Histatin 5 and human lactoferrin inhibit biofilm formation of a fluconazole resistant *Candida albicans* clinical isolate

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Abstract: *Candida albicans* is the most important fungal pathogen that causes infections in humans. Biofilms are hard-to-treat structures due to their high antifungal resistance. Saliva is a fluid that contains antimicrobial substances acting as the first-line of defense against pathogens, and its immune components may be potential tools for the discovery of new treatments against candidiasis. To evaluate the activity of histatin 5 and human lactoferrin against biofilm formation. A fluconazole-resistant *Candida albicans* clinical isolate was used as the model microorganism. Morphogenesis was evaluated by differential counting. Biofilm quantification was performed by XTT reduction assay. Thickness and topography of biofilms were assessed through confocal laser scanning microscopy (CLSM). Histatin 5 inhibited yeast-to-hyphae transition in a dose-dependent manner, while the effect of human lactoferrin on this process was inversely proportional to its concentration. Both compounds were able to significantly inhibit biofilm metabolic activity. Histatin 5 reduced biofilm thickness. Histatin 5 and human lactoferrin exhibited *in vitro* cytotoxicity against a fluconazole-resistant *Candida albicans* biofilm, which points to the potential application of these compounds in the treatment of biofilms formed by this fungus, especially in resistant infections.

Key words: biofilm, *Candida albicans*, fluconazole resistance, histatin 5, lactoferrin.

INTRODUCTION

Candida albicans is the most important etiological agent of candidiasis, which can present as a superficial or invasive infection (Wille et al. 2013). The variation in the pathogenicity of the fungus occurs due to an imbalance between the fungal virulence factors and the immunological status of the host, which may be of major concern, considering the growing number of immunocompromised patients in the population in recent decades, especially HIV-infected individuals (Naglik et al. 2003, Challacombe et al. 2006).

Dimorphism and biofilm formation are considered extremely significant virulence attributes in *C. albicans*, contributing significantly to the establishment of infection. The transition between

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planktonic yeast cells to hyphae and pseudohyphae facilitates the invasion of host tissues at early stages of the infection (Gow et al. 2002, Kumamoto and Vinces 2005). Biofilm formation by *C. albicans* is considered an important virulence factor since this structure is able to develop on medical devices, such as central venous catheters, urinary catheters, and mechanical heart valves, among others, enabling the spread of the microorganism into the bloodstream and thus to all organs of the human body, also creating the need to remove such devices and thus interfering directly with patient survival (Blankenship and Mitchell 2006, Tumbarello et al. 2007). Moreover, biofilm-grown *C. albicans* cells show greater antifungal resistance than planktonic cells, making it difficult to eradicate this structure by conventional drug treatments and urging the development of new antifungal strategies (Chandra et al. 2008).

Histatins are peptides abundant in the saliva. At least 12 peptides of this family have been identified and, among them, histatin 5 is thus far the most studied (Peters et al. 2010). It is a constitutive peptide produced and secreted by all major salivary glands, therefore, reaching high concentrations in saliva, maintaining its physiological range between 10 and 300 μg/mL (Puri and Edgerton 2014). Lactoferrin is an iron-binding glycoprotein produced by mucous cells of the oral epithelium, being found in high concentrations in the secretions of several mucous surfaces, including in saliva (González-Chávez et al. 2009). The antifungal activity of lactoferrin is related to its chelating ability, and, unlike histatin 5, its concentration does not change in HIV infection (Lourenço et al. 2013).

Knowing the antifungal properties of salivary immune components allows for the discovery of new approaches for treating candidiasis. Furthermore, biofilm formation is one of the most important features related to the establishment of candidiasis due to the high antifungal resistance of biofilms. Thus, the aim of this study was to evaluate the effects of histatin 5 and human lactoferrin on *C. albicans* biofilm formation, as well as its thickness and topography.

MATERIALS AND METHODS

YEAST STRAIN AND GROWTH CONDITIONS

A *C. albicans* strain isolated from the oral mucosa of a pediatric HIV patient known to be resistant to fluconazole (MIC > 256 μg/mL) was used in this study (Braga-Silva et al. 2007). Yeasts were subcultured and maintained on Sabouraud dextrose agar at 4 °C. Before any experimental procedures, the yeasts were subcultured into YPD medium (yeast extract 1%, peptone 2%, and dextrose 2%) (Difco Bacto, Sparks, NE, USA) and incubated at 37 °C for 48 h.

COMPOUNDS

Histatin 5 (GenScript, Piscataway, NJ, USA) was reconstituted in 1 mM phosphate-buffered saline (PBS; pH 7.2), reaching a final concentration of 1.0 mg/mL. Human lactoferrin (Sigma-Aldrich, St. Louis, MO, USA) was resuspended in distilled sterilized water to a final concentration of 1.0 mg/mL. Both proteins were stored at -20 °C immediately after preparation. The highest concentrations of the proteins used in the subsequent experiments (150 μg/mL for histatin 5 and 500 μg/mL for human lactoferrin) were chosen due to their lack of toxicity, since these concentrations are physiologically found within human secretions. All reagents and stains were purchased from Sigma-Aldrich, unless otherwise stated.

YEAST-TO-HYPHAE TRANSITION

The effect of histatin 5 and human lactoferrin on *C. albicans* yeast-to-hyphae transition was evaluated as described previously, with slight modifications (Braga-Silva et al. 2007). Briefly, 2×10^6 cells were washed three times in 1 mM PBS and then incubated in 1.0 mL fetal bovine serum (FBS;

Cultilab, São Paulo, Brazil) in the presence of serial dilutions of histatin 5 or human lactoferrin for 3 h at 37 °C, with agitation (75 rpm). Cell viability was assessed by the trypan blue exclusion method. A Neubauer chamber was used for differential counting, and the percentage of germ tubes was determined. This procedure was performed four times, and a minimum of 500 cells were counted in each experiment.

BIOFILM METABOLIC ACTIVITY

The effect of histatin 5 and human lactoferrin on *C. albicans* biofilm metabolic activity was evaluated as described previously, with slight modifications (Thein et al. 2007). Briefly, 1×10^6 cells were plated onto a 96-well flat-bottomed microtiter plate and incubated at 37 °C for 90 min, with gentle agitation (75 rpm). The supernatant was removed, and the wells were gently washed twice with PBS to remove non-adherent cells. YNB medium (BD, Franklin Lakes, NJ, USA) supplemented with 100 mM glucose containing serial dilutions of histatin 5 (range 150–0.2 µg/mL) or human lactoferrin (range 500–0.4 µg/mL) was added to the plates containing the cells and incubated at 37 °C for 48 h. After incubation, the supernatant was removed, and the cells were gently washed twice in PBS to remove non-adherent cells. Biofilm quantification was performed by the XTT reduction assay, and the metabolic activity of the cells was measured spectrophotometrically at 492 nm (Fluostar Optima; BMG Labtech, Offenburg, Germany).

EVALUATION OF BIOFILM THICKNESS AND TOPOGRAPHY

Biofilm thickness and topography were evaluated by confocal laser scanning microscopy (CLSM) in the presence of histatin 5 or human lactoferrin. The methodology described by Lattif et al. (2010), with slight modifications, was used in order to allow biofilm formation on 8-well borosilicate plates (Lab-Tec, Rochester, NY, USA). Yeast cells were washed twice and resuspended in YNB medium supplemented with 100 mM glucose. Then, 8×10^6 cells were added to the wells of the borosilicate plates and incubated for 90 min at 37 °C. After incubation, the cells were gently washed twice with PBS, and YNB medium supplemented with 100 mM glucose in the presence or absence of histatin 5 at 150 µg/mL or human lactoferrin at 500 µg/mL was then added to the wells. The plates were incubated for 48 h at 37 °C, and the formed biofilms were washed twice with PBS. Afterwards, they were incubated with a solution containing the fluorescent dyes 2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenyl quinolinium iodide (FUN-1; 10 µM) and Alexa Fluor 488 conjugate of concanavalin A (ConA-Alexa Fluor 488; 25 µg/mL) (both purchased from Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA) for 40 min at 37 °C in the dark. Images were captured by the laser confocal scanning microscope LEICA TCS SP5 AOBS and analyzed by FIJI and LAS AF Lite software (Leica Microsystems, Wetzlar, Germany).

STATISTICAL ANALYSIS

Experiments were performed at least three times, and the results are expressed as mean values ± standard deviation. Data were analyzed by Student's *t*-test, and a probability level of 5% ($p < 0.05$) was considered significant.

RESULTS AND DISCUSSION

Among pathogenic fungi that affect humans, *C. albicans* is the species most frequently associated with biofilm development, notably on medical devices (Bonhomme and D'Enfert 2013). In this context, we assessed the effect of histatin 5 and human lactoferrin on *C. albicans* biofilm formation through a methodology proposed by Thein et al. (2007), based on XTT salt reduction. The results revealed that histatin 5 decreased the metabolic activity of the biofilm by 14% at 1.1 µg/mL, reaching

41% of inhibition at the highest concentration used (150 µg/mL) (Figure 1a). Studying the effect of histatin 5 on *C. albicans* and *C. glabrata* biofilms, Konopka et al. (2010) showed that histatin 5 was able to decrease biofilm metabolic activity by 50% at concentrations ranging from 5.1 µg/mL to 21 µg/mL. The difference between these results and the data obtained in our study may reflect the genetic variability among *C. albicans* clinical isolates.

The influence of histatin 5 on biofilm formation may be a consequence of its effect on the yeast-to-hyphae transition. Histatin 5 interfered with the process in a dose-dependent manner, inhibiting morphogenesis by 24%, 25%, and 36% at 37.5, 75, and 150 µg/mL, respectively. Vylkova et al. (2011) showed that starved *C. albicans* cells may acquire amino acids from the extracellular medium and catabolize them as a primary carbon source. The deamination resulting from this process leads to the production of ammonia and extracellular alkalization, which stimulates the fungal morphogenesis. Dur31 is a polyamine transporter associated with amino acid uptake in *C. albicans*, and it is also likely to transport histatin 5 (Kumar et al. 2011), which then may act as a competitive inhibitor of amino acids, decreasing their uptake and impairing morphogenesis induced

by a subsequent raise in pH, a process which in turn inhibits biofilm formation itself (Mayer et al. 2012).

Human lactoferrin significantly affected the metabolic activity of *C. albicans* biofilms (Figure 1b). Treatment with lactoferrin at 1.9 µg/mL led to a decrease of biofilm viability by 18%, reaching 46% of inhibition at 500 µg/mL. Interestingly, the effect of this protein on morphogenesis was inversely proportional to its concentration: it inhibited morphogenesis by 21%, 18%, and 7% at concentrations of 125, 250, and 500 µg/mL, respectively. Iron chelation is the main mechanism related to the antimicrobial and antibiofilm activity of lactoferrin (Ammons and Copié 2013). Nevertheless, some evidence suggests a more complex interaction between lactoferrin, yeast-to-hyphae transition, and biofilm formation, since iron deprivation promotes morphogenesis through *efg1* expression. Hence, iron chelation would facilitate biofilm formation and establishment (Hameed et al. 2008).

The topography of biofilms formed in the presence of 150 µg/mL histatin 5 or 500 µg/mL human lactoferrin was assessed by CLSM. After biofilm formation in the presence or absence of the compounds, the yeast cells were stained with FUN-
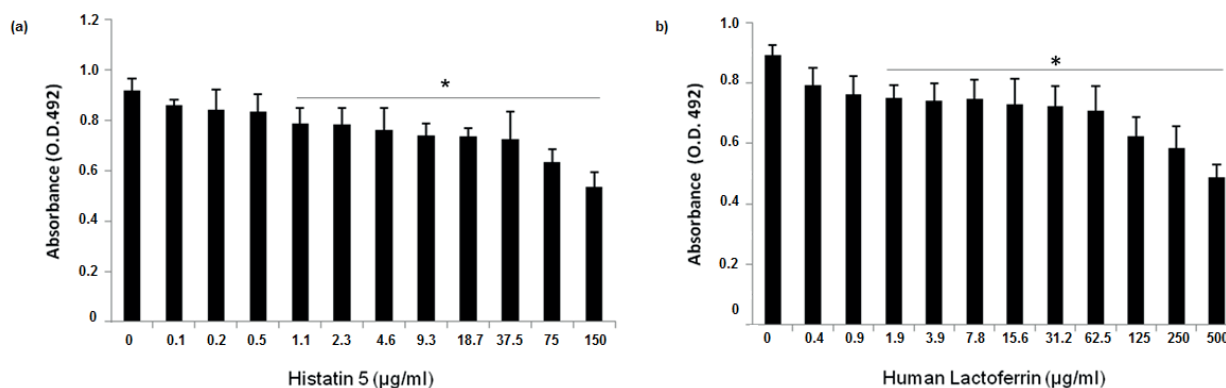

Figure 1 - Effect of histatin 5 (**a**) and human lactoferrin (**b**) on the metabolic activity of biofilm formed by a fluconazole-resistant *C. albicans* clinical isolate. After adhesion, yeast cells were washed twice with PBS 10 mM pH 7.2 and incubated with histatin 5 for 48 h at 37 ºC. Cell viability was measured by the reduction of XTT salt. *$p < 0.05$.

1, a red fluorescent dye that stains the cytoplasm of metabolic active cells, and ConA-Alexa Fluor 488, a green fluorescent dye that stains the extracellular matrix. The results revealed meaningful structural changes in biofilm formation under the influence of histatin 5 or human lactoferrin. The control system, in which the biofilm was formed in absence of the compounds, presented a uniform organization of fungal cells covering the entire surface of the substrate, with a high amount of both yeast and hyphal cells (Figure 2a). On the other hand, the biofilm formed in the presence of histatin 5 or human lactoferrin presented a heterogeneous arrangement, with isolated cell clumps that did not cover the entire surface of the substrate. Furthermore, in these treated systems, mainly yeast cells of a round shape were observed, and hyphae or germ tubes were barely present (Figure 2b, c).

Quantitative analysis of the transmitted fluorescence of biofilms formed in the presence or absence of histatin 5 and human lactoferrin, and the results are summarized in Figure 2d. The data obtained show a significant decrease in the cells' metabolic activity when the biofilms were developed in the presence of histatin 5 at 150 µg/mL or human lactoferrin at 500 µg/mL (56% and 54%, respectively). Nonetheless, a significant reduction in the fluorescence emitted by ConA-Alexa Fluor 488 was not observed when the biofilms were incubated with histatin 5 at 150 µg/mL or lactoferrin at 500 µg/mL, suggesting that these compounds did not affect the ability of treated cells to produce extracellular matrix.

Extracellular matrix production in *C. albicans* biofilms is regulated by two transcriptional factors: *rlm1* plays an essential role in the development of
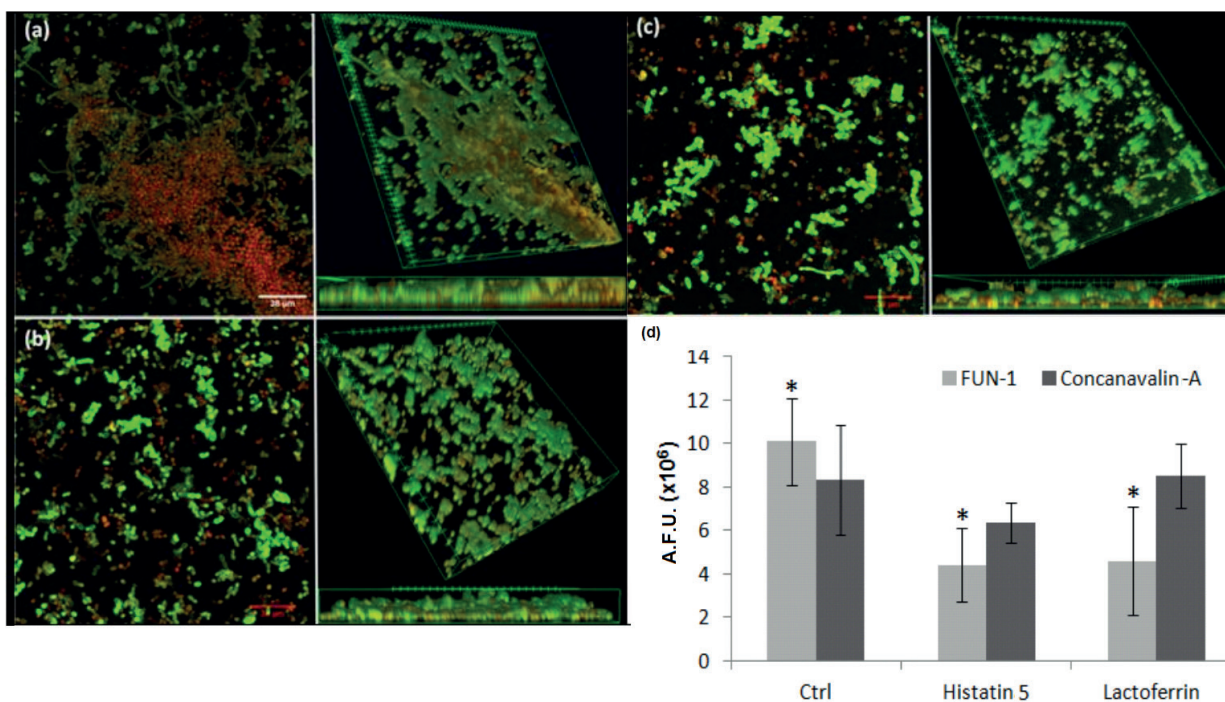

Figure 2 - CLSM in front view (right side) and 3D reconstruction (left side), showing the topography of fluconazole-resistant *C. albicans* biofilms. Biofilms were built for 48 h in absence of the compounds (**a**), in presence of histatin 5 at 150 µg/mL (**b**) and in presence of human lactoferrin at 500 µg/mL (**c**). Biofilms were stained with FUN-1, that stains the cytoplasm of metabolic active cells, and ConA-Alexa Fluor, that stains extracellular matrix. Quantitative analysis of arbitrary fluorescence units (A.F.U.) emmited by FUN-1 and Con-A alexa fluor 488 transmitted by fluconazole-resistant *C. albicans* clinical isolate biofilms that were formed for 48 h in presence of histatin 5 at 150 µg/mL or human lactoferrin at 500 µg/mL. *p < 0.05 (**d**).

β-1,3-glucan matrix (Nett et al. 2011), while *zap1* acts as a negative regulator that inhibits specific genes (*gca1, gca2,* and *adh5*) involved in biofilm maturation and matrix development (Nobile et al. 2009). Upregulation and downregulation of these transcriptional factors by exogenous substances may modulate matrix production (Paulone et al. 2017). Further studies should be conducted to clarify whether histatin 5 and human lactoferrin affect these factors. A combined action on *rlm1* and *zap1* or a lack of such action would justify the results related to fluorescence emitted by ConA-Alexa Fluor 488.

The evaluation of the thickness of biofilms formed in the presence of histatin 5 or human lactoferrin was performed using LAS AF Lite software. The treatment with histatin 5 at 150 μg/mL significantly decreased biofilm thickness by 41.2%. On the other hand, human lactoferrin did not influence biofilm thickness.

In summary, our study points to a potential application of histatin 5 and human lactoferrin in the development of new *C. albicans* antibiofilm treatments, since both compounds were able to inhibit biofilm formation of a fluconazole-resistant *C. albicans* strain. The exogenous administration of these proteins could become a viable therapeutic alternative, overcoming toxicity issues, considering the constitutive and physiological characteristics of the tested compounds.

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AUTHOR CONTRIBUTIONS

JARC and DCM conceived and designed the experiments, analyzed and interpreted data, performed experiments and wrote the manuscript; CAA performed the experiments; MBP conceived and designed the experiments; RMAS conceived and designed the experiments, analyzed and interpreted data, revised the manuscript and gave final approval of the version to be published. JARC and DCM contributed equally and should be considered as first author.

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