

Exopolysaccharide Matrix of Developed *Candida albicans* Biofilms After Exposure to Antifungal Agents

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This study aimed to evaluate the effects of fluconazole or nystatin exposure on developed *Candida albicans* biofilms regarding their exopolysaccharide matrix. The minimal inhibitory concentration (MIC) against fluconazole or nystatin was determined for *C. albicans* reference strain (ATCC 90028). Poly(methylmethacrylate) resin (PMMA) specimens were fabricated according to the manufacturer's instructions and had their surface roughness measured. Biofilms were developed on specimens surfaces for 48 h and after that were exposed during 24 h to fluconazole or nystatin prepared in a medium at MIC, 10 x MIC or 100 x MIC. Metabolic activity was evaluated using an XTT assay. Production of soluble and insoluble exopolysaccharide and intracellular polysaccharides was evaluated by the phenol-sulfuric method. Confocal laser scanning microscope was used to evaluate biofilm architecture and percentage of dead/live cells. Data were analyzed statistically by ANOVA and Tukey's test at 5% significance level. The presence of fluconazole or nystatin at concentrations higher than MIC results in a great reduction of metabolic activity ($p < 0.001$). At MIC or 10 x MIC, fluconazole showed high amounts of intracellular polysaccharides ($p < 0.05$), but did not affect the exopolysaccharide matrix ($p > 0.05$). The exposure to nystatin also did not alter the exopolysaccharide matrix at all the tested concentrations ($p > 0.05$). Biofilm architecture was not affected by either of the antifungal agents ($p > 0.05$). Nystatin promoted higher proportion of dead cells ($p < 0.05$). It may be concluded that fluconazole and nystatin above the MIC concentration reduced the metabolic activity of *C. albicans* biofilms; however, they were not able to alter the exopolysaccharide matrix and biofilm architecture.

Key Words: antifungal agents, *Candida albicans*, biofilm, denture bases.

INTRODUCTION

A large percentage of denture wearers are affected by *Candida*-associated denture stomatitis (CADS), with *C. albicans* identified as the main pathogen of this condition (1). One of the factors contributing to the virulence of *C. albicans* is its ability to attach to biotic and/or abiotic surfaces and growing in communities known as biofilms (2). These structured communities, encased in a matrix of exopolysaccharide, display unique characteristics that confer survival advantages over their

planktonic counterparts (3,4).

It is known that there are many ecologic advantages in forming biofilms, including protection from the environment, resistance to physical and chemical stress, nutrient availability and metabolic cooperation (5). Additionally, from the perspective of infection, the most important feature of biofilm growth is its high resistance to antifungal agents, which can be up to 100-fold greater than that of planktonic cells (4-7). The mechanisms contributing to this increased antifungal resistance are not well established, although

several possibilities have been investigated. These possibilities include a lower metabolic rate of cells within biofilms (8), presence of an exopolysaccharide matrix (9-11), persistent cells (3) and biofilm three-dimensional conformation (12).

The antifungal agents most commonly used to treat patients with CADS are azoles or polyenes (8,9) and the mechanism of actions of these antifungal agents is directly related to alterations of the biofilm's metabolic and structural levels (5-7). The azoles, such as fluconazole, act as a fungistatic agent affecting the ergosterol biosynthesis and, consequently, the fungal cell permeability (7). In contrast, the polyenes, which include nystatin, are fungicidal agents and cause fungal cell death interfering in ergosterol-containing membranes and also by oxidative damage (9).

Considering the mechanism of action of fluconazole (7), it would be natural to expect that after the exposure to fluconazole, developed biofilms could maintain their architecture, but probably with particular changes in cell metabolism. Likewise, it is expected the complete disruption of developed biofilms after administering nystatin (9).

Additionally, considering that the antifungal dose required for biofilm eradication usually exceeds the minimal inhibitory concentration (MIC) recommended for eradication of its planktonic counterparts (5,6), it is expected that concentrations higher than MIC could be another point affecting the biofilm architecture. So far, however, it is not fully understood whether antifungal agents at concentrations higher than MIC could actually interfere on biofilms at metabolic levels and/or biofilm organization, particularly in the exopolysaccharide matrix. Therefore, the aim of this study was to evaluate the exopolysaccharide matrix of developed *C. albicans* biofilms after exposure to fluconazole or nystatin.

MATERIAL AND METHODS

Experimental Design

This *in vitro* study had a randomized and blinded design. MIC was determined for *C. albicans* reference strain (ATCC 90028) against fluconazole or nystatin. Poly(methylmethacrylate) (PMMA) resin discs (10 mm x 2 mm) were fabricated according to the manufacturer's directions and had their surface roughness measured. Biofilms of *C. albicans* were allowed developing on specimen surface for 48 h. Next,

the biofilms were exposed during 24 h to fluconazole or nystatin prepared in a medium at MIC, 10 x MIC or 100 x MIC. Metabolic activity was evaluated using an XTT colorimetric assay. Production of exopolysaccharide and intracellular polysaccharides were evaluated using the phenol-sulfuric method. Confocal laser scanning microscopy was used for architecture analysis and percentage of dead/live cells. Statistical analysis was performed considering as study factors the antifungal agent and its concentrations.

Specimen Preparation

Discs of water bath PMMA resin (Lucitone 550; Denstply, Rio de Janeiro, RJ, Brazil) were fabricated in accordance with the manufacturer's directions. Next, the specimens were ground using progressively smoother aluminum oxide papers (320, 400 and 600 grits) in a horizontal polisher (model APL-4; Arotec, São Paulo, SP, Brazil) and immersed in purified water for 12 h at 35°C for residual monomer release (13).

The surface roughness of specimens was measured using a profilometer (Surfcoorder SE 1700; Kosaka Laboratory Ltd, Kosaka, Japan) with a 0.01 µm precision, calibrated at sample length of 0.8 mm, 3.2 mm percussion of measure and 0.5 mm/s. Three readings were made for each specimen and a mean value was calculated. The average surface roughness obtained was 0.32 ± 0.05 µm. After that, the specimens were disinfected with 70% alcohol, ultrasonicated for 20 min to remove any residues, and randomly assigned to groups according to antifungal agent and their respective concentrations.

C. albicans Suspensions

C. albicans reference strain (ATCC 90028) was aerobically cultured on Sabouraud Dextrose Agar (Difco Laboratories, Detroit, MI, USA) at 35°C for 24 h. A loopful of the growth was inoculated into Yeast Nitrogen Base broth (YNB; Difco Laboratories) supplemented with 50 mM glucose and incubated for 18 h at 35°C in an orbital shaker (model NT 151; Kline Shaker, Nova Técnica Laboratory, São Paulo, SP, Brazil).

Thereafter, cells were harvested and washed twice with phosphate buffered saline (PBS; pH 7.2), suspended in YNB supplemented with 100 mM glucose and optically adjusted to a density of approximately 10^7 cells/mL.

Minimal Inhibitory Concentration (MIC)

MIC of fluconazole (reference powder; Sigma-Aldrich Co., St. Louis, MO, USA) or nystatin (reference powder; Sigma-Aldrich) against *C. albicans* was determined based on M27-A3 standards (14). Tests were performed in triplicate, on three different occasions.

Biofilm Development

Discs were placed inside sterilized 24-well plates and 2 mL of *C. albicans* suspensions were added. This set was incubated for 90 min at 35°C in an orbital shaker. After the adhesion phase, specimens were washed twice with PBS and exposed to YNB supplemented with 100 mM glucose for 48 h of biofilm development. The specimens were washed with PBS and the medium was changed every 24 h. After that, medium containing fluconazole or nystatin was prepared in YNB supplemented with 100 mM glucose at MIC, 10 x MIC or 100 x MIC. Plates were incubated for 24 h at 35°C in an orbital shaker. After exposure, biofilms were evaluated for their metabolic activity, polysaccharide production and architecture. All tests were performed in four replicates, on three different occasions.

Metabolic Activity

The metabolic activity was measured using an adapted XTT assay (15). Briefly, XTT (Sigma-Aldrich) was dissolved in PBS supplemented with 200 mM of glucose (1.0 mg/mL) and mixed to a menadione solution (0.4 mM). The developed biofilms exposed to fluconazole or nystatin were washed twice and transferred to new plates containing the XTT solution. This set was protected from light and incubated at 35°C for 3 h. The solution of each well was centrifuged and the supernatant was transferred to a 96-well plate. The colorimetric changes were measured at 492 nm using a plate reader (Spectra-MAX 340; Molecular Devices Ltd., Sunnyvale, CA, USA).

Polysaccharide Extraction

For polysaccharide extraction, the developed biofilms exposed to fluconazole or nystatin were washed twice and transferred to plastic tubes containing PBS. This set was sonicated at 7 W for 30 s. An aliquot of this solution was centrifuged (10,000 g for 5 min at 4°C) and

the supernatant containing exopolysaccharide soluble (EPS) was transferred to another tube named EPS. To the pellet, an aliquot of 1 M NaOH was added for the exopolysaccharide insoluble (EPI) extraction. The tube was agitated for 15 min, centrifuged and the supernatant was transferred to another tube named EPI. Finally, an aliquot of 1 M NaOH was added to the residual pellet for the intracellular polysaccharide (IP) extraction (16). The tube was heated for 15 min at 100°C, centrifuged and the supernatant was transferred to another tube named IP. To the tubes EPS, EPI and IP, 3 volumes of cold ethanol were added and maintained for 30 min at -20°C. Next, the tubes were centrifuged and pellets were washed twice with cold 75% ethanol. Precipitated polysaccharides were resuspended in 1 M NaOH and total carbohydrate was estimated by the phenol sulfuric method (17), using glucose as standard. Results were normalized to the dry weight of biofilm.

Biofilm Architecture

For architecture analysis, the developed biofilms exposed to fluconazole or nystatin were stained using SYTO-9 and propidium iodide (Live/Dead BacLight viability kit; Molecular Probes, Eugene, OR, USA) and incubated during 20 min protected from light at 35°C. Images of stained biofilms were captured using a confocal scanning laser microscopy (CSLM; Leica Microsystems CMS, Mannheim, Germany). A series of images were obtained at 1- μm intervals. At least five representative optical fields were captured for each specimen. COMSTAT software was used to analyze the bio-volume ($\mu\text{m}^3/\mu\text{m}^2$), average thickness (μm), diffusion distance (μm) and dead/live cells ratio (%) (18).

Statistical Analysis

All analyses were performed using the SAS software (SAS Institute Inc., version 9.0; Cary, NC, USA) with the level of significance set at 5%. The normality of error distribution and the degree of non-constant variance were checked for each response variable using the SAS/LAB package and data were transformed as suggested by the software: metabolic activity, EPS, EPI and IP (log10), bio-volume and average thickness (square root) and roughness coefficient (exponentiation, y^2). All data were analyzed using two-way ANOVA, considering as study factors the antifungal agent and its concentrations.

RESULTS

MIC values obtained for fluconazole and nystatin were 0.5 µg/mL and 2.0 µg/mL, respectively. A significant reduction in metabolic activity was observed for *C. albicans* biofilms developed in the presence of fluconazole or nystatin at concentrations higher than MIC ($p < 0.05$). However, no statistically significant differences were observed between 10 x MIC or 100 x MIC for fluconazole ($p > 0.05$). The great reduction was found for biofilms in the presence of nystatin, decreasing 80% at 10 x MIC and 95% at 100 x MIC (Fig. 1).

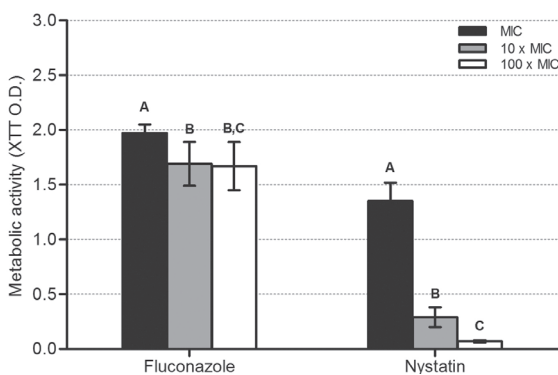


Figure 1. Metabolic activity for *C. albicans* biofilms developed on PMMA surface in the presence of fluconazole or nystatin at MIC, 10 x MIC or 100 x MIC concentrations. Different letters indicate that are significant differences between fluconazole or nystatin tested concentrations ($p < 0.05$).

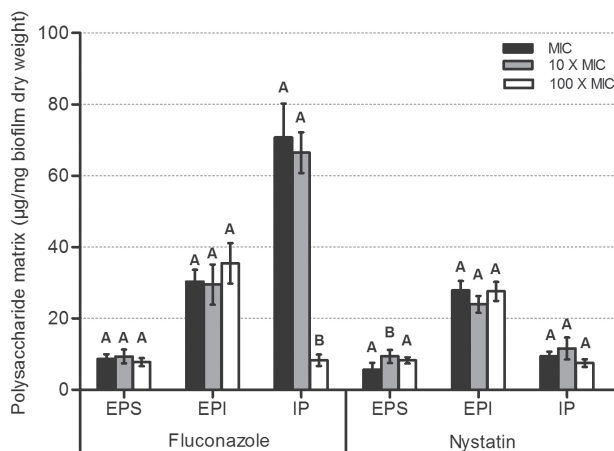


Figure 2. Polysaccharide matrix (µg/mg biofilm dry weight) for *C. albicans* biofilms developed on PMMA surface in the presence of fluconazole or nystatin at MIC, 10 x MIC or 100 x MIC concentrations. Different letters indicate significant differences between fluconazole or nystatin tested concentrations ($p < 0.05$).

The amount of EPS and EPI from biofilm matrix was not affected by the presence of fluconazole at all concentrations ($p > 0.05$). Still for this antifungal, high amounts of IP at MIC and 10 x MIC were found. For nystatin, there were no differences for EPI and IP amounts at all investigated concentrations ($p > 0.05$, Fig. 2).

The architecture of *C. albicans* biofilms was not affected by the presence of fluconazole or nystatin at all evaluated concentrations ($p > 0.05$), as shown by the parameters of bio-volume, average thickness and diffusion distance. In contrast, dead/live cells ratio was significantly higher for fluconazole at 100 x MIC ($p < 0.05$), and for nystatin at 10 x MIC and 100 x MIC ($p < 0.05$, Fig. 3) relative to the other concentrations.

In CLSM images, the live cells appear as green, while the dead cells appear as red. In the z-slices of *C. albicans* biofilms was observed a great amount of black spaces between cells in the presence of fluconazole at 10 x MIC and 100 x MIC (Fig. 4). Additionally, it was possible to identify higher amounts of hyphae forms at MIC concentration (Fig. 4A) and dead cells at 100 x MIC (Fig. 4C). For nystatin, was observed a more compact biofilm, with smaller black spaces and large amount of dead cells at all concentrations (Figs. 4D-4F).

DISCUSSION

Considering the mechanism of action of

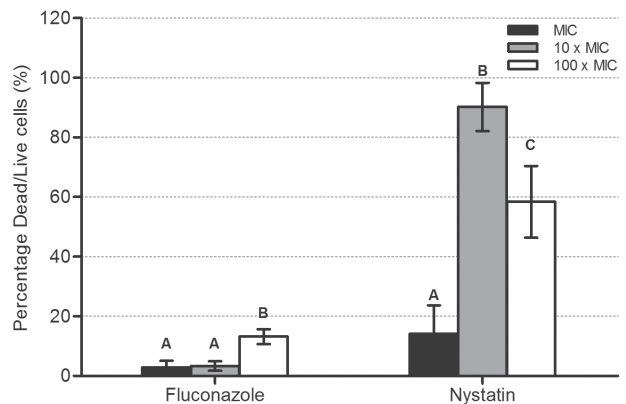


Figure 3. Dead/live cells ratio (%) for *C. albicans* biofilms developed on PMMA surface in the presence of fluconazole or nystatin at MIC, 10 x MIC or 100 x MIC concentrations. Different letters indicate significant differences between fluconazole or nystatin tested concentrations ($p < 0.05$).

fluconazole and nystatin, it is expected particular changes in the cellular metabolism and architecture after their exposure to developed *C. albicans* biofilm. Also, it is known that the antifungal dose required for biofilm eradication usually exceeds the MIC (5,6). However, it is not fully evaluated yet if fluconazole or nystatin at concentrations higher than MIC could actually interfere on biofilm at metabolic levels, exopolysaccharide production and/or biofilm organization. Thus, the obtained results suggest a global understanding about biofilm behavior when exposed to antifungal agents commonly used in clinical practice.

The administrated concentration and availability of fluconazole or nystatin in oral cavity is an important factor that should be considered in the treatment of CADS (8,9). It is known that after swallowing a suspension of fluconazole, a concentration of up to 97.99 $\mu\text{g}/\text{mL}$ can be detected and after 24 h the bioavailability may be reduced to 2.25-4.52 $\mu\text{g}/\text{mL}$ (19). Another important point to highlight is that the half-life of fluconazole ranges from 27 to 37 h (20), indicating that after administration it

can be found in saliva for up to 24 h. In contrast, the concentration of nystatin has a rapid decrease with time after topical administration (21). However, this antifungal agent is capable of eliciting a longer post-antifungal effect (PAFE), with the highest values ranging from 0.43 to 21.05 h (22). Thus, it is important to notice that in the present study was considered a compatible time of exposure and concentrations to those used in clinical practice.

In order to verify the biofilm viability after fluconazole or nystatin exposure, in the present study was evaluated *C. albicans* biofilm metabolic activity using the XTT colorimetric method (6,15). By this method, the results revealed that both fluconazole and nystatin reduced the metabolic activity of *C. albicans* biofilms, but they were not eradicated at concentrations higher than MIC. For fluconazole, no difference was observed in susceptibility at 10 x MIC or 100 x MIC concentrations, suggesting that 10 x MIC was sufficient to render the biofilm to a dormant stage, which corroborates with other studies (6,7). For nystatin, a great reduction was

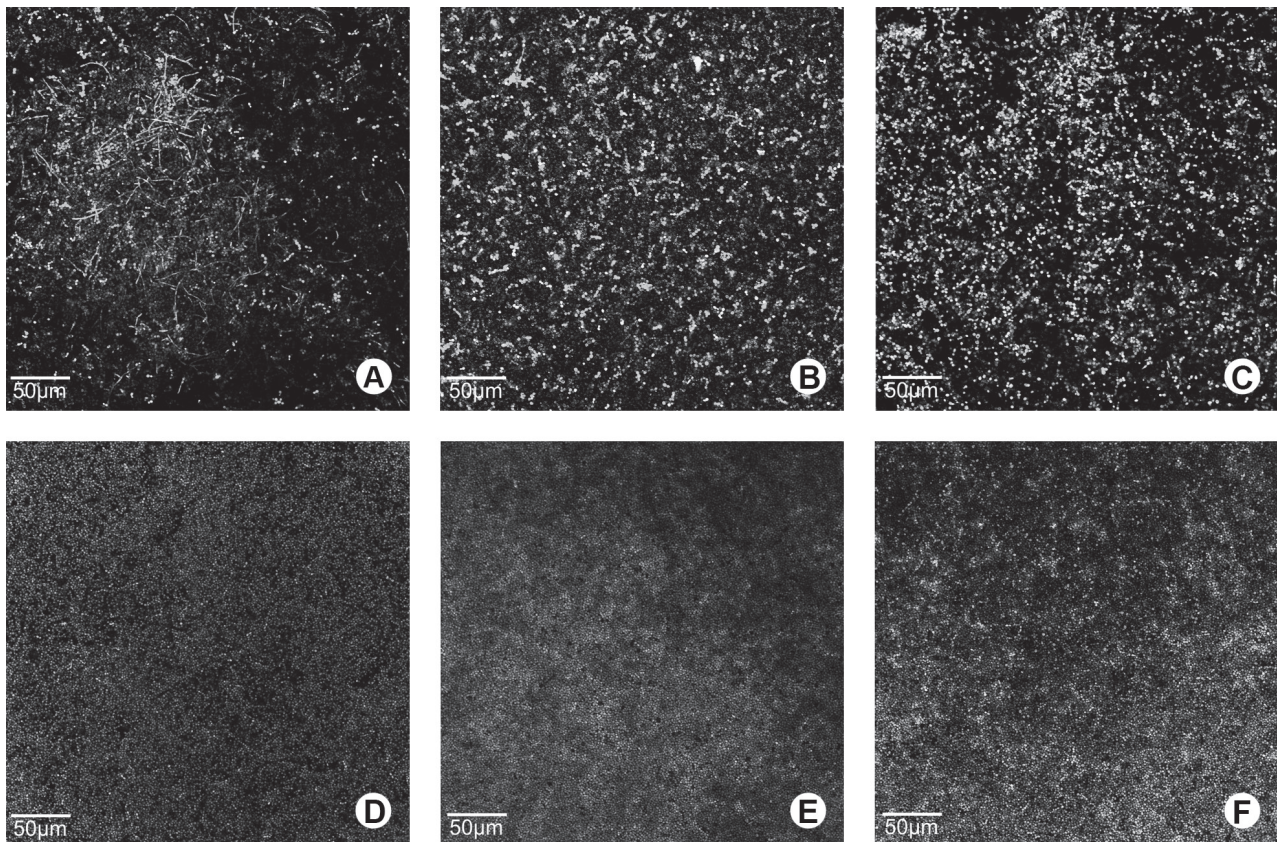


Figure 4. Confocal z-slices images of *C. albicans* biofilms developed on PMMA surface in the presence of fluconazole (A: MIC, B: 10 x MIC and C: 100 x MIC) or nystatin (D: MIC, E: 10 x MIC and F: 100 x MIC).

observed at 10 x MIC concentration and, possibly, complete biofilm eradication at 100 x MIC concentration. These results are justified by the fungicidal mechanism of action of this drug. Nystatin is capable to intercalate into ergosterol-containing membranes, a component of the fungal membrane, forming channels and destroying the proton gradient in the cell with leakage of cytoplasmic content, resulting in fungal death (9,21,22).

Considering that the biofilm matrix is one of the main components related to the biofilm architecture (3,4), in the present study was evaluated the exopolysaccharide and intracellular polysaccharide of biofilms after exposure to fluconazole or nystatin. For fluconazole, it was observed that the exopolysaccharide matrix was not affected at all tested concentrations. It is possible to speculate that the continuous presence of the drug at the different tested concentrations could stimulate the production of extracellular glucans, which were deposited in the matrix. It is known that these extracellular glucans are important for biofilm resistance and act by sequestering antifungals such as fluconazole, rendering cells resistant to their action and preventing its intracellular penetration (23).

Another important finding was the great amount of intracellular polysaccharide at MIC and 10 x MIC found in the presence of fluconazole, while less intracellular polysaccharide was observed at 100 x MIC. It is known that fluconazole is able to deform the cell nucleus and increase the number of vacuoles (20), which are correlated to the fluconazole mechanism of action, inhibiting the ergosterol biosynthesis (9). With this inhibition, toxic substances that are ergosterol precursors and also polysaccharides could accumulate in the cell, probably in these vacuoles, explaining our results of IP for MIC and 10 x MIC. However, at a concentration higher than 100 x MIC, it is possible to speculate that many cells suffered lyses, resulting in the lower IP values observed.

Considering that one of the assumptions of this study was the possibility of modifying the architecture of developed biofilms after antifungal exposure, it was possible to observe that both drugs did not alter the bio-volume, average thickness and diffusion distance at all the tested concentrations. These findings may be explained, at least in part, by the polysaccharide matrix results. Even reducing metabolic activity and possibly the number of cells, the architectural parameters represented not only the cell volume, but the volume occupied by the matrix and biomass of dead cells. In addition,

the small changes could be associated with the time of antifungal exposure, i.e. 24 h, and the fact that the biofilm was already grown when the antifungal therapy was initiated. Additionally, for the percentage of dead/live cells were observed higher amounts after nystatin exposure as confirmed by the obtained confocal images. This result complements and explains the reduction in metabolic activity, justifying the capacity of nystatin to cause fungal cell death (9,21,22). Although the small changes were defined in the architectural parameters, images obtained by confocal microscopy showed some morphological aspects, such as the presence of black spaces between cells and the hyphal forms after exposure to fluconazole. It is possible to suggest that black spaces may be occupied by exopolysaccharide, which explains the high amount of formed matrix. Additionally, the large number of hyphal forms presented at MIC concentration was also an important finding. It is known that the ability to form hyphae is an important virulence attribute of *C. albicans* (4). In this context, it is possible that continuous exposure of biofilms to low concentrations of fluconazole stimulated cells dimorphism, which could actually affect secretion of hydrolytic enzymes and thereby increase biofilms pathogenicity (24,25).

Within the limitations of this study, it may be concluded that both fluconazole and nystatin reduced the metabolic activity of *C. albicans* biofilms; however, they were not able to alter the exopolysaccharide matrix and biofilm architecture.

RESUMO

Este estudo avaliou o efeito da exposição de fluconazol ou nistatina a biofilmes de *Candida albicans* desenvolvidos, considerando a matriz de polissacarídeos extracelulares. Inicialmente uma cepa referência de *C. albicans* (ATCC 90028) foi submetida ao teste de concentração inibitória mínima (CIM) utilizando-se o fluconazol ou nistatina como agentes antifúngicos. Após, espécimes foram confeccionados em resina acrílica de polimetilmetacrilato (PMMA) de acordo com as recomendações do fabricante e tiveram sua rugosidade de superfície padronizada. Após, biofilmes de *C. albicans* foram desenvolvidos na superfície dos espécimes durante 48 h. Em seguida, os biofilmes foram expostos a fluconazol ou nistatina nas concentrações de CIM, 10 x CIM ou 100 x CIM, por 24 h. A atividade metabólica dos biofilmes foi avaliada pelo teste de XTT. A produção de polissacarídeos extracelulares solúveis e insolúveis, bem como dos polissacarídeos intracelulares foi avaliada pelo método fenol-sulfúrico. A arquitetura dos biofilmes e proporção de células vivas e mortas foi investigada utilizando-se microscopia confocal a laser. Os resultados foram analisados por ANOVA seguido do teste de Tukey, utilizando-se o nível de significância de 5%. A presença do fluconazol ou nistatina em concentrações maiores que CIM

resultaram em uma redução significativa da atividade metabólica ($p < 0,001$). Nas concentrações de CIM e $10 \times$ CIM, biofilmes expostos ao fluconazol apresentaram quantidades significativas de polissacarídeos intracelulares ($p < 0,05$), enquanto não houve alterações na quantidade de polissacarídeos extracelulares ($p > 0,05$). A presença de nistatina também não alterou a matriz de polissacarídeos extracelulares em todas as concentrações investigadas ($p > 0,05$). A arquitetura dos biofilmes não foi afetada por ambos os agentes antifúngicos, em qualquer concentração testada ($p > 0,05$). A nistatina apresentou maior proporção de células mortas ($p < 0,05$). Conclui-se que tanto para o fluconazol quanto para a nistatina, concentrações maiores que CIM reduziram a atividade metabólica dos biofilmes de *C. albicans*; no entanto, tais concentrações não alteraram a matriz de polissacarídeos extracelulares nem a arquitetura dos biofilmes.

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