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Paracoccidioides species are dimorphic fungi that initially infect the lungs but can also spread throughout the body. The spreading infection is most likely due to the formation of a biofilm that makes it difficult for the host to eliminate the infection. Biofilm formation is crucial for the development of infections and confines the pathogen to an extracellular matrix. Its presence is associated with antimicrobial resistance and avoidance of host defenses. This current study provides the first description of biofilm formation by *Paracoccidioides brasiliensis* (Pb18) and an analysis of gene expression, using real-time PCR, associated with 3 adhesins and 2 hydrolytic enzymes that could be associated with the virulence profile. Biofilm formation was analyzed using fluorescence microscopy, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Metabolic activity was determined using the XTT reduction assay. *P. brasiliensis* was able to form mature biofilm in 144 h with a thickness of 100 μm. The presence of a biofilm was found to be associated with an increase in the expression of adhesins and enzymes. GP43, enolase, GAPDH and aspartyl proteinase genes were over-expressed, whereas phospholipase was down-regulated in biofilm. The characterization of biofilm formed by *P. brasiliensis* may contribute to a better understanding of the pathogenesis of paracoccidioidomycosis as well as the search for new therapeutic alternatives; while improving the effectiveness of treatment.

Introduction

The incidence of systemic fungal diseases has been growing worldwide in recent years, highlighting fungal diseases as an important topic in the field of medicine. Paracoccidioidomycosis (PCM) is a systemic mycosis with higher incidence in Latin America. The majority of cases occur in Brazil, which has the highest concentration of endemic areas, with more than 80% of the reported cases occurring in Brazil. The etiological agent of PCM is *Paracoccidioides* spp.[1,2] This genus is composed of 2 species, *Paracoccidioides lutzii* and *P. brasiliensis*; the latter is sub-classified into 3 different phylogenetic groups.[1,2]

An occupational predisposing factor for acquiring PCM is a work environment that exposes the population to soil, e.g., work on plantations in rural areas.[3] In such an environment, propagules from the mycelial form have access to the lungs.

The ability of microorganisms to adapt to environmental changes is of fundamental importance, as this ability enables pathogens to survive and cause disease. Tissue necrosis generates hypoxic conditions, and both host and pathogens adapt to survive. The adaptations of fungi to hostile environments with low

oxygen levels are underexplored, especially because granulomas appear to have a hypoxic environment.[4]

Pathogenic fungi, such as *Paracoccidioides* spp, have multiple virulence factors that can damage the host. A necessary step in colonization and the development of disease involves the ability of microorganisms to adhere to host surfaces. Adherence is a widely distributed biological phenomenon, which enables microorganisms to colonize their particular habitats. Many fungi, especially pathogenic fungi, are able to adhere to host tissue, the first step in the process of biofilm formation.[5,6] One important event during infection by *Paracoccidioides* spp. is adherence to pulmonary epithelial cells. Several proteins of this fungus (adhesins) have been shown to be ligands of the extracellular matrix in studies performed primarily with lung epithelial cells.[7–10] Various molecules have been described as adhesins in *Paracoccidioides* spp The 43-kDa glycoprotein is involved in *Paracoccidioides* adhesion,[11] as are the 30-, 32- and 54-kDa forms.[8,12] The glycoprotein gp43 and the 30-kDa protein are able to bind laminin.[11,13] Enolase is a fibronectin-binding protein in *Paracoccidioides* spp. that is also present in the cytoplasm and the cell wall, but at a higher levels in the cell wall, suggesting that it performs

additional functions related to the glycolytic pathway.[8,12] *In vitro* studies have shown that the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) adhesin may be involved in the pathogenesis of the fungus because this molecule is capable of mediating the ability of the fungus to enter the cell.[14,15]

Additionally, certain hydrolytic enzymes, such as phospholipase, are used by various microorganisms to invade host tissues.[16] These molecules have been described in *P. brasiliensis* and may play an important role in invasion by this fungus.[16–18] *P. brasiliensis* is also known to produce a variety of proteinases.[19,20] The aspartyl proteinases, also known as acid proteases, constitute one of the 4 superfamilies of proteolytic enzymes. They are generally similar to pepsin and show specificity for preferential cleavage at peptide bonds between hydrophobic amino acid residues.[19]

Biofilms are a form of natural microbial growth that is important in the development of infections. They serve as niches for pathogens and are associated with high levels of resistance to antimicrobial agents.[21] The growth and antibiotic resistance of microorganisms differ based on whether they are located in biofilms or in planktonic form.

Fungi of many types have demonstrated the ability to colonize surfaces and form biofilms (Cryptococcus neoformans, Rhodotorula espécies, Aspergillus fumigatus, Malassezia pachydermatis, Histoplasma capsulatum, Pneumocystis species, Coccidioides immitis, Fusarium species, Saccharomyces cerevisiae, Trichosporon asahii, Zygomycetes, Blastoschizomyces and more recently Trichophyton rubrum and Trichophyton mentagrophytes). Most of the previous studies have focused on biofilms of Candida albicans, but other species of Candida, other yeasts and filamentous fungi are known to form biofilms.[22–25] Most importantly, fungal biofilms are of clinical importance, particularly in the context of chronic diseases.[26] For biofilms to successfully form in a host, microorganisms must first adhere to target tissues and concurrently obtain essential nutrients for growth and development. Recently, an in vitro study demonstrated the efficiency with which Histoplasma capsulatum var. capsulatum forms biofilms on abiotic surfaces.[23] Biofilm formation has been associated with the expression of various adhesins.[27] Therefore, the current study aimed to verify the ability of P. brasiliensis to form a biofilm and to investigate the gene expression of several adhesins that could be associated with the biofilm formation of this pathogen.

Results

Infection of pneumocytes and alveolar macrophages by *P. brasiliensis*

The notion that *P. brasiliensis* can form biofilms is based on experiments showing that the fungus can appear in clumps that strongly resemble the formation of a fungal mass, which is highly characteristic of biofilms. Infections of *P. brasiliensis* (Pb18) yeast cells in pneumocytes and macrophages were evaluated by confocal laser microscopy and In Cell Analyzer 2000. Pb18 was able to adhere to pneumocytes, as fungal masses were attached to several areas of these cells (**Fig. 1B**). The same pattern of interaction

occurred with phagocytic cells, in which an agglomeration of fungal cells was observed around the alveolar macrophage (**Fig. 1D**). **Figure 1A and C** show uninfected macrophages and A549 cells, respectively.

Kinetics of biofilm formation by *P. brasiliensis*

All biofilm standardization was performed with *P. brasiliensis* (Pb18), the strain used in virulence studies. The kinetics of biofilm formation by *P. brasiliensis* was measured on polystyrene microtiter plates using the XTT reduction assay to determine the amount of metabolic activity. The formation of a biofilm was consistent after 144 h of incubation at 37°C in a CO_2 incubator. The initial formation of a biofilm was observed after 7 h. This initial period included pre-adhesion, during which *P. brasiliensis* (Pb18) became attached to the plastic surface in a monolayer arrangement. Over a period of 48 – 120 h, an increase in the biofilm was observed, and the metabolic activity of the biofilm, measured using the XTT reduction assay, increased over time as the cellular mass increased. During the maturation stage (48 – 144 h), the architecture of the *P. brasiliensis* biofilm became more complex (**Fig. 2**).

### Biofilm morphology of *P. brasiliensis*

The morphology of the *P. brasiliensis* biofilm was evaluated using calcofluor white fluorescence microscopy, SEM and CLMS. The *P. brasiliensis* biofilm showed an intense blue coloration, derived from the binding of fluorochrome to the fungal cell wall (**Fig. 3**). SEM revealed a highly organized network of fungal cells in the form of a biofilm and an extracellular matrix (**Fig. 4**). The Pb18 biofilm consisted of a dense network of yeast; an orthogonal image was analyzed to determine the thickness and architecture of the biofilm. The sections of the 3-dimensional image (**Fig. 5B and D**) showed that the *P. brasiliensis* biofilm had a thickness of approximately 100 μm, as observed by CLMS.

Gene expression analysis using real-time PCR (qRT-PCR)

The gene expression of certain adhesins and enzymes of *P. brasiliensis* was compared between the biofilm and planktonic conditions. The expression of GP43, GAPDH and aspartyl proteinase was significantly higher in the biofilm when compared to the planktonic condition (**Fig. 6**). The expressions of aspartyl proteinase, GAPDH, GP43 and enolase were 10, 9, 2.5 and 1.5 times greater in the biofilm, respectively, than in the planktonic form. The expression of phospholipase was less in the biofilm when compared to the planktonic form.

Discussion

Biofilm formation has been described for various fungi, including *Candida* species, *C. neoformans*, *C. immitis*, *A. fumigatus*, *Fusarium solani* and, more recently, *H. capsulatum*.[21,23–25] Adhesion is a biological process that allows various organisms to colonize their habitats. The organisms that form biofilms may grow differently in the planktonic and biofilm forms. The aim of the present research was to investigate the ability of *P. brasiliensis*
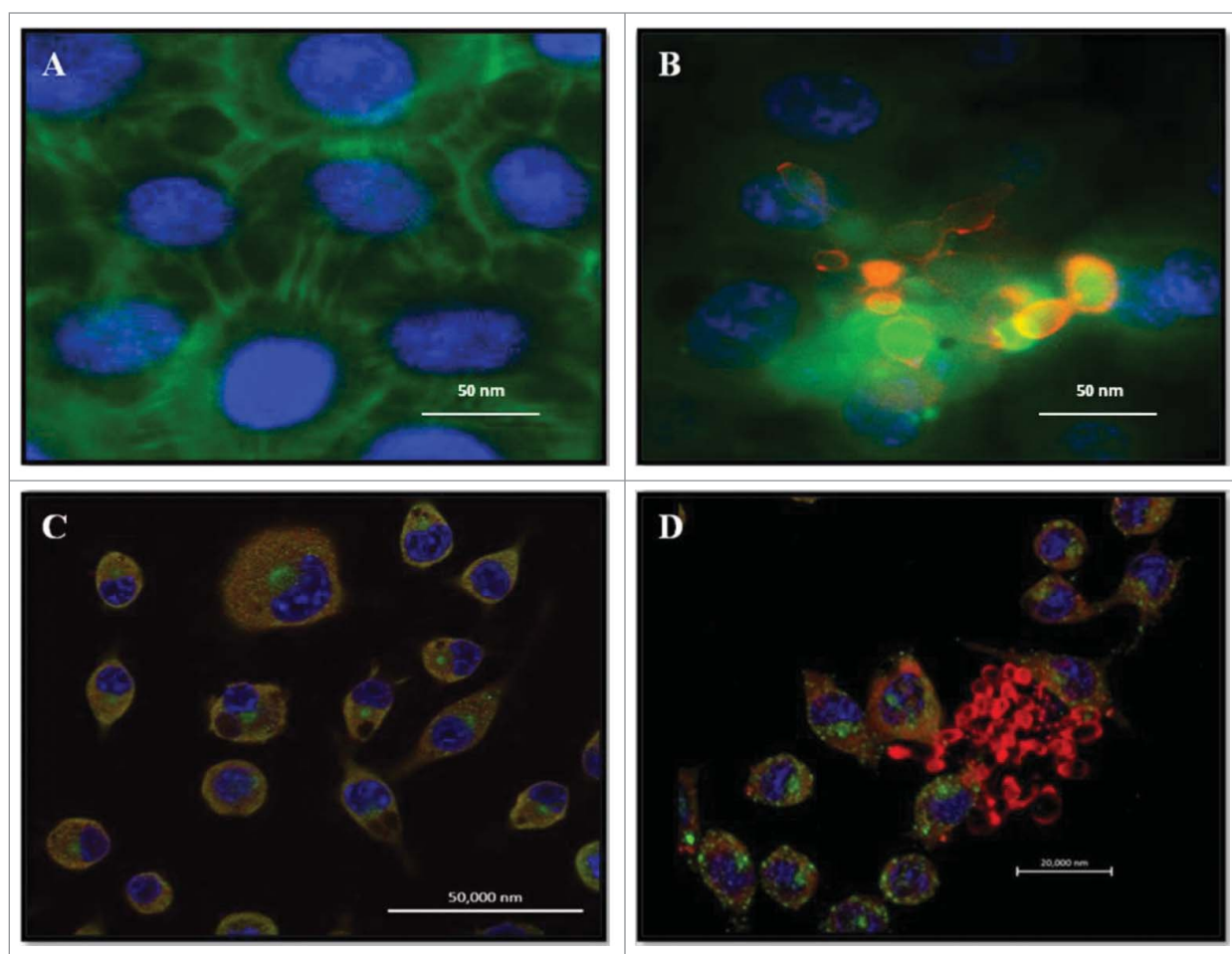
Figure 1. Double Immunofluorescence for epithelial cell line A549 and alveolar macrophages murine AMJ2-C11. (**A**) Uninfected A549 cells. (**B**) A549 cells infected with *P. brasiliensis*. A549 cells were labeled with FITC-phalloidin (green), *P. brasiliensis* stained with anti-cell free antibody and Alexa Fluor® 594 conjugate and nucleus was labeled with DAPI (blue). (**C**) Uninfected macrophages. (**D**) Macrophages infected with *P. brasiliensis*. Macrophages were immunolabeled with the primary antibodies anti- cytoplasmic protein and secondary conjugated Alexa Fluor R 488 (green), and *P. brasiliensis* immunolabeled with anti-cell-free antibody (red) and secondary conjugated Alexa Fluor ® 594, and nucleus was labeled with DAPI (blue) (Zeiss LSM 510 Meta Confocal Microscope).

to form a biofilm and to analyze the expression of genes encoding adhesins and hydrolytic enzymes that could be associated with biofilm formation.

In our study, *P. brasiliensis* was able to form a biofilm *in vitro* under hypoxic conditions. Studies by Bonhomme et al.[40] and Stichternoth & Ernst,[41] have indicated that hypoxia is required for *C. albicans* to form biofilms.
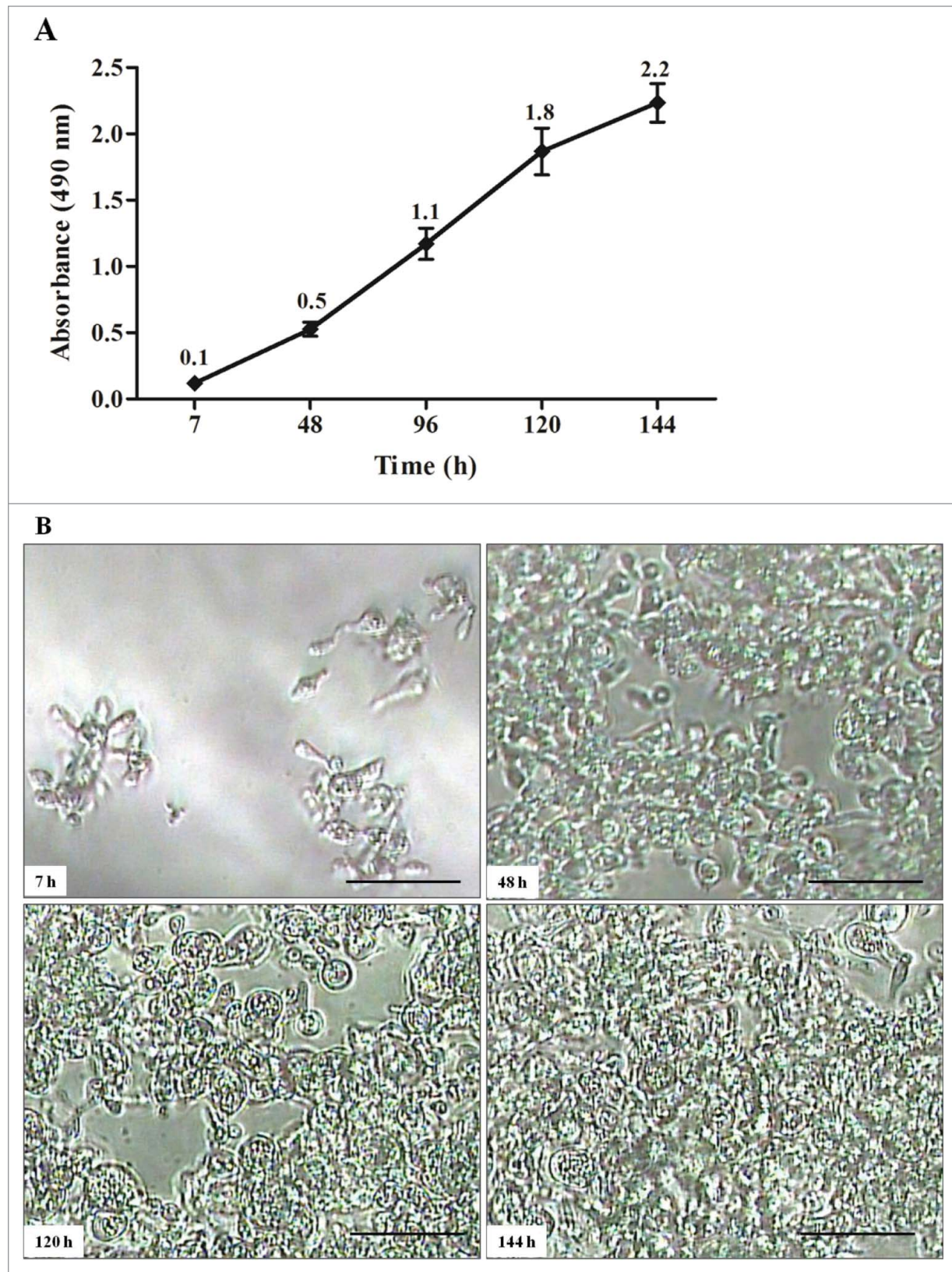
The kinetics of biofilm formation by the Pb18 strain showed that the biofilm initially formed before 7 h had elapsed, which included the adhesion period. Mature biofilms were produced from 120 to 144 h. *P. brasiliensis* is a slowly growing fungus. In terms of the time required for formation, Pb18 shows a longer delay and slower growth when compared with other fungi. In contrast, growth is more rapid in the dimorphic fungus, *H. capsulatum* (72 h), and in *C. albicans* (24 h).[23,42] The thickness of the mature biofilm was approximately 100 μm, which is greater than that found for other fungi, such as *C. neoformans,* which exhibited a biofilm thickness of 76 μm, as demonstrated by

Martinez et al.[43] The metabolic activity of the biofilm increased over time as the cellular mass increased.
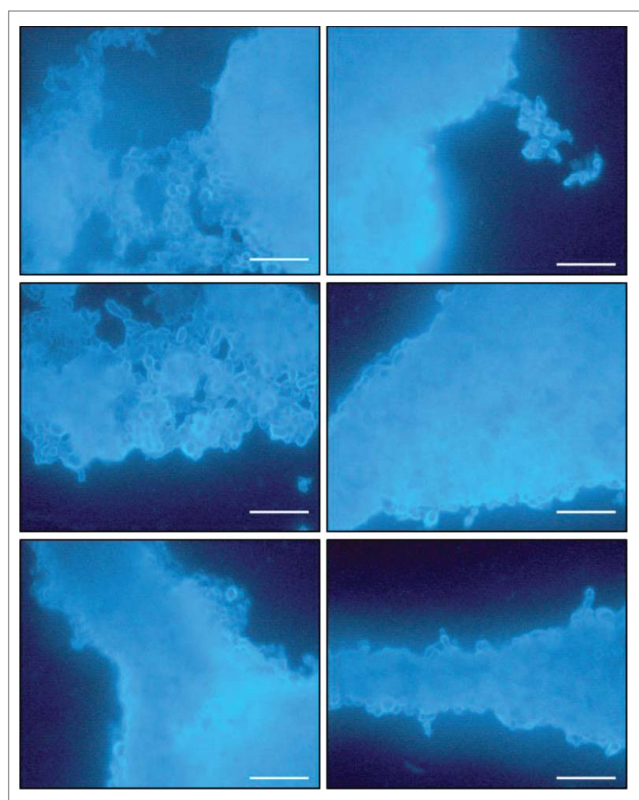
The Pb18 fungal cells may also form an agglomeration resembling a biofilm. However, this structure occurs in *ex vivo* cells, shown in **Figure 1B and D**. Pb18 formed fungal masses in epithelial cells (**Fig. 1B**) and in phagocytic cells (**Fig. 1D**). It was thought that this fungus has the ability to form biofilm, since histopathological findings found fungal mass granulomas. This aspect may be similar to that observed in histopathological observations of organized granulomas, with compact aggregates of macrophages, epithelial cells and masses of yeasts.[44] The same pattern was observed for *H. capsulatum*, which formed a ring of fungal cells in phagocytic cells. This structure may result from a particular type of interaction of this fungus with the tissue. Biofilm formation may contribute to a chronic state of this disease.[23]

The up-regulation of selected genes was demonstrated using qRT-PCR. The results allowed the identification of a potential adhesin of *P. brasiliensis*. Adhesins have an important role in

Figure 2. Kinetics of biofilm formation by *P. brasiliensis* in microdilution plates. (**A**) Measurements determined by the XTT reduction assay. Each point represents the mean of 3 measurements of absorbance at 490nm on a microtiter reader (iMarkTM Microplate Reader; BIO-RAD). (**B**) Kinetics monitored by Microphotograph taken using a camera attached to an inverted microscope. Bars = 30 nm for all panels.

Figure 3. Images of mature biofilms of *P. brasiliensis* (Pb 18), formed after incubation for 144 h at 37°C 40× magnification. Images were acquired by Fluorescence microscopy and the fungi biofilms were stained by Calcofluor White Stain reagent (Fluka ®). All scale bars are 125 μm.

biofilm hinders the action of antifungal agents, and biofilms can become a reservoir of cells that show resistance to certain drugs. Therefore, there is a considerable interest in the persistence of infection due to biofilms in infectious diseases.

Aspartyl proteinase and phospholipase were up-regulated and down-regulated, respectively, in the *P. brasiliensis* biofilm when compared with the planktonic form. The results of the present study are consistent with previous research by Ramage et al.[27] and Nailis et al.,[46] in which an increased expression of aspartyl proteinase and a decreased expression of phospholipase were demonstrated in a *C. albicans* biofilm. Ramage et al.[27] reported that the production of proteinase by *C. albicans* aided and established adhesion, invasion and tissue destruction and may be related to the severity of disease because the expression of this enzyme was significantly higher in mature biofilms.

Proteinases produced by *P. brasiliensis*, *A. fumigatus*, *T. rubrum*, and *C. neoformans* have been described in previous studies and are of great importance because they may split the major components of the basement membrane *in vitro* and are, therefore, potentially relevant for the spread of this fungus.[19,49,50] Additionally, these proteins are hydrolytic enzymes capable of either hydrolyzing large substrates into small units for transportation into the cell to serve as a nutrient or for degrading tissue and facilitating colonization or invasion, serving as a virulence factor for the pathogen.

The current paper is the first study of *P. brasiliensis* biofilms. Additional studies are required to determine the role of these biofilms *in vivo* in association with pathogenesis.

Materials and Methods

Microorganism

Experiments were performed using a clinical isolate of *P. brasiliensis* (Pb18). The strain was cultivated in Fava-Netto at 37°C for 5 d in an atmosphere with a reduced level of oxygen (5% CO_2). This strain was isolated from a case of paracoccidioidomycosis (PMC) and then maintained at the Faculty of Medicine, University of São Paulo, Brazil. This strain is considered a highly virulent isolate (Pb18 isolate), as described in the intraperitoneal infection of susceptible, genetically homogeneous B10A mice.[28] Pb18 also presents a high level of adhesion.[29]

Preparation of inoculum

A suspension of *P. brasiliensis* (Pb18) was prepared in PBS and was adjusted to 10^8 cells/ml for the infection assay based on observations performed with a Neubauer counting chamber.

Antisera and reagents

A cell-free antigen was obtained using Pb18 and was prepared as described elsewhere.[30] Approximately 300 mg of the fungus was added to 1 ml of sterile PBS. This mixture was vortexed for 30 seconds and centrifuged at 400 g for 1 minute. The supernatant (cell-free antigen) was collected, aliquoted and stored at

biofilm formation, as demonstrated by *C. albicans*, in which ALS genes exhibited increased expression during the formation of biofilms.[45] The real-time PCR performed in the current study detected the expression of GP43, GAPDH, aspartyl proteinase and phospholipase in both planktonic form and biofilm growth, but at different levels. The GP43, GAPDH and aspartyl proteinase genes were significantly ($P \leq 0.05$) overexpressed in the biofilm, whereas phospholipase was downregulated. These results are consistent with data demonstrating an increased expression of adhesins in *Candida* biofilms,[46] the most studied fungal biofilm, as well as in bacterial studies.[47]

Various studies have indicated that the 43-kDa glycoprotein is involved in *Paracoccidioides* adhesion.[11,29] In the present study, GP43 was up-regulated in biofilm formation, demonstrating that this glycoprotein could be involved in adhesion and biofilm formation ($P < 0.05$).

A remarkable level of expression of the GAPDH adhesin was observed; almost 10-fold greater in the biofilm form when compared to the planktonic form ($P < 0.05$). Studies performed by Bailão and collaborators,[14] demonstrated that this adhesin was also upregulated in the yeast form based on recovery of the compound from infected mice.

Verstrepen and Klis,[48] also emphasized the remarkable ability of fungi to express adhesins and form biofilms. This characteristic is of great medical importance, because the presence of a mature

Figure 4. SEM images of mature biofilms of *P. brasiliensis* (Pb 18), in different dimensions, formed after incubation for 144 h at 37°C (**A–D**). In the arrows in **Figure 4D** showing the presence of extracellular matrix.

−20°C. The Bradford method (BioRad, São Paulo,SP, Brazil) was used to quantify the protein concentration and the samples were analyzed using the SDS-PAGE method. A polyclonal antibody produced against *P. brasiliensis* cell-free antigen was prepared as described in Mendes-Giannini et al.[5] Rabbits were inoculated with injections of 1.0 ml of antigen mixed with 1.0 ml of complete Freund´s adjuvant. After 3 months of injections of antigen, the rabbits were bled, and the fractions of antisera were separated by precipitation with ammonium sulfate and stored at − 70°C.

The following antibodies were used in this current study: Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, USA), Alexa Fluor® 594 goat anti-rabbit IgG (Molecular Probes, Invitrogen, USA) and Alexa Fluor® 488 goat anti-mouse IgG (Molecular Probes, USA). Fluorescein isothiocyanate (FITC)-labeled phalloidin and all other reagents were purchased from Sigma-Aldrich, USA.

Macrophages and epithelial cell line A549 culture

Macrophages, alveolar line AMJ2-C11 (ATCC CRL-2456), were cultivated overnight in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Brazil), supplemented with 10% fetal calf serum (Cultilab, Brazil). Cultures of a human lung adenocarcinoma, cell line A549, were obtained from the American Type Culture Collection (ATCC-Rockville, MD). These cells were seeded in Ham's F-12 medium supplemented with 10% fetal calf serum.

Infection assay

Macrophages and continuous epithelial cell line A549 cells were cultured at 36.5°C in 24-well plates with the well bottoms covered with coverslips. The cultures were adjusted to 1×10^5 cells per well. A total of 1×10^5 Pb18 yeast-phase cells/ml were added to the cells to obtain a yeast/macrophage ratio of 1:1. After infection, these cover slips were fixed with 4% paraformaldehyde and submitted to immunofluorescence. The coverslips were permeabilized with a solution of Triton X-100, 0.5% in PBS, for 20 minutes. The coverslips were then washed, the "cell-free" antiserum was added, and the samples were incubated at room temperature for 1 hour, washed again, and conjugated anti-rabbit labeled with Alexa 594 (INVITROGEN) at a ratio of 1:300 was added. Phalloidin-FITC conjugate (SIGMA) at a ratio of 1:100 at 4°C was added and the specimens sat overnight. The specimens were again washed, 100 μl DAPI (4 '- 6 – diamidino – 2 – phenylindole) was added for 10 minutes to mark the cores, washed with PBS, mounted on slides with buffered glycerol, and examined using a confocal microscope (Zeiss LSM 510 Meta Confocal Microscope).

The cultures were then incubated for 7 h at 36.5°C to observe adhesion. The assay was analyzed using conventional fluorescence microscopy and scanning confocal laser microscopy (CLMS) (Zeiss LSM 510 Meta Confocal Microscope, Carl Zeiss, Jena, Göttingen).

Biofilm assay

The assay was performed as described by Silva et al.[31] with slight modifications. Initially, 500 μl of a culture of 1×10^8 cells/ml in saline was added to the wells of a 24-well plate (TPP, Trasadingen, Switzerland) and covered with coverslips. The plates were incubated at 37°C for 7 h in 5% CO_2 for biofilm pre-adhesion. After pre-adhesion for 7 h, the supernatant was removed from each well. Subsequently, 1000 μL of mFUM (Modified Fluid Universal Medium – Guggenheim et al.[32]) was added to each well and the plates were further incubated for 144 h. The culture medium was renewed every 48 hours. After biofilm formation for 144 h, the supernatant was again removed, and the wells were rinsed using 400 μl of PBS. Six wells were filled with a sterile medium as a control. All assays were repeated at least 3 times.. To characterize the biofilm, measurements of biofilm metabolic activity were made using XTT. At the structural level, *P. brasiliensis* biofilm formation was characterized
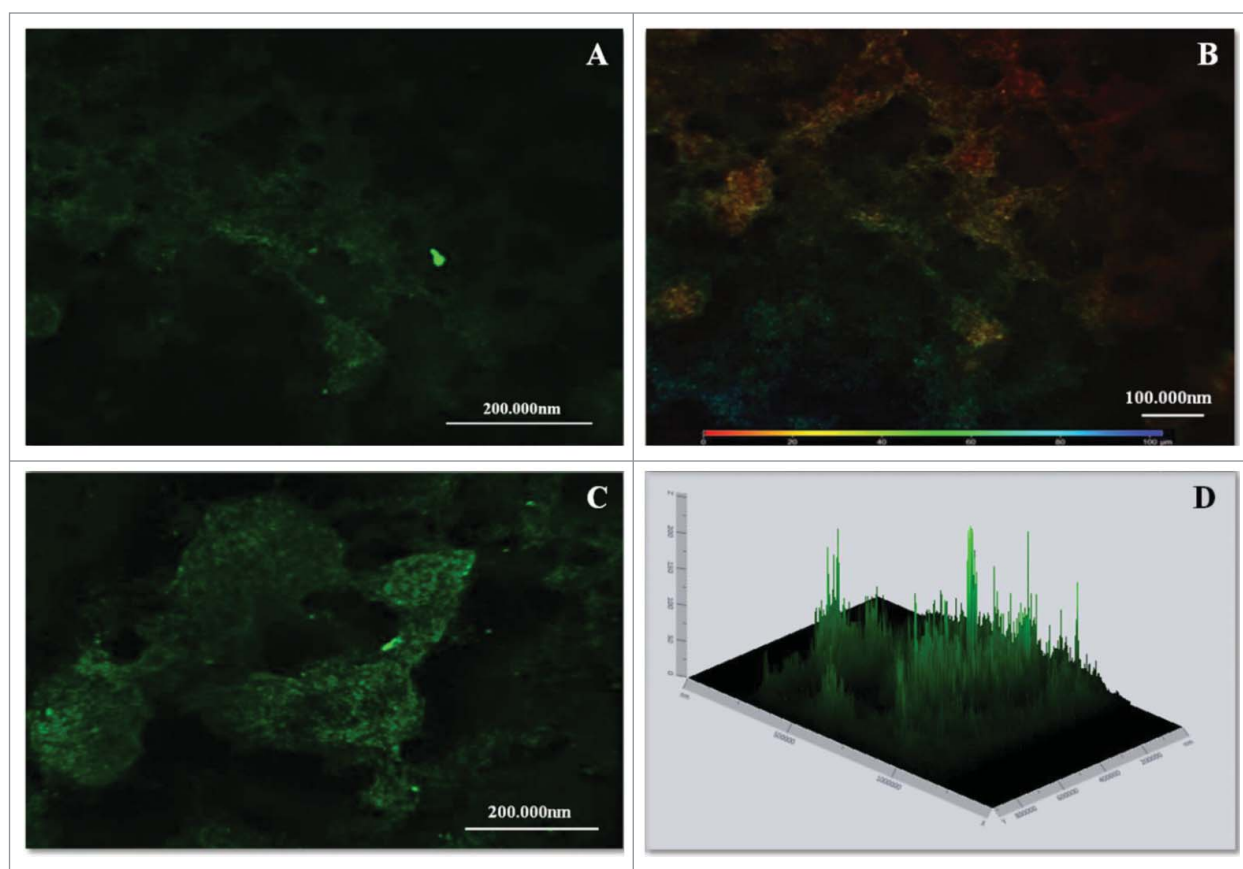
Figure 5. CLSM images of mature biofilms of *P. brasiliensis (144 h)*. Fluorescence labeling of *P. brasiliensis* biofilms. (**A and C**) Biofilm was immunolabeled with primary antibodies anti-cell-free and secondary conjugated Alexa Fluor ® 488. (**B**) Scale depth image A showing the thickness of the biofilm. (**D**) Projection of biofilm formation of *P. brasiliensis* 2.5D (Zeiss LSM 510 Meta Confocal Microscope).

using Calcofluor White Stain (Fluka®, São Paulo, SP., Brazil) and scanning electron microscopy (SEM), and biofilm measurements were performed using confocal laser scanning microscopy (CLSM).

Measurement of biofilm metabolic activity

A quantitative measurement of *P. brasiliensis* biofilm formation was obtained from the XTT reduction assay. For this measurement, 50 μL of XTT salt solution (1 mg ml^{-1} in PBS) and 4 μl of menadione solution (1 mM in ethanol; Sigma-Aldrich, São Paulo, SP., Brazil) were added to each well of the microtiter plates and incubated at 37°C for 3 h, resulting in a colorimetric reaction that is correlated with cell viability. This reaction was measured using a microtiter reader (iMarkTM Microplate Reader; BIORAD, Brazil) at 490 nm. In all assays, culture media were included as negative controls.[31–33]
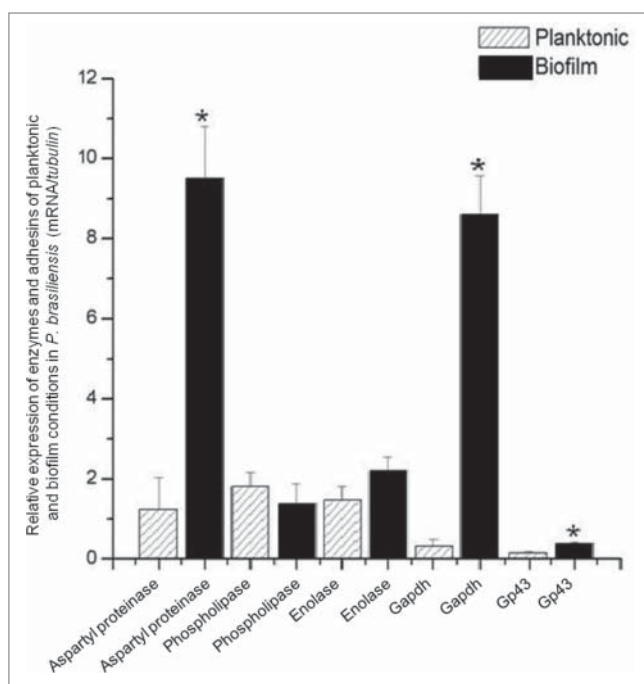
Fluorescence microscopy

For fluorescence microscopy, coverslips with biofilm were stained with Calcofluor White Stain reagent (1g/L – Fluka®). To prepare the coverslips, 50 μl of dye were added. The coverslips were carefully removed from each well and transferred to a slide for observation under a fluorescence microscope. Calcofluor is a non-specific fluorochrome that binds to cellulose and chitin in the fungal cell wall. This staining procedure provides a rapid method for the detection of yeasts and pathogenic fungi.[34] A range of wavelengths, from 300 to 440 nm, can be used for excitation. Emission occurred at a wavelength of 355 nm.

Scanning electron microscopy

Biofilms formed on coverslips were processed as described by Morris et al.[35] and modified by Ells and Truelstrup Hansen.[36] Briefly, the specimens were washed 3 times with PBS to remove planktonic cells and then fixed with 1% glutaraldehyde in 0.2 M sodium cacodylate buffer for 18 h at 4°C. After three PBS washes of 10 min each, the biofilms that still adhered to the coverslips were fixed with 1% osmium tetroxide for 2 h. The specimens were washed with PBS and dehydrated with an increasing gradient of ethanol, from 50% to 100% ethanol, at room temperature. The samples were dried using the critical point method in a Samdri 780A desiccator (Rockville, MD, USA) using CO_2. Topographic features of the biofilms were analyzed with a Zeiss-Leica/440 SEM at the Institute of Chemistry of Sao Carlos, University of Sao Paulo, using a voltage of 25 kV and a 10 mm working distance.

Figure 6. Relative expression of adhesins/ enzymes of *P. brasiliensis* during growth in biofilm and planktonic conditons, both in low oxygen tension using real-time PCR (qRT-PCR). Statistical analyzes were performed by Student's t-test, $P < 0.05$.

Gene	Primers
GP43	Sense 5′-CTTGTCTGGGCCAAAAACTC-3′
	Antisense 5′-GCCAGGGTTTGTTTGACTGT-3′
Enolase	Sense 5′-TAGGCACCCTCACTGAATCC-3′
	Antisense 5′-GCTCTCAATCCCACAACGAT-3′
Phospholipase	Sense 5′-TGTTGGTGCGATCAAAAGAC-3
	Antisense 5′-GGATACGACGTCGCCACTAT-3
Aspartyl Proteinase	Sense 5′-AAAGGAAACACGGAAACACG-3
	Antisense 5′-CGTTCCTGAGACGGTGGTAT-3
GAPDH	Sense 5′-AAATGCTGTTGAGCACGATG-3′
	Antisense 5′-CTGTGCTGGATATCGCCTTT-3′
β-tubulin (Housekeeping gene)	Sense 5′-TGGCCACTTTCTCTGTCGTTC3′
	Antisense 5′-CAGGGTGGCATTGTATGGCT3′

observed under UV light. Two independent RNA samples were prepared for use in the experiments. First-strand cDNA synthesis was performed using 1 μg/μl RNA and the enzyme Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA).[37]

Gene expression analysis for real-time PCR (qRT-PCR)

The expression levels of 5 genes involved in various cellular functions of *P. brasiliensis* were measured and described during growth in biofilm and planktonic forms, both under low oxygen tension. Quantitative real-time PCR (qRT-PCR) was performed for genes using the primer constants shown in **Table 1**. The concentrations of primers used were adjusted to 0.5 μM for improved amplification efficiency. The quantity of cDNA used was 1 μl, and 12.5 μl of Maxima®SYBR Green (Fermentas, São Paulo, SP., Brazil) was added. The final volume was adjusted to 25 μl. PCR was performed with a starting temperature of 50°C for 2 minutes, followed by 10 minutes at 95°C, then 40 cycles at 95°C for 15 seconds, followed by annealing and synthesis at 60°C for 1 minute. The reactions were performed in triplicate using an Applied Biosystems 7500 thermal cycler. Variations in mRNA expression were calculated using the $2^{-\Delta CT}$ formula, where ΔCT is the difference between the targets and the housekeeping gene β-tubulin in accordance with previous studies performed by several authors. The data were analyzed using the $2^{-\Delta \Delta C_T}$ method.[38] The specific primers for qRT-PCR were designed using the Primer 3 software.[39]

Statistical analysis

The data were analyzed using the Origin 6.0 software (Origin Lab Corporation, Northampton, MA). A P value ≤ 0.05 was considered statistically significant.

Indirect immunofluorescence

After biofilm formation, the biofilm was fixed with 4% paraformaldehyde, washed in PBS, and permeabilized in 0.5% Triton X-100 for 30 minutes. After permeabilization, anti-*P. brasiliensis* (cell-free) serum was added for 1 h, unbound antibodies were removed by washing with PBS, and Alexa Fluor®488 goat anti-rabbit IgG was then added for 1 h. The biofilm was then washed 3 times with PBS and analyzed under confocal laser scanning microscopy (LSM 510 – META, Zeiss). For the adhesion assay, the same procedure was used, but the secondary antibody was Alexa Fluor®594 goat anti-rabbit IgG, added for 1 h. FITC-labeled phalloidin was then added for 1 h and observed using confocal microscopy.

RNA isolation and cDNA synthesis

RNA extraction was performed from mature biofilms (144h) and planktonic cells (144h). Planktonic cells were cultured in mFUM in bottles. Every 48 hours, the cells in suspension were withdrawn and centrifuged, then placed in fresh medium in a new flask for a period of 144 hours. After 144 hours, the biofilm and planktonic cells were washed and centrifuged. The resulting pellet was stored in Ultra freezer for subsequent RNA extraction. Total RNA was obtained by the addition of TRIZOL® (Invitrogen, Carlsbad, CA, USA) after growing the Pb18 strain in both the biofilm and planktonic forms under 5% CO_2 conditions. Total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA). RNA quality (i.e., the presence of discrete 18S and 28S rRNA peaks) was determined in 1.5% agarose gels in 1× TBE buffer for 2 h at 100 V. The gels were stained with GelRed and

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