference between them, nor did the API groups (P+L+) of the two biofilm ages ($\rho \geq 0.942$) (Fig. 3).

## Metabolic activity (XTT reduction assay)

ANOVA on ranks demonstrated a significant effect of biofilm age ($\rho < 0.001$), groups of treatment ($\rho < 0.001$), and the interaction between them ($\rho = 0.026$). For 24-h biofilm, a significant difference ($\rho < 0.001$) of absorbance values was verified when API groups (P+L+) with all Cur concentrations were compared with control (P−L−). No significant difference ($\rho \geq 0.102$) was observed among control (P−L−), samples

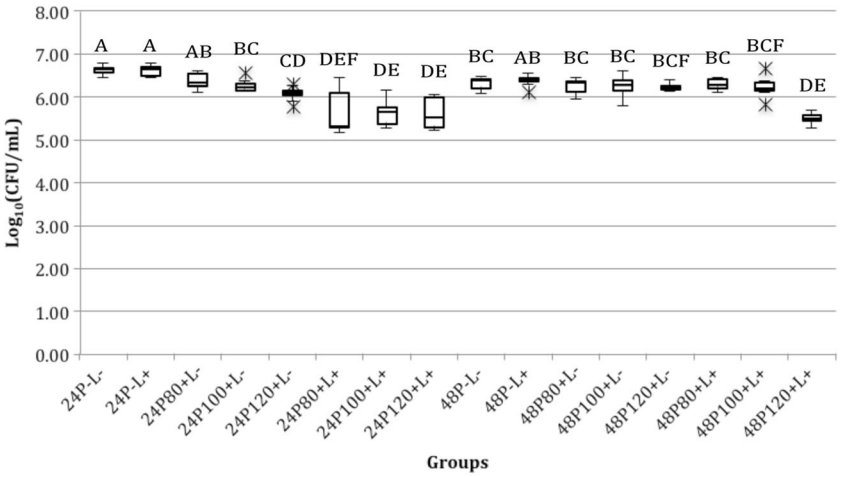


**Fig. 1** Summary of $\log_{10}$CFU/mL values of *C. albicans*. Box plots show the median (*dash*), first and third quartiles (*box*), highest and lowest values (*error bars*), and outliers (*asterisk*). 24P−L−: control group of 24-h multispecies biofilm. 24P−L+: 24-h multispecies biofilm treated with light only (37.5 J/cm$^2$). 24P80+L−: 24-h multispecies biofilm treated with Cur only at 80 μM. 24P100+L−: 24-h multispecies biofilm treated with Cur only at 100 μM. 24P120+L−: 24-h multispecies biofilm treated with Cur only at 120 μM. 24P80+L+: 24-h multispecies biofilm submitted to API with Cur at 80 μM. 24P100+L+: 24-h multispecies biofilm submitted to API with Cur at 100 μM. 24P120+L+: 24-h multispecies biofilm submitted to API with Cur at 120 μM. 48P−L−: control group of 48-h multispecies biofilm. 48P−L+: 48-h multispecies biofilm treated with light only (37.5 J/cm$^2$). 48P80+L−: 48-h multispecies biofilm treated with Cur only at 80 μM. 48P100+L−: 48-h multispecies biofilm treated with Cur only at 100 μM. 48P120+L−: 48-h multispecies biofilm treated with Cur only at 120 μM. 48P80+L+: 48-h multispecies biofilm submitted to API with Cur at 80 μM. 48P100+L+: 48-h multispecies biofilm submitted to API with Cur at 100 μM. 48P120+L+: 48-h multispecies biofilm submitted to API with Cur at 120 μM. *Different letters* denote significant difference ($P < 0.05$) according to the post hoc test

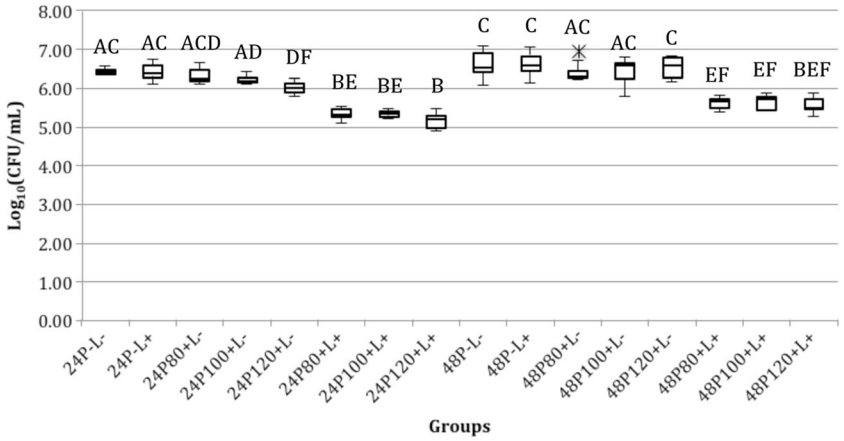


**Fig. 2** Summary of $\log_{10}$CFU/mL values of *C. glabrata*. Box plots show the median (*dash*), first and third quartiles (*box*), highest and lowest values (*error bars*), and outliers (*asterisk*). 24P−L−: control group of 24-h multispecies biofilm. 24P−L+: 24-h multispecies biofilm treated with light only (37.5 J/cm$^2$). 24P80+L−: 24-h multispecies biofilm treated with Cur only at 80 μM. 24P100+L−: 24-h multispecies biofilm treated with Cur only at 100 μM. 24P120+L−: 24-h multispecies biofilm treated with Cur only at 120 μM. 24P80+L+: 24-h multispecies biofilm submitted to API with Cur at 80 μM. 24P100+L+: 24-h multispecies biofilm submitted to API with Cur at 100 μM. 24P120+L+: 24-h multispecies biofilm submitted to API with Cur at 120 μM. 48P−L−: control group of 48-h multispecies biofilm. 48P−L+: 48-h multispecies biofilm treated with light only (37.5 J/cm$^2$). 48P80+L−: 48-h multispecies biofilm treated with Cur only at 80 μM. 48P100+L−: 48-h multispecies biofilm treated with Cur only at 100 μM. 48P120+L−: 48-h multispecies biofilm treated with Cur only at 120 μM. 48P80+L+: 48-h multispecies biofilm submitted to API with Cur at 80 μM. 48P100+L+: 48-h multispecies biofilm submitted to API with Cur at 100 μM. 48P120+L+: 48-h multispecies biofilm submitted to API with Cur at 120 μM. *Different letters* denote significant difference ($P < 0.05$) according to the post hoc test

treated only by light (P−L+), and those treated only by Cur (P+L−) for all concentrations. When 48-h biofilm was evaluated, API (P+L+) also resulted in a significant difference ($\rho < 0.001$) compared with control (P−L−) for all Cur concentrations. No significant difference ($\rho \geq 0.992$) was observed when control (P−L−), P−L+, and P+L− groups were compared for all Cur concentrations. For the two biofilm ages (24 and 48 h), no significant difference ($\rho \geq 0.996$) was verified among API (P+L+) groups when Cur concentrations were compared. Furthermore, absorbance values of 24-h biofilm were significantly different than those of the 48-h biofilm when controls (P−L−) were compared ($\rho < 0.001$) (Fig. 4).

### Total biomass quantification

ANOVA on ranks demonstrated a significant effect for biofilm age ($\rho < 0.001$) and groups of treatment ($\rho < 0.001$) but not for the interaction between them ($\rho = 0.836$). API (P+L+) resulted in significant difference ($\rho < 0.001$) compared with control (P−L−) for all Cur concentrations, which showed no significant difference among them ($\rho \geq 0.331$). No significant difference ($\rho \geq 0.233$) was verified among control (P−L−), P−L+, and P−L+ groups for all Cur concentrations (Fig. 5).

### *Biofilm analysis by CLSM*

The images obtained by CLSM (Figs. 6 and 7) show that after the applications of API mediated by Cur at 120 μM [P+L+ (24 h) and P+L+ (48 h)] (Figs. 6e and 7c) on multispecies biofilm formed on the resin test specimens in 24 and 48 h, there was a visible increase in the number of cells marked with red fluorescence, indicating microbial cell damage, when compared with their respective controls P−L− (24 h) and P−L− (48 h) (Figs. 6a and 7a). Furthermore, in group P+L− (24 h) with Cur at 120 μM, presented in Fig. 4c, a visible increase in the cells with red fluorescence was observed when compared with group P−L− (24 h) (Fig. 6a). The cross section of biofilms showed thickness of 20, 19, 17, 23.5, and 22 μm, with reference to groups P−L− (24 h), P+L− (24 h), P+L+ (24 h), P−L− (48 h), and P+L+ (48 h), respectively (Figs. 6b, d, f and 7b, d).

## Discussion

The results of this study demonstrated that the application of API was a significant factor in all the assessment performed (quantification of colonies, metabolic activity, and total biomass) in multispecies biofilm, as well as for biofilm age (24 and 48 h), which was not significant only for *C. albicans*. Thus, for all the evaluations performed, the results obtained for the 48 h biofilm differed significantly from those of 24-h biofilm except for the quantification of *C. albicans* colonies. Moreover, the interaction of these factors (biofilm age and groups of treatment) was also significant for all the evaluations performed, except for biofilm biomass. By means of colony quantification, it was verified that API significantly

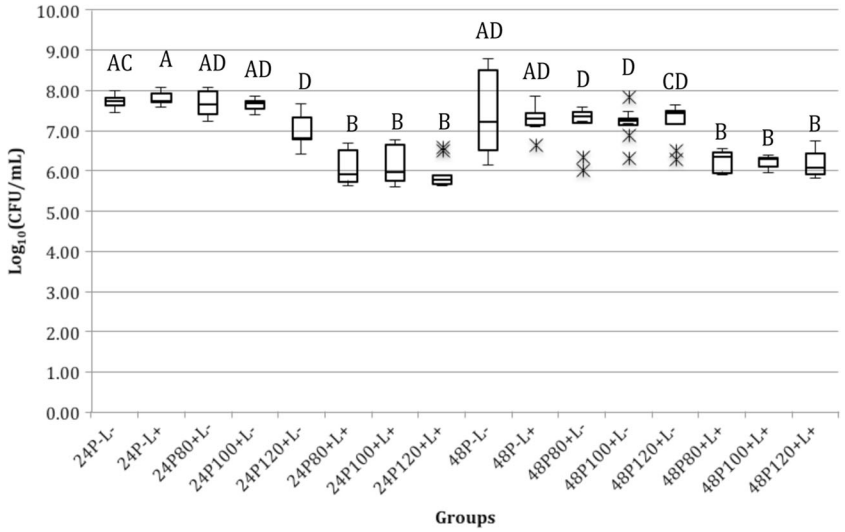


**Fig. 3** Summary of $\log_{10}$CFU/mL values of *S. mutans*. Box plots show the median (*dash*), first and third quartiles (*box*), highest and lowest values (*error bars*), and outliers (*asterisk*). 24P−L−: control group of 24-h multispecies biofilm. 24P−L+: 24-h multispecies biofilm treated with light only (37.5 J/cm$^2$). 24P80+L−: 24-h multispecies biofilm treated with Cur only at 80 μM. 24P100+L−: 24-h multispecies biofilm treated with Cur only at 100 μM. 24P120+L−: 24-h multispecies biofilm treated with Cur only at 120 μM. 24P80+L+: 24-h multispecies biofilm submitted to API with Cur at 80 μM. 24P100+L+: 24-h multispecies biofilm submitted to API with Cur at 100 μM. 24P120+L+: 24-h multispecies biofilm submitted to API with Cur at 120 μM. 48P−L−: control group of 48-h multispecies biofilm. 48P−L+: 48-h multispecies biofilm treated with light only (37.5 J/cm$^2$). 48P80+L−: 48-h multispecies biofilm treated with Cur only at 80 μM. 48P100+L−: 48-h multispecies biofilm treated with Cur only at 100 μM. 48P120+L−: 48-h multispecies biofilm treated with Cur only at 120 μM. 48P80+L+: 48-h multispecies biofilm submitted to API with Cur at 80 μM. 48P100+L+: 48-h multispecies biofilm submitted to API with Cur at 100 μM. 48P120+L+: 48-h multispecies biofilm submitted to API with Cur at 120 μM. *Different letters* denote significant difference ($P < 0.05$) according to the post hoc test

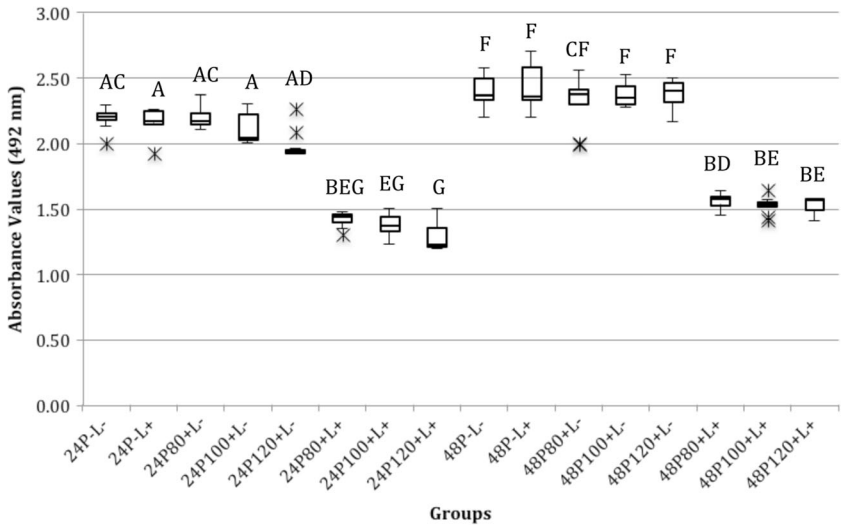


**Fig. 4** Summary of metabolic activity of multispecies biofilm. Box plots show the median (*dash*), first and third quartiles (*box*), highest and lowest values (*error bars*), and outliers (*asterisk*). 24P−L−: control group of 24-h multispecies biofilm. 24P−L+: 24-h multispecies biofilm treated with light only (37.5 J/cm$^2$). 24P80+L−: 24-h multispecies biofilm treated with Cur only at 80 μM. 24P100+L−: 24-h multispecies biofilm treated with Cur only at 100 μM. 24P120+L−: 24-h multispecies biofilm treated with Cur only at 120 μM. 24P80+L+: 24-h multispecies biofilm submitted to API with Cur at 80 μM. 24P100+L+: 24-h multispecies biofilm submitted to API with Cur at 100 μM. 24P120+L+: 24-h multispecies biofilm submitted to API with Cur at 120 μM. 48P−L−: control group of 48-h multispecies biofilm. 48P−L+: 48-h multispecies biofilm treated with light only (37.5 J/cm$^2$). 48P80+L−: 48-h multispecies biofilm treated with Cur only at 80 μM. 48P100+L−: 48-h multispecies biofilm treated with Cur only at 100 μM. 48P120+L−: 48-h multispecies biofilm treated with Cur only at 120 μM. 48P80+L+: 48-h multispecies biofilm submitted to API with Cur at 80 μM. 48P100+L+: 48-h multispecies biofilm submitted to API with Cur at 100 μM. 48P120+L+: 48-h multispecies biofilm submitted to API with Cur at 120 μM. *Different letters* denote significant difference ($P < 0.05$) according to the post hoc test

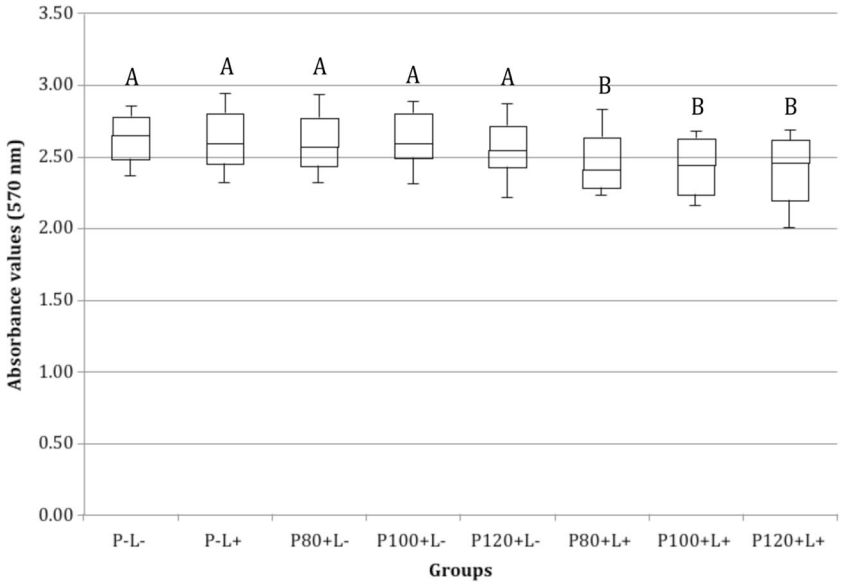


**Fig. 5** Summary of total biomass of 24- and 48-h multispecies biofilms. Box plots show the median (*dash*), first and third quartiles (*box*), highest and lowest values (*error bars*), and outliers (*asterisk*). P−L−: control group. P−L+: biofilms treated with light only (37.5 J/cm$^2$). P80+L−: biofilms treated with Cur only at 80 μM. P100+L−: biofilms treated with Cur only at 100 μM. 24P120+L−: biofilms treated with Cur only at 120 μM. P80+L+: biofilms submitted to API with Cur at 80 μM. P100+L+: biofilm submitted to API with Cur at 100 μM. P120+L+: biofilms submitted to API with Cur at 120 μM. *Different letters* denote significant difference ($P < 0.05$) according to the post hoc test for the factor groups of treatment

reduced the viability of *C. albicans*, *C. glabrata*, and *S. mutans* for the two biofilm ages (24 and 48 h). All Cur concentrations (80, 100, and 120 μM) evaluated showed no significant differences in microbial photoinactivation among them, except for *C. albicans* in 48-h biofilm, which was photoinactivated only by the concentration of Cur at 120 μM. This result demonstrated that in 24-h biofilm, all the species evaluated were susceptible to API using the three concentrations of Cur evaluated. Moreover, in 48-h biofilm, *C. albicans* was less susceptible to photoinactivation in comparison with *C. glabrata* and *S. mutans*, which showed similar susceptibility to API. This difference in susceptibility to API may be justified by the different phases of *C. albicans* biofilm formation. Chandra et al. [57] showed that the *C. albicans* biofilm progresses in three distinct phases of development in a polymethylmethacrylate model: initial phase (0 to 11 h), intermediate phase (12 to 30 h), and maturation phase (38 to 72 h). In the first hours of the initial phase, they observed that the majority of the *C. albicans* cells were in the form of blastospores adhered to the surface of the polymethylmethacrylate strips. Close to 11 h, the *C. albicans* colonies appeared as thick strips of fungal growth, due to the cell growth and aggregation along the areas of surface irregularities. The intermediate phase of development was characterized by the appearance of predominantly extracellular material, similar to a turbid pellicle covering the fungal colonies. During the maturation phase, the quantity of extracellular material increases with the time of incubation, and the *C. albicans* cells were completely enveloped within this material, constituting the biofilm. Therefore, the lower susceptibility of *C. albicans* to API in multispecies biofilms of 48 h may be justified by the phase of maturation being more resistant than the intermediate phase reached by the 24-h biofilm.

Since $\log_{10}$ reduction cannot be calculated due to nonparametric analysis of the data, the reduction of microbial species could be estimated as observed in data shown in the box plot figures. For *C. albicans* (Fig. 1), considering all samples shown in box plots, it is possible to observe for API groups (biofilm of 24 and 48 h) a reduction near to 0.5 $\log_{10}$ compared with all samples from the controls (biofilm of 24 and 48 h). For *C. glabrata*, Fig. 2 shows that samples of 24 h submitted to API demonstrated a reduction near to 1 $\log_{10}$ compared with the control (biofilm of 24 h). On the other hand, although a significant difference was also verified, the reduction for 48-h samples observed in the API group compared with control was near to 0.5 $\log_{10}$. For *S. mutans*, samples of 24 h submitted to API showed a reduction near to 1.5 $\log_{10}$ compared with control as observed in Fig. 3. However, for 48 h, although a significant difference was verified, Fig. 3 shows a high variation in the control group, which values are close to API group. Thus, a reduction could not be estimated. A previous study cultivated the 48-h biofilm with the same three species on polystyrene plate and found reductions from 1.19 $\log_{10}$ to 2.39 $\log_{10}$ after API mediated by Photodithazine (PDZ) [43]. Another study, using the same 48-h multispecies biofilm of the present investigation and PDZ as a photosensitizer, demonstrated no significant difference between samples submitted to API and untreated control when 1 and 3 API

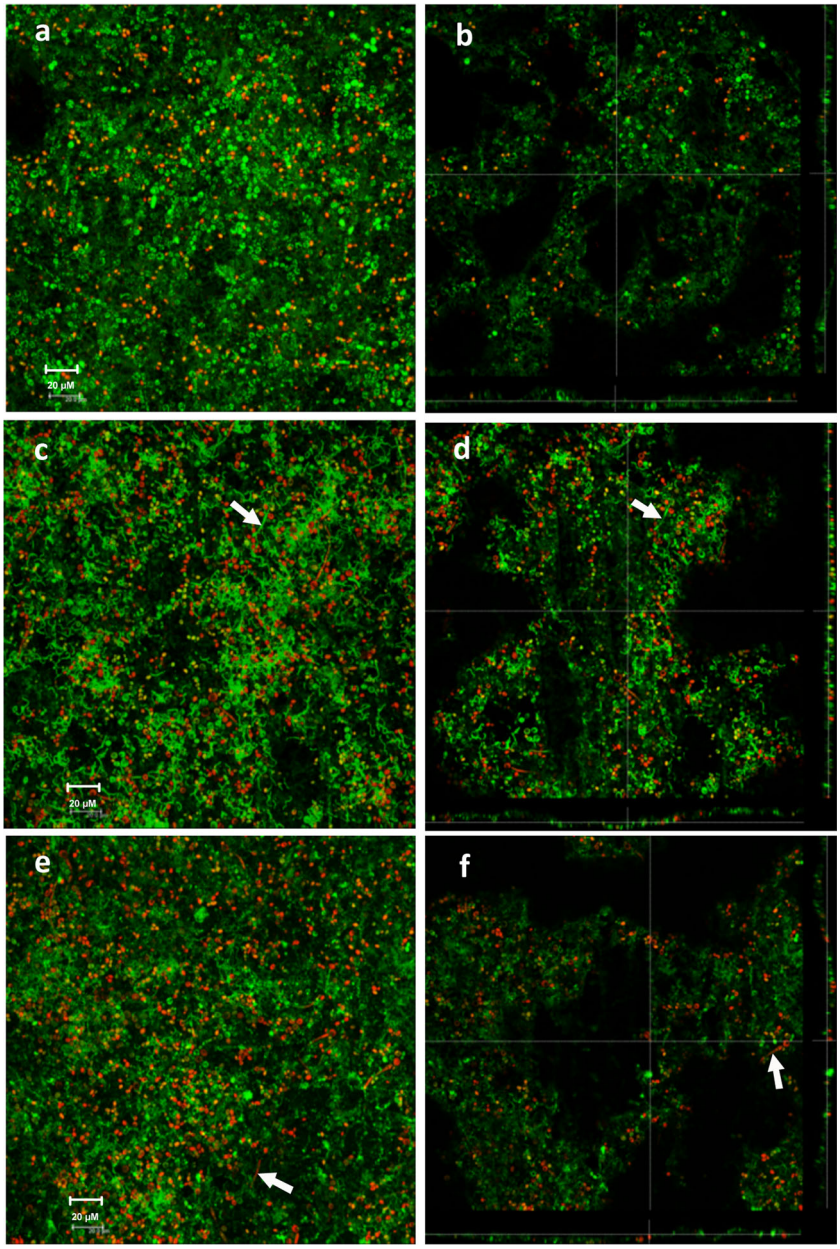


**Fig. 6** CLSM image of multispecies biofilms grown on acrylic resin samples stained with fluorochromes SYTO-9 and PI. Red cells are considered death (PI) and green cells are considered live (SYTO-9). **a** Image of 24-h biofilm from the control group (P−L− (24 h)). **b** Cross sections and side views of 20 μm (*yellow line*) thick biofilm from group P−L− (24 h). **c** Image of biofilm from group P+L− (120 μM of Cur without LED light) (P+L− (24 h)). **d** Cross sections and side views of 19.0 μm (*yellow line*) thick biofilm of group P+L− (24 h). **e** Image of 24-h biofilm after API (P+L+ (24 h)). **f** Cross sections and side views of 17.0-μm (*yellow line*) thick biofilm from group P+L+ (24 h). *White arrows* show filamentous form of *C. albicans* (hyphae/pseudohyphae)

applications were performed [62]. Therefore, the results of the present investigation could be considered more relevant than this previous study. However, it is important to emphasize that the $\log_{10}$ reduction cannot be calculated in this investigation.

Furthermore, in the absence of LED light (P+L−), it was verified that the higher concentration of Cur (120 μM) promoted significant reduction in cell viability of the microorganisms in the 24-h biofilm and so did the concentration of 100 μM in *C. albicans*. This result is in agreement with those of studies that have suggested that the application of Cur alone may present an antimicrobial effect [46–48, 63]. A study conducted by Garcia-Gomes et al. [63] confirmed the antifungal effect of Cur when verifying that the concentration of 50 μM of Cur, in the absence of light, was capable of inhibiting the growth of *C. albicans*. Therefore, the association of Cur with light has been proposed to potentiate its antifungal action [41]. In the 48-h biofilm, the microorganisms *C. glabrata* and *S. mutans* presented a reduction in cell viability for the three concentrations of Cur when associated with LED light, whereas for *C. albicans*, the reduction in $\log_{10}$(CFU/mL) values was obtained only for the highest concentration used. According to the scanning electron microscopy and confocal microscopy images obtained by Seneviratne et al. [60], *C. albicans* forms thicker biofilms that present tridimensional characteristics and multiple layers of biofilm, when compared with that of

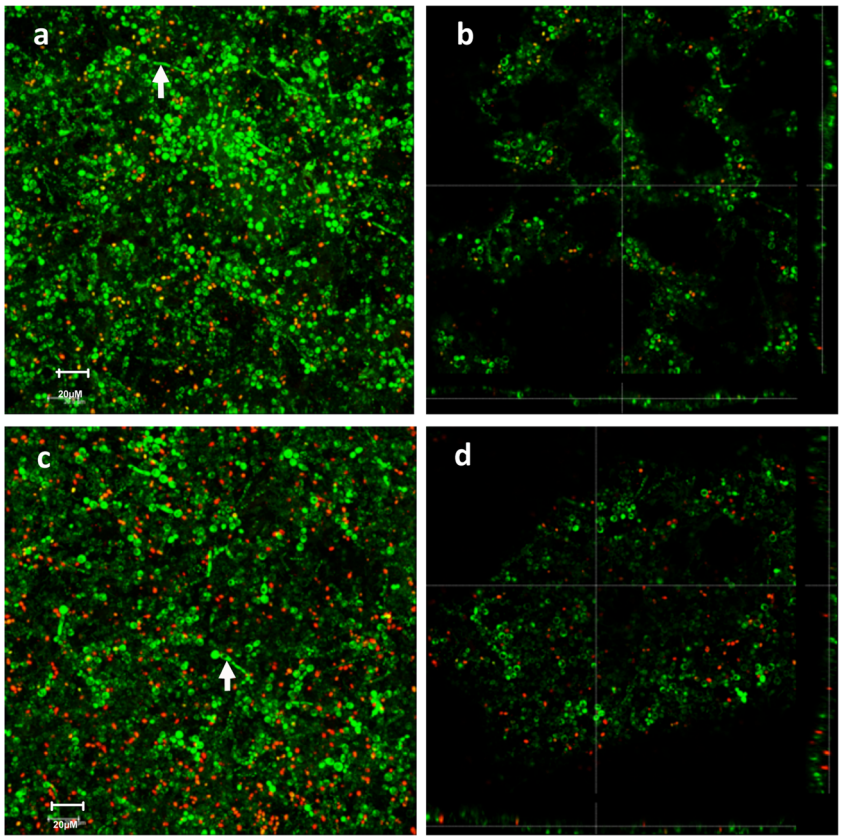


**Fig. 7** **a** CLSM image of 48-h biofilm from the control group (P−L− (48 h)). **b** Cross sections and side views of 23.5-μm (*yellow line*) thick biofilm from group P−L− (48 h). **c** Image of 48-h biofilm from group P+L+ (120 μM of Cur and LED light). **d** Cross sections and side views of 22.0-μm (*yellow line*) thick biofilm from group P+L+ (24 h). *White arrows* show filamentous form of *C. albicans* (hyphae/pseudohyphae)

*C. glablata*, which produces thin, compact biofilms. Therefore, the more robust architecture of the biofilm formed by *C. albicans* may have been a relevant factor that made it difficult for the PS Cur to act on the biofilm. In addition, for the 48-h biofilm, differing from the 24-h biofilm, none of the concentrations of Cur in the absence of light (P+L− with 80, 100, and 120 μM) was capable of reducing the cell viability of the microorganisms evaluated. Therefore, it may be suggested that the higher resistance of the 48-h biofilm made it difficult for API to act on the microorganisms.

Moreover, the different concentrations of Cur promoted no significant difference with regard to the effectiveness of API. In the study of Dovigo et al. [41], the photodegradation of Cur was evaluated, in which it was shown that for the concentration of 20 μM, light doses higher than 5.28 J/cm$^2$ did not promoted an increase in the effectiveness of API. This occurred because as from 4 min (time with reference to the dose of 5.28 J/cm$^2$), there was an increase in the photodegradation of Cur (20 μM) and reduction in the production of reactive oxygen species, thus stabilizing the phototoxic effect of Cur. In the present study, much higher concentrations of Cur (80, 100, and 120 μM) were used for API than those that were used in the study of Dovigo et al. [41]. However, no statistical difference between the concentrations used was verified. This may have occurred because the light dose of 37.5 J/cm$^2$ had probably not been sufficient to completely activate all the molecules of the PS present in these more concentrated solutions, and therefore, a complete photodegradation of the PS may not have occurred, in addition to a restricted production of reactive oxygen species. The possibility of complete photodegradation not having occurred is due to the fact that during the experimental phase, no complete photowhitening of the Cur solutions was observed after illumination with LED. Thus, the limited production of reactive oxygen species may have attained a threshold of phototoxicity, and therefore, the higher concentrations were not more efficient for the reduction in cell viability when compared with the lower concentration. Taking into consideration the limitations of this study, higher light doses were not evaluated due to the long time interval of illumination necessary for these doses. The LED appliance available for the study has an intensity of 22 mW/cm$^2$, and to obtain a light dose of 37.5 J/cm$^2$, a period of 29 min of illumination was required. Therefore, longer times of illumination may make the application of the therapy clinically unfeasible. However, further studies are necessary, which evaluate different light doses for illuminating high concentrations of Cur, using more powerful appliances to diminish the time of illumination.

The results of biofilm metabolic activity (XTT assay) showed that both 24-h and 48-h biofilm were susceptible to API, confirming the results obtained in the colony quantification assays. For the biofilms of both ages (24 and 48 h), the three concentrations of Cur with light were capable of promoting a reduction in cell metabolism. Although no significant difference was observed among the three concentrations, the highest reduction was 40.62 % for the group API (P+L+) mediated by the concentration of 120 μM of Cur. This result is in agreement with the findings of Quishida et al. [43], who also verified a significant reduction in metabolic activity of multispecies biofilm of *C. albicans*, *C. glabrata*, and *S. mutans* after API mediated by the PS Photodithazine for all the concentrations evaluated. Differently from the quantification of colonies, the presence of Cur at 120 μM in the absence of light was not capable of reducing the cell metabolism for the younger biofilms (24 h). Although this result differs from that of CFU/mL, it suggests that the antimicrobial action of Cur alone is not as effective in reducing the cell metabolism as is its association with light. For the control group (P−L−), greater metabolic activity of the 48-h biofilm was verified when compared with that of the 24-h biofilm, suggesting significantly greater viability of the more mature biofilm. In spite of this, as with the CFU/mL test, the action of API on 48-h biofilm did not differ from that of the 24-h biofilm, suggesting similar susceptibility of both biofilm ages to the photodynamic effects. This difference observed in the metabolic activity of the controls and API groups for the biofilms of different ages may be justified by the fact that the XTT assay was performed immediately after treatment of the biofilm so that there was no possibility of recovery of the cells affected by photodynamic action, producing similar absorbance values for the 24- and 48-h biofilms, differently to that observed for the control groups. Whereas for the quantification of colonies, during the period of 48 h of incubation for the growth of colonies, some cells may recover from phototoxic damages and become viable. Therefore, it is important to complement the viability tests performed. The advantages of the XTT assay are the rapidity of the test and the possibility of working with whole biofilm, without rupturing it.

The results of the violet crystal test demonstrated that the 48-h biofilm presented significantly higher biomass values in comparison with the 24-h biofilm, as expected, due to the maturity of the 48-h biofilm. Moreover, API mediated by the three concentrations of Cur reduced the total biomass of biofilm in comparison with the control (P−L−). According to these results, API was not only capable of reducing the cell viability and cell metabolism but was also efficient for reducing the total biomass of biofilms formed on the acrylic resin test specimens. This result is in agreement with those obtained by Dovigo et al. [26], with monospecies biofilms from clinical isolates of *Candida* spp., which presented reduction in biomass from 52.3 to 64.1 % after photoinactivation with Cur. However, Quishida et al. [43] verified no significant reduction in total biomass of multispecies biofilm of *C. albicans*, *C. glabrata*, and *S. mutans* after API mediated by Photodithazine. This difference in the results may be attributed to the photosensitizers used, suggesting that Cur presents a greater capacity for disarticulation of the biofilm structure.

The CLSM images showed that after API with Cur 120 μM, there was a visible increase in cells stained red when compared with their respective controls (24 and 48 h), suggesting that there may be cell photodamage and penetration of the fluorochrome PI into the cell. This result is in agreement with those observed by Quishida et al. [43], who also verified a visible increase in red cells after API mediated by Photodithazine in multispecies biofilm of *C. albicans*, *C. glabrata*, and *S. mutans*. Moreover, after API with CUR 120 μM, the 24-h biofilm visibly presented a higher number of red cells when compared with the 48-h biofilm. As regards the biofilm thickness, the control group (P−L−) of 24-h biofilm presented a thickness of 20 μM, while the control group (P−L−) of 48-h biofilm presented a thickness of 23.5 μM, showing an increase in thickness of the 48-h biofilm. In a previous study [43], when biofilm with the same microbial species were formed in wells of a polystyrene plate, the thickness of 48-h biofilm was 19 μM, thinner than that of the biofilm in the present study, possibly due to the difference in the substrates used for biofilm adherence and formation. Furthermore, the 24-h biofilm treated only with Cur 120 μM showed a visible increase in the quantity of red cells when compared with the control group (P−L−) of 24 h, suggesting the antimicrobial activity of Cur even in conditions of the absence of illumination in a less mature biofilm [33–35]. However, no quantitative analysis of the images obtained was performed, which limits interpretation of the images.

Although the results showed a significant difference after API, they could not be extrapolated to a clinical scenario. The in vitro biofilm is cultivated with a high concentration of microorganisms ($10^7$–$10^8$ CFU/mL) compared with microbial load found in clinical infections ($10^4$–$10^5$ CFU/mL for *Candida* spp. and *S. mutans*) [8]. Furthermore, the synergism among the species in a multispecies biofilm may favor the microorganisms against the antimicrobial therapy. This issue needs further investigations. In addition, few studies are available evaluating the efficacy of API against multispecies biofilm. Therefore, the results obtained in this study may direct future studies in order to improve methods and strategies against complex biofilms.

## Conclusion

According to the results of this study and considering its limitations, it can be concluded that both 24-h and 48-h biofilms were susceptible to API mediated by Cur in the concentrations

evaluated and light dose of 37.5 J/cm$^2$. No difference in the photoinactivation of the biofilm was verified among the concentrations of Cur evaluated. However, no other doses of light and pre-illumination times were evaluated.

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**Compliance with ethical standards**

**Conflict of interest** None declared

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