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Original Article

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Oxidative and nitrosative stress responses during macrophage–*Candida albicans* biofilm interaction

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Abstract

Candida albicans is an important source of device-associated infection because of its capacity for biofilm formation. This yeast has the ability to form biofilms which favors the persistence of the infection. Furthermore, the innate immune response has a critical role in the control of these infections and macrophages (Mø) are vital to this process. An important fungicidal mechanism employed by Mø involves the generation of toxic reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI). The interaction between biofilms and these immune cells, and the contribution of oxidative and nitrosative stress, that is determinant to the course of the infection, remains elusive. The aim of this study was to investigate this interaction. To this purpose, two models of Mø-biofilms contact, early (model 1) and mature (model 2) biofilms, were used; and the production of ROS, RNI and the oxidative stress response (OSR) were evaluated. We found that the presence of Mø decreased the biofilm formation at an early stage and increased the production of ROS and RNI, with activation of ORS (enzymatic and nonenzymatic). On the other hand, the interaction between mature biofilms and Mø resulted in an increasing biofilm formation, with low levels of RNI and ROS production and decrease of OSR. Dynamic interactions between Mø and fungal biofilms were also clearly evident from images obtained by confocal scanning laser microscopy. The prooxidant-antioxidant balance was different depending of *C. albicans* biofilms stages and likely acts as a signal over their formation in presence of Mø. These results may contribute to a better understanding of the immune-pathogenesis of *C. albicans* biofilm infections.

Key words: *Candida albicans*, biofilms, macrophages, oxidative stress, yeast.

Introduction

Candidiasis is an infection caused by a group of yeasts of the *Candida* genus, usually *Candida albicans*. This fungus is responsible for widespread disease and the incidence has been increasing particularly among patients with immune systems compromised by human immunodeficiency virus infection, organ transplantation, and/or chemotherapy for cancer.[1,2] Numerous factors have been suggested to be virulence attributes for *C. albicans* and includes secretion of extracellular hydrolytic enzymes, surface recognition molecules, and phenotypic switching; allowing evasion of host immune response.[3,4]

Recently, the biofilm formation by *Candida* has been associated with the progression and persistence of the infection.[5,6] Among fungi, different *Candida* species are the most common colonizers of superficial devices like dental implants, as well as on indwelling medical, frequently associated with biofilm formation. These biofilms are organized communities of microorganisms with an extracellular polymeric substance; being the most common mode of microorganisms' growth in nature.[7] This type of growth has been reported to be more resistant in antifungal treatment and immune response than their planktonic (free-living) counterparts.[8,9] Biofilm formation usually begins with pioneer cells attaching to animate or inanimate surface. Once established, these cells grow and divide to form microcolonies, then a phase of cell filamentation and proliferation is induced and finally, macrocolonies are formed in mature biofilms.[10,11] Studies with different products, metabolites, and conditions of culture have demonstrated a close relationship between oxidative stress and biofilm formation.[12–15]

The interaction between *C. albicans* and its host cells is characterized by a complex interplay between the expression of fungal virulence factors, which results in adherence, invasion and cell damage, and the host immune system, that responds by secreting pro-inflammatory cytokines, activating antimicrobial functions and killing the fungal pathogen.[16,17] Macrophages (Mø) are very important in the control of infections. These cells are capable of phagocytosing and killing microorganisms and secrete a variety of soluble factors, including cytokines and chemokines, and oxidative metabolites such as reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI).[17–20] Although interactions occurring between *C. albicans* and the host immune system have been well investigated for *Candida* planktonically grown, less information is currently available for such interactions in a biofilm environment.[21,22]

The objective of this study was to analyze, through of two *in vitro* different models, the interaction between Mø and early (model 1) or mature (model 2) biofilms. We demonstrated that a co-culture of mature *C. albicans* biofilms with Mø enhances the ability of this pathogen to form biofilms and that this activity is mediated by oxidative metabolites stress, and dependent of stage of biofilm formation.

Methods

Strains and macrophage cells line

C. albicans NCPF 3153 (National Collection of Pathogenic Fungi, Bristol, UK – strain no. 1) and *C. albicans* azole-resistant strain (strain no. 2) that overexpresses the transporter genes CDR1, CDR2, and MDR1 were used and strains were classified as strong biofilm producer, according to the following classification (4 × ODc) < OD.[13,15,23] Before use, yeasts were plated onto Sabouraud dextrose agar (SDA, Difco, Detroit, MI, USA) and incubated overnight at 37°C to ensure purity and viability. For long-term storage, yeasts stocks were kept at −80°C suspended in Sabouraud Dextrose Broth (SDB, Difco) with 15% (v/v) glycerol as cryoprotectant.[13,15,24]

A murine Mø-like cell line (RAW 264.7, Sigma-Aldrich Co, St. Louis, MO, USA) was used. This was maintained in Roswell Park Memorial Institute medium (RPMI) 1640 medium without phenol red (Sigma-Aldrich) containing 10% v/v heat-inactivated fetal bovine serum (FBS; Greiner Bio-One, Frickenhausen, Germany) and 0.1% v/v gentamicin (Pfizer Inc., NY, USA) at pH = 7.2.[25,26]

Experimental design

Model 1, early biofilm: To obtain monolayers of Mø, 6×10^4 cells were dispensed aseptically into a 96-wells plate (Greiner Bio-One). After 2 h of incubation (37°C, 5% CO_2), plates were washed with cold RPMI 1640 (pH = 7.2) to remove nonadherent cells. The cells attached on the plate were incubated with *C. albicans* strains at a yeast/Mø ratio of 3/1 in a humid atmosphere of 95% air and 5% CO_2 for 24 h at 37°C, and the different parameters of oxidative stress were evaluated[25] (Fig. 1A).

Mϕ monolayer was >90 % pure according to morphologic analysis or non-specific esterase staining (Sigma-Aldrich). Viability assessed by the Trypan blue (Sigma-Aldrich) exclusion test was greater than 98% in all experiments.[25,26] As positive controls 1μg/ml lipopolysaccharides (LPS) from *Escherichia coli* (055:B5; Sigma-Aldrich) were used.[25,27]

Biofilm formation for 24 h on plates without Mø was used as biofilm formation control.[13,15] Heat-killed

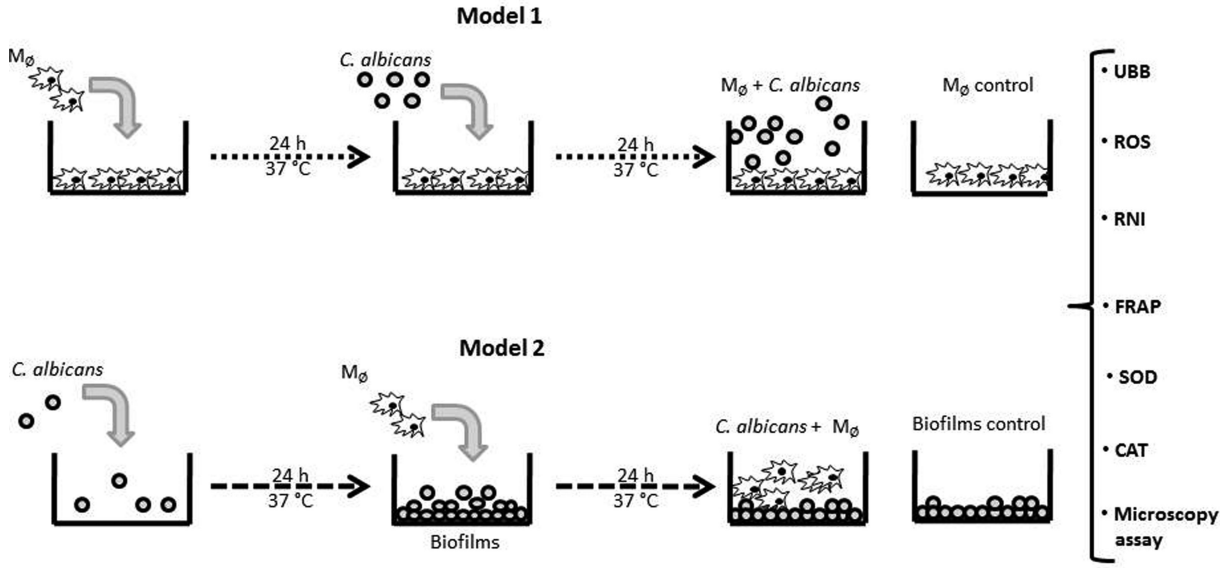

Figure 1. Model host–pathogen interaction. Model 1, early *C. albicans* biofilms over macrophages (Mø). Model 2, *C. albicans* mature biofilms and Mø were co-culture. Different parameters as reactive oxygen species (ROS), reactive nitrogen intermediates (RNI), levels of total antioxidant defences (FRAP), the enzymes superoxide dismutase (SOD), catalase (CAT) and microscopy assays were performed.

C. albicans (Caϕ)(100°C/15 min) at a 1:10,000 ratio (Mϕ+Caϕ) was used as nonforming biofilms control, but with the ability to stimulate host cells.[4,27]

Model 2, mature biofilms: Mature *C. albicans* biofilms formation were adapted from a method of O'Toole and Kolter,[28] which is based on the ability of microorganisms to form biofilms on solid surfaces.[12–15] Briefly, 100 μl of 1 × 10^9 colony-forming units per milliliter (cfu/ml) suspension in cold RPMI 1640 were seeded into flat-bottomed 96-well microplates (Greiner Bio-One) pretreated with FBS (50%) at 37°C for 30 min. Following fungi adhesion (90 min), each well was washed with cold RPMI 1640 to remove nonadhered cells, and 200 μl of fresh medium was added. Microplates were incubated for 24 h without shaking to allow the mature biofilms formation (37°C, 5% CO_2). After incubation, the plates were washed twice with cold RPMI 1640 and the supernatants were eliminated. Then, Mø (6 × 10^4 cells) or RPMI 1640 (culture control) were aggregated and incubated for 24 h (37°C, 5% CO_2). After incubation, biofilm formation and oxidative stress metabolites were evaluated. Biofilm formation for 24 h on plates without Mø was used as unstressed negative control and wells were prepared with the same pre-treatment contained RPMI 1640 as negative control (Fig. 1B).[13–15]

Mϕ cells were pretreated with chloroquine for 15 min prior to infection that is a known lysosomotropic agent that increases lysosomal pH and inhibits lysosome-autophagosome fusion. Chloroquine stock solutions (0.05 mM C6628, Sigma-Aldrich) were made in double deionized water and sterilized by filtration with an Acrodisc syringe filter with a 0.2 μm Tuffryn membrane (Pall Life Sciences, NY, USA).[30] Viability assessed by the Trypan blue exclusion test was greater than 98% in all experiments.[25,26]

Quantification of biofilm and microscopy assay

The total biomass of the biofilm formed was quantified by staining with Crystal violet (CV) (1% w/v) for 5 min.[28] After incubation, the plates were washed three times with 200 μl of phosphate-buffered saline (PBS), and CV was extracted from the biomass with a detaining solution: ethanol/acetone (70:30). Optical density (OD) was measured at 595 nm by using a multi label reader (Tecan Sunrise Model, TECAN, Männedorf, Switzerland). The biomass of the formed biofilms was expressed in biofilm biomass unit (BBU), which was arbitrarily defined as 0.1 OD_{595} equal to 1 BBU. Correlation study between cfu/ml with BBU was determined.[12–15,29]

The Mϕ-biofilms co-cultures were observed by optical, fluorescence microscopy and confocal scanning laser microscopy (CSLM) as described below. Model 1 and 2 were formed on small glass covers (12 mm Ø; Menzel Deckgläser, Braunschweig, Germany) placed in the wells of a 24-well microtiter plate (Greiner Bio-One). Previously, all approaches were prescreened at 100–400× magnification using an inverted optical microscope (Ax overt 40 C, Carl Zeiss MicroImaging GmbH, Göttingen, Germany). For morphological evaluation of Mϕ with biofilms co-cultures, cells were observed by optical and fluorescence

microscopy. Stained cells were observed at 600–800× magnification with an Axioplant fluorescent microscope equipped with a digital camera (DXM1200, Nikon, Hamamatsu, Japan). For fluorescence microscopy and CSLM assay, material was fixed with methanol (500 μl) at −20°C for 10 min prior washing with 500 μl Tris buffer saline (TBS, pH 7) 3 times for 5 min. It was blocked with blocking solution for 1 h at 25°C to fix cells. The cells were incubated with LEAF Purified Anti-mouse CD45 Antibody (25 μl) (Biolegend-LEAF ^{TM} Purified anti-mouse CD45) for 90 min at 25°C and then, with the secondary antibody (25 μl) (Invitrogen-Alexa Fluor® 546 anti-IgG / orange-red reacting with heavy IgG chains, Invitrogen, Paisley, UK) for 90 min at 25°C. It was washed two times with TBS (500 μl) for 5 min. Subsequently, *C. albicans* was stained using fluorescein isothiocyanate (FITC)-labeled anti-*Candida* polyclonal antibody (Meridian Life Science, Saco, Memphis, TN, USA).[27]

Samples were observed using an Olympus FluoView FV1000 CSLM) Olympus Latin America, Miami, FL, USA) equipped with objectives UPlanSApo20 × /0.75(NA), 60 × /1.4(NA) and 100 × /1.40 oil UIS2 Olympus oil immersion lens. Optical sections were acquired at 0.5-μm intervals for the total thickness of biofilms.[13–15] For each sample, images from three randomly selected positions were obtained and analyzed independently. The thickness of *C. albicans* biofilm was assessed using COMSTAT software.[15]

Assays for oxidative and nitrosative metabolites

The supernatant of the different Mϕ-biofilms co-cultures was separated for ROS and RNI stress metabolites. ROS production was detected in the supernatant (1 mg/ml) by the reduction of nitro blue tetrazolium (NBT, Sigma-Aldrich) to form an insoluble precipitate (dark blue diformazan) at 37°C for 30 min, so that the formed blue diformazan is proportional to the generated ROS. To stop the reaction, 0.02 ml of hydrochloric acid (0.1 M) was added. Optical density was measured at 540 nm (Tecan Sunrise Model), and results were expressed as OD_{540nm}/BBU (ROS/BBU).[13–15]

RNI production was determined using the Griess reagent, which was measured as nitrite by a microplate assay method.[25–27] Optical density was measured at 540 nm, and results were expressed as the nitrite concentration value/BBU (RNI/BBU).[26,30–32]

Nonenzymatic and enzymatic antioxidant activity

The oxidative stress response (OSR), which include both enzymatic and nonenzymatic mechanisms, and particularly, the enzymes superoxide dismutase (SOD) and catalase (CAT) were evaluated in Mϕ-biofilms co-cultures (models 1 and 2). The total OSR was evaluated by the ferric reducing antioxidant potency (FRAP) assay of the cultures thought of its ability to reduce Fe^{+3} to Fe^{+2}, which reacted with Trypiridiltriazine (TPTZ, Sigma-Aldrich) to give a blue complex that absorbed at 593 nm. The supernatants (10 lb) were mixed with 25 μl of 10 mM TPTZ in 40 mM HCl, 25 μl and 20 mM $FeCl_3.6H_20$ plus 250 μl of 300 mM acetate buffer (pH 3.6). Standard $FeS0_4$ diluted at different concentrations was employed in order to plot concentration (μM of Fe^{+2}) versus OD. The results were expressed as FRAP/BBU.[15,31,32]

The specific enzymatic activity of SOD was assayed photochemically based on the ability of SOD to inhibit the reduction of NBT by the generated superoxide radical through the illumination of riboflavin at the presence of oxygen and the electron donor methionine. Results were expressed as SOD/BBU.[13–15]

Likewise, to quantify CAT activity, the supernatants were treated with 50 μl of PBS, 40 μl of 0.2 M H_2O_2, and 200 μl of 0.2 M $K_2Cr_2O_7$ solution in glacial acetic acid. The curve of the reaction was made with different concentrations of pure CAT plus the reagents mentioned above. The OD was determined at 570 nm and the results were expressed as as CAT/BBU.[13,31]

Statistical analysis

All experiments were performed by triplicate in three independent experiments. The data presented are the cumulative results of all experiments performed, expressed as means plus standard deviations and were statistically analyzed by using a one-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons. Differences between means were assessed with a P value $<.05$ being considered statistically significant.

Results

To examine the interactions between host immune cells (Mø) and early or mature *C. albicans* biofilms, two different co-cultures, models 1 and 2, respectively, were used (Fig. 1).

At early stages of biofilm formation, the presence of Mø resulted in a diminution of the biofilms formed in both strains, however this effect was more evident in strain 1 than in strain 2 (2 vs. 1.4 times - $^{**}P \leq .005$). In addition, Mø activated with heat-killed yeast (Mϕ + Caϕ) shown similar values than Mø activated with LPS; obviously non-biofilm formation was observed (Fig. 2A). Although the Mϕ

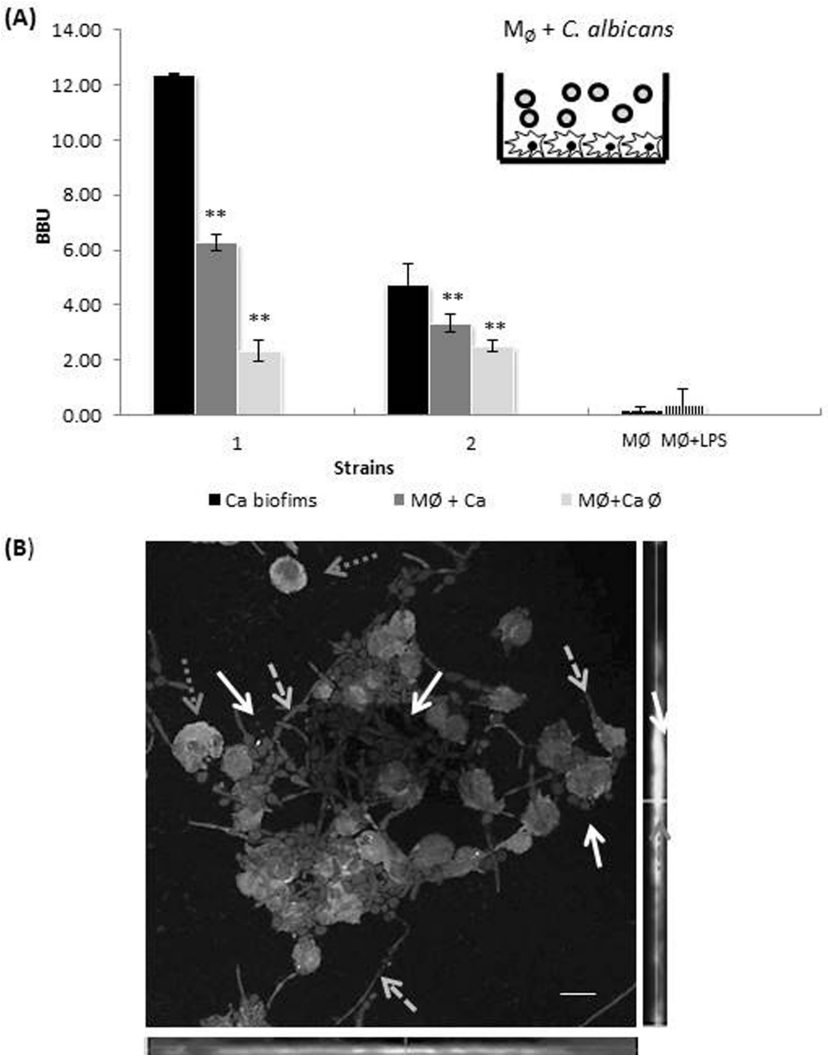

Figure 2. Model 1, co-culture of macrophages (Mø) with early *C. albicans* biofilms. (A) Quantification of biofilms by Crystal violet (CV) staining expressed in biofilm biomass units (BBU). The data are presented as the mean ± SD for three independent experiments, each in triplicate. $^{**}P \leq 0.005$ denote statistical significance with untreated biofilms. (B) Confocal scanning laser microscopy (CSLM) images of early *C. albicans* biofilms (strain 1). Green channel show yeast cells (white arrows) and hyphal forms (light grey arrows). Dark grey arrows show the Mø. The lowest images and the right margin of each panel correspond to the X/Z and Y/Z planes, respectively. Scale bar: 10 μm.

do not form biofilms, they were stained with CV giving a background color and therefore were included in Figure 2.

The architecture of this model was evaluated using CSLM, which can provide information about the morphological composition and organization yeast cells and Mϕ and their spatial localization. Figure 2B shows laser scanning fluorescence images for XY (top) and XZ (bottom) of sessile cells (green) of *C. albicans* biofilms stained with FITC-labeled anti-*Candida* polyclonal antibody, which reveal that biofilm formation for strain 1 was 12.5 ± 5 μm. The presence of yeast cells, blastopores (white arrows) and hyphae (light blue arrows) was observed. The hazy yellow appearance was due to diffuse staining of the Mø that were observed in the bottom of the plate. In an early step of biofilms formation of *C. albicans* incubated with Mø clearly it was observed that there was phagocytosis of green-stained fungal cells within red-stained Mø. The Mø- *C. albicans* co-culture by optical (inset) and fluorescence microscopies is shown in Figure 3A and with heat-killed *C. albicans* in Figure 3B.

Reactive oxygen species production has been demonstrated to be involved in the biofilm genesis, and it plays a fundamental role in the regulation of this biological process. The ROS generation at early biofilms in contact with Mø was higher than that observed in Mø-only cultures in both strains (increased 10 and 7 times the ROS levels) and was greater for strain 2 than strain 1 ($^{**}P \leq .005$). In addition, the incubation with heat-killed *C. albicans* strains 1 and 2

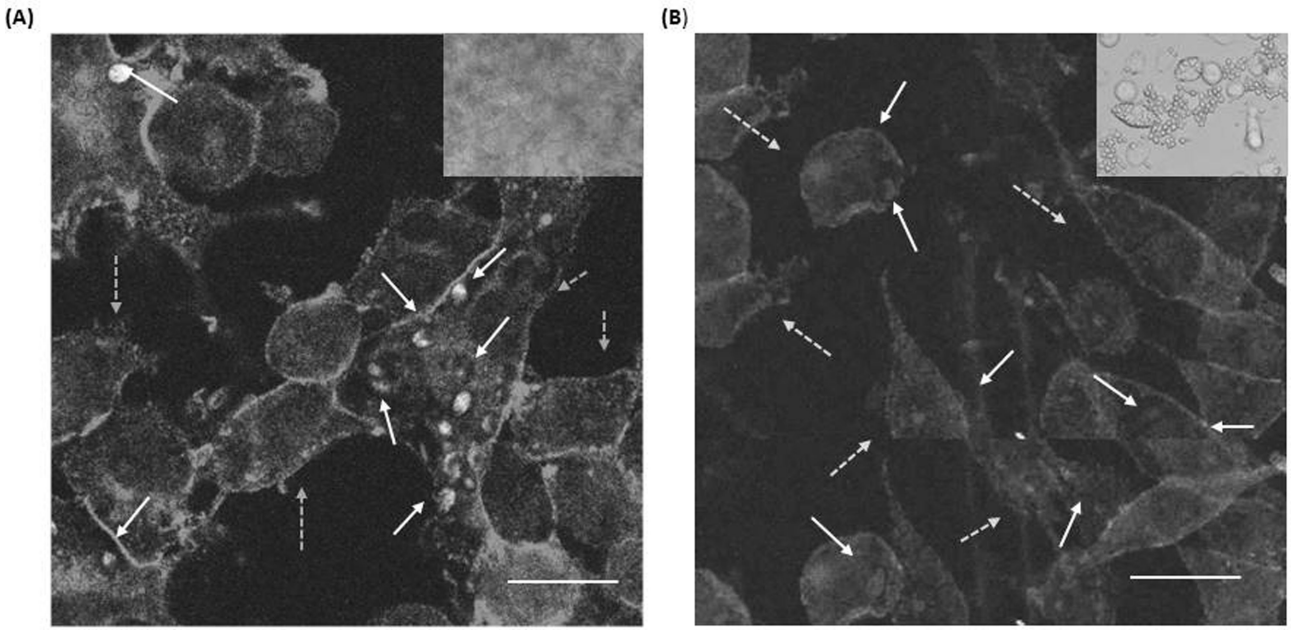

Figure 3. Images of fluorescence and optical (inset) microscopies of macrophages (Mø)-*C. albicans* coculture. (A) Strain 1 of *C. albicans*. (B) Heat-killed *C. albicans*. White arrows show yeast cells and light grey arrows the Mø. Scale bar: 10 μm.

increased the ROS levels, respectively, but these were fewer than those produced in the co-cultures of Mø- *C. albicans* (Fig. 4A).

It has been demonstrated that other cytotoxic product of stress such as RNI, that including nitric oxide, modulates biofilm formation. Therefore, the levels of this metabolite in our models were evaluated (Fig. 4B). In a similar manner as observed with ROS, when the biofilm is formed on Mø, the levels of RNI were higher than those observed in control cultures for both strains 1 and 2 ($^{**}P \leq .005$). Strain 2 showed higher levels of nitrosative stress than control and heat-killed *C. albicans* (Fig. 4B), but the increased with respect to control was greater in strain 1 (5 time vs. 2.2).

A reduction in the oxidative defenses can result in oxidative stress; the total OSR determinate by FRAP, and the activity of specific enzymes, such as SOD and CAT, were evaluated in our models (Fig. 5). The levels of FRAP in Mø-early biofilm co-cultures of strains 1 and 2 were higher than those found in control cultures ($^{*}P \leq .05$). In addition, in these cultures, the SOD activity was incremented with respect to Mø alone cultures ($^{*}P \leq .05$), but less CAT activity was observed. At the incubation with heat-killed *C. albicans*, this treatment resulted in an increment of OSR, especially in CAT activity (Fig. 5C).

At model 2, the aggregated of Mø to mature biofilms resulted an increasing 4.5 times in the BBU in comparison to the control biofilms for strain 1 ($^{**}P \leq .005$). This increase was observed at strain 2 as well, but it was less marked (Fig. 6A). The BBU of the *C. albicans* biofilm formed at the presence of Mϕ pretreated with chloroquine was similar to that of *C. albicans* biofilms alone in both strains. However, this BBU was lower than that observed in biofilms formed in presence of Mø untreated.

To determine the localization and depth of Mø and the architecture, morphological composition and organization of hydrated biofilms of *C. albicans*, we used imaging reconstruction by CSLM (model 2). The laser scanning fluorescence images shows the XY (top), XZ (bottom), and ZY (left) planes of sessile cells (green) with yeast cells (white arrows) and hyphal elements (light blue arrows) of *C. albicans* biofilms and very few Mø at the top of biofilms (red arrows). The red-spotted appears diffusely, suggesting that the extracellular biofilm material could partially cover the Mø. The biofilms thickness was 60–70 μm (Fig. 6C). Figure 6B and D show pseudo-color images, according the multicolor scales, where observed the Mø inside of *C. albicans* biofilms. The yeast cells appear in dark blue in a complex morphology with blastopores and hyphae imbibed in the extracellular polymeric substance, while the Mø in turquoise. Mø were localized mostly at the top layers and were unable to inside in basal layers of biofilms. Interestingly, these Mø did not appear to phagocytose *C. albicans* cells because, the pretreated Mø with chloroquine shown similar values than biofilms alone (Fig. 6A).

The production of ROS in the co-culture of model 2 was expressed as the ratio between stress metabolites and the

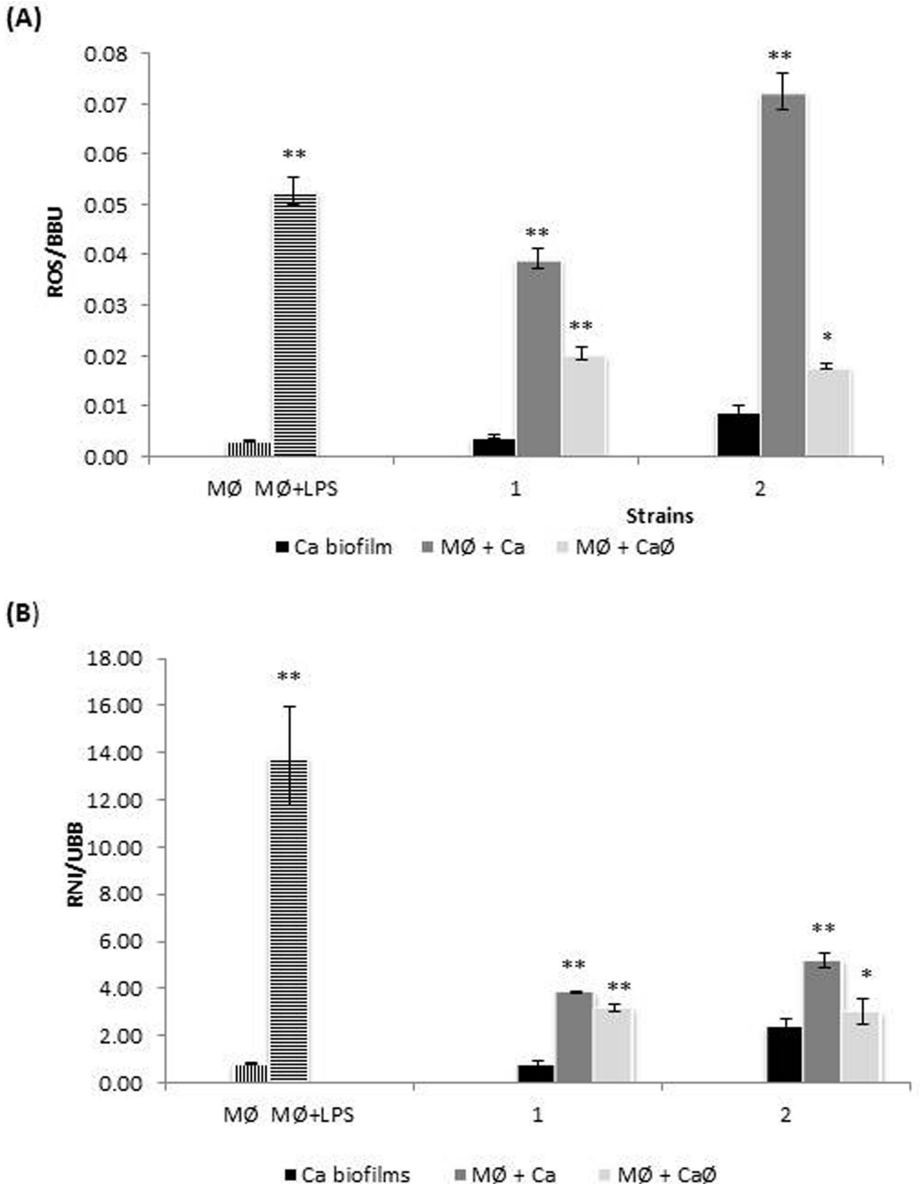

Figure 4. Metabolites of cellular stress in Model 1. (A) ROS/BBU ratio determined by a nitro blue tetrazolium (NBT) assay. (B) RNI/BBU ratio determined by Griess reagents. Mø incubated with lipopolysaccharides (LPS) from *E. coli* (positive control) and macrophage (Mø) with RPMI 1640 medium (negative control). Error bars represent the standard deviations of the means of three independent experiments, each in triplicate. $^{*}P \leq 0.05$ and $^{**}P \leq 0.005$ denote statistical significance with Mø only cultures (control).

biofilm (ROS/BBU; Fig. 7A). The ROS levels were decreased significantly compared that control level in co-culture of both strains, although ROS levels in strain 2 were higher than those induced in cultures with strain 1. No significant differences at the levels of ROS were found between biofilms alone and co-culture of Mø treated with chloroquine. Similar profile to ROS production was found when the levels of RNI were studies (Fig. 7B).

In both strains, the antioxidant system (OSR) decreased significantly compared with control biofilms or Mø treated with lysosomotropic agent ($^{*}P \leq .005$). The level of CAT in the co-cultures with Mø was similar to the control. However, different of CAT activity with Mø treated with chloroquine was found in strain 2, where the levels were elevated (Fig. 5D). SOD activity was undetectable by our methodology in this model.

Discussion

Extensive research has focused on understanding genetic and biochemical aspects of biofilm development and antifungal drug resistance of fungal pathogen.[5–9] However, less

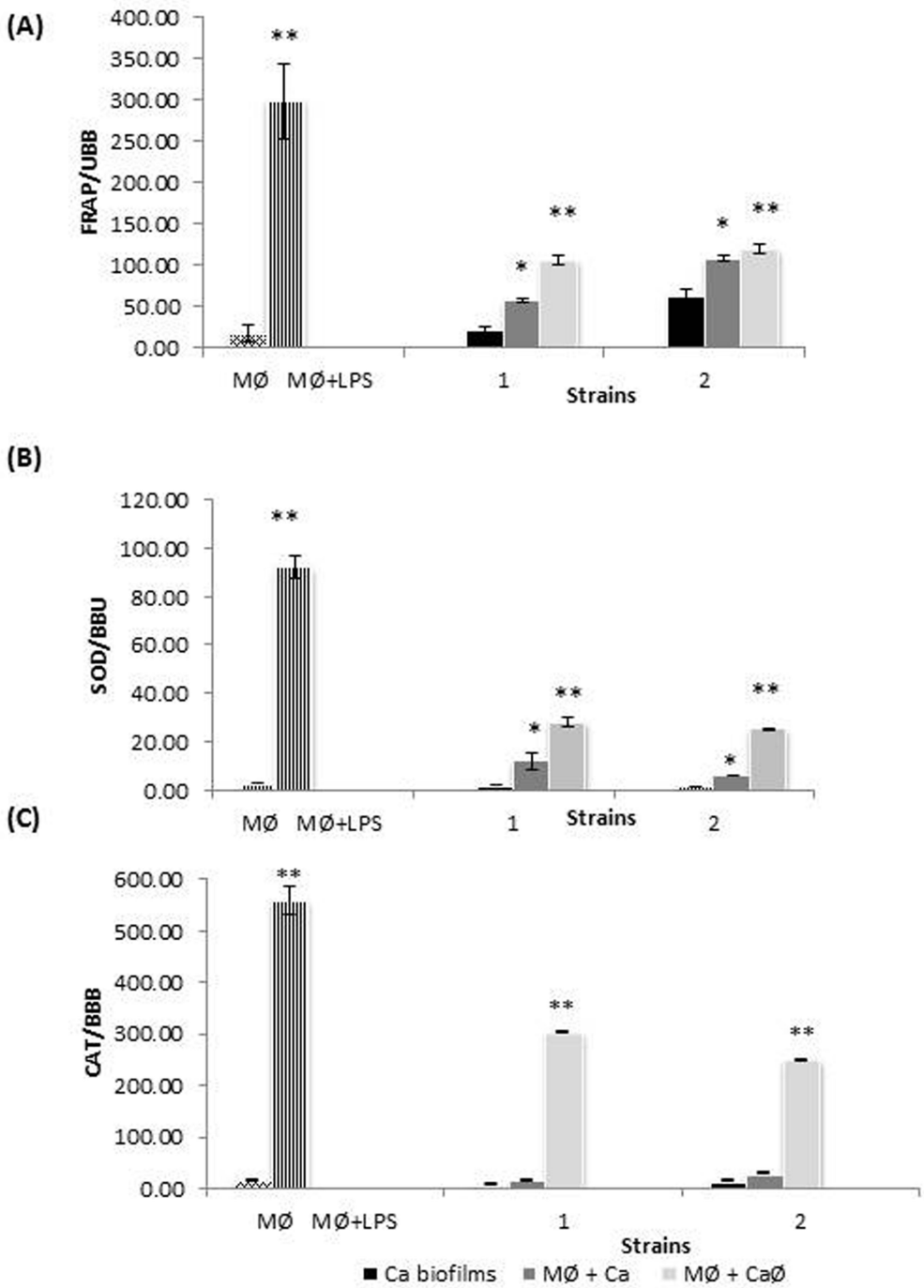

Figure 5. Antioxidant defenses in *C. albicans* biofilms in Model 1. (A) FRAP/BBU. (B) SOD /BBU and (C) CAT/BBU. Macrophages (Mø) incubated with lipopolysaccharides (LPS) from *E. coli* (positive control) and Mø with RPMI 1640 medium (negative control). Error bars represent the standard deviations of the means of three independent experiments, each in triplicate. $^{*}P \leq 0.05$ and $^{**}P \leq 0.005$ denote statistical significance with macrophage only cultures (control).

research have been concentrated on the interaction and effects of the host immune response on *C. albicans* biofilms pathogenesis.[21,22,33,34] There are dynamic interactions that occur between host, which immune cells mechanisms and an array of cytotoxic soluble factors play an important role, and pathogenic fungi like *C. albicans*, which have evolved robust adaptive responses to evade this response and survival in their hosts.[4,16,25–27,33–35] *C. albicans* must adapt to diverse niches they occupy in their host and the dynamic environmental; the success dependent upon their ability to

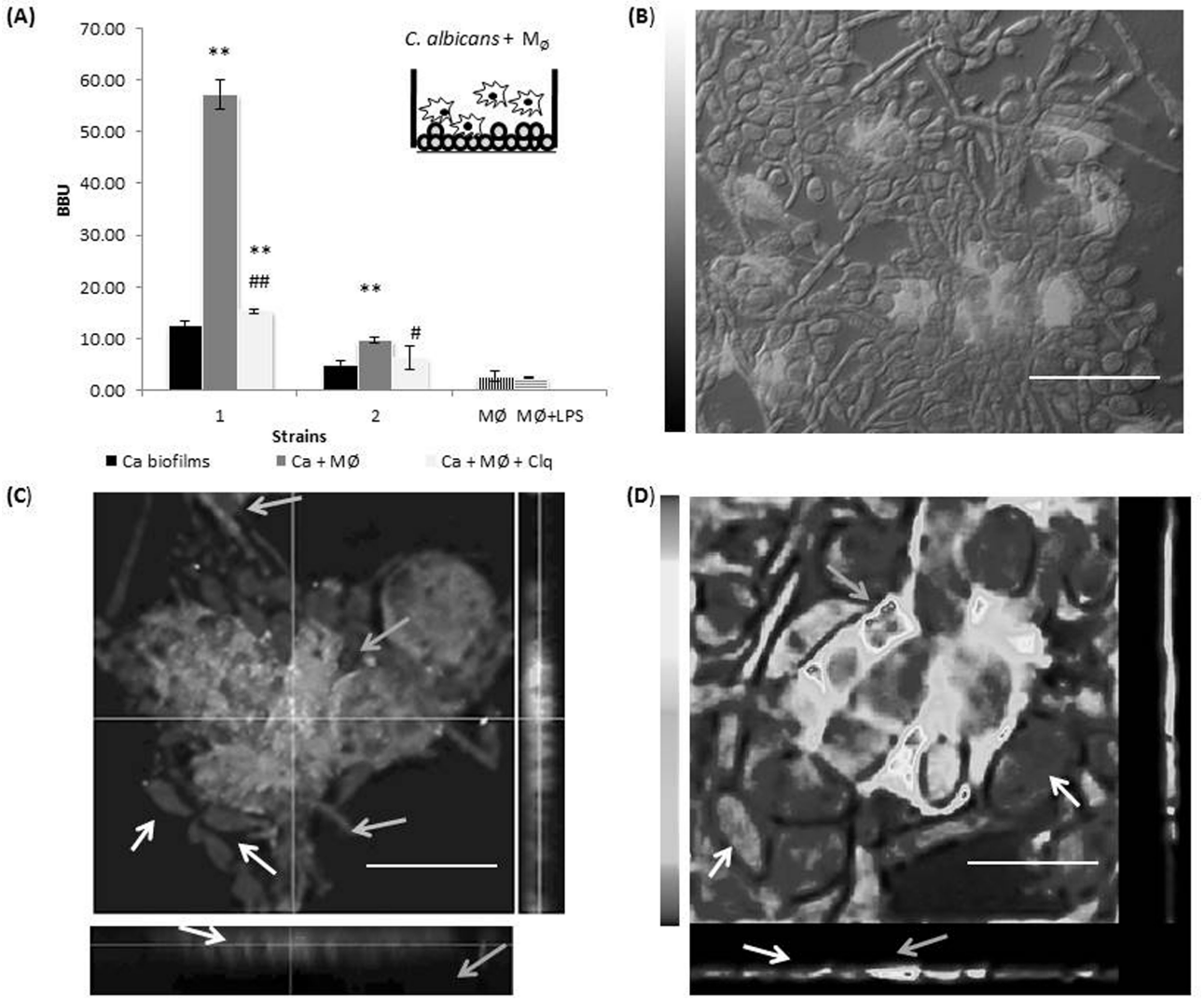

Figure 6. Model 2, mature biofilms in co-culture with macrophages (Mø). (A) Aggregated of Mø to mature biofilms and quantification by Crystal violet (CV) staining expressed in biofilm biomass units (BBU). Mø were pre-treated with chloroquine, as phagocytosis inhibitor, and Mø with RPMI 1640 medium as negative control. The data are presented as the mean ± SD for three independent experiments, each in triplicate. $^{**}P \leq 0.005$ denote statistical significance with untreated biofilms; # $P \leq 0.05$ and ## $P \leq 0.005$ denote statistical significance between biofilm treated with Mø untreated versus Mø plus chloroquine. (B) Pseudo-color imaging reconstruction by confocal scanning laser microscopy (CSLM). (C-D) CSLM images for XY (top), XZ (bottom) and ZY (left) of sessile cells with yeast cells (white arrows) and hyphal forms (light grey arrows) of *C. albicans* biofilms and Mø at the top of biofilms (dark grey arrows) without being able to penetrate inside. (D) Pseudo-color image of *C. albicans* biofilms with Mø where the deep is represented according the multicolor scale. Scale bar: 10 μm.

adapt to the different stresses, such as osmotic, oxidative, and nitrosative stresses, among others factors.[16,18,19,36]

Candidiasis is usually associated with the colonization of medical devices, such as dental implants, catheters, heart valves, vascular bypass grafts, ocular lenses, artificial joints, and central nervous system shunts, which can act as substrates for biofilm growth. However, the clinical rates of infection depend significantly of implant location.[5,9,37] Several steps are necessary to the formation of biofilms, initiating with the interaction of the surface (model 1) until the presence of a mature biofilms (model 2). In these steps, the organization, metabolites, enzymes, and so forth, are different; therefore, the interaction with immune cells on these steps also could be different. Mature biofilms are more resistant than young biofilms to the treatment with antimicrobials and to immune system, and it is possible that biofilms formation may be a defense mechanism through which *C. albicans* protects itself against host immune response.[18,36–39] We investigated whether interactions between Mø and *C. albicans* biofilms, at different stages of maturation, lead to a differential production of metabolites stress, which could be involved

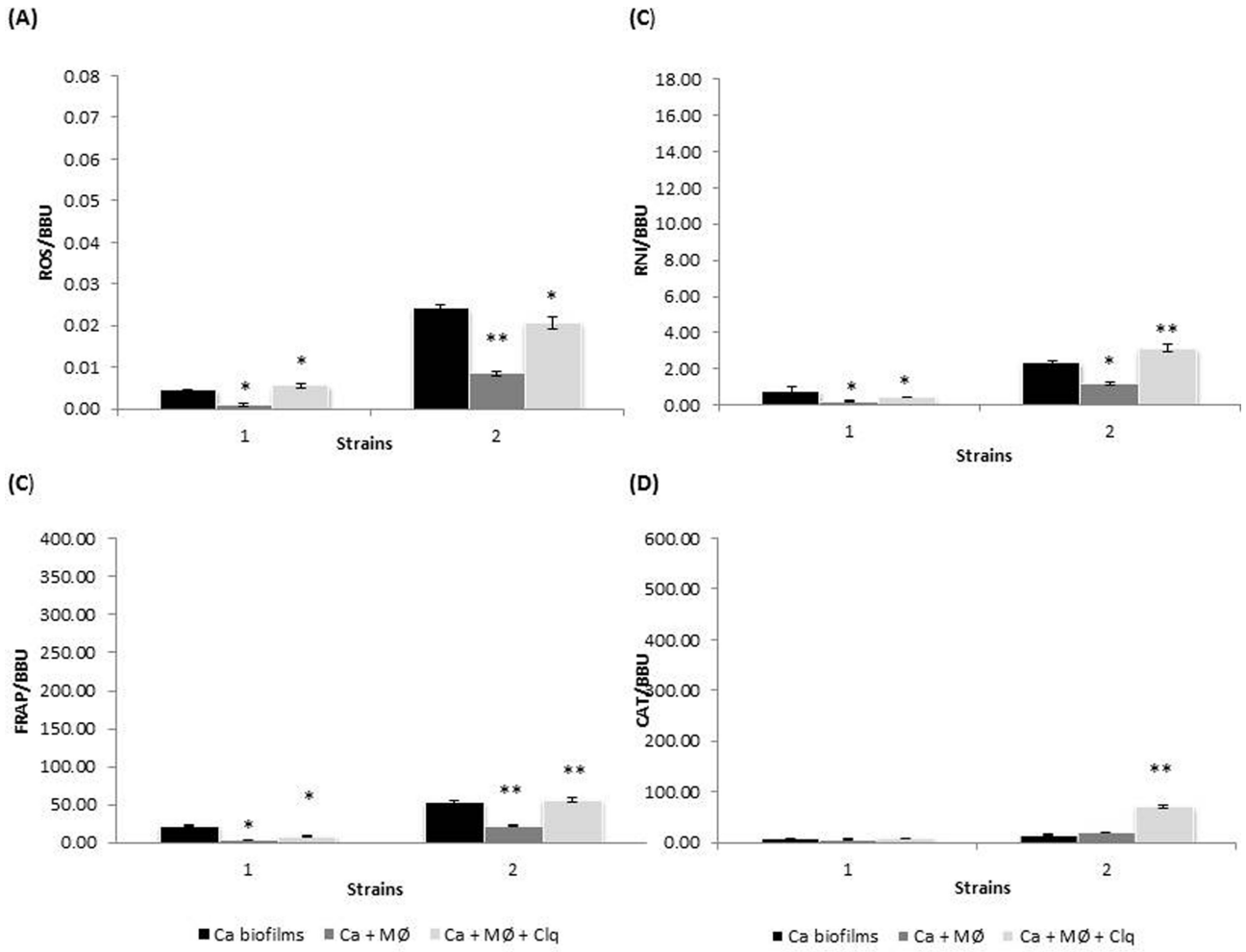

Figure 7. Metabolites of cellular stress and antioxidant defenses in Model 2. (A) ROS/BBU ratio determined by a nitro blue tetrazolium (NBT) assay. (B) RNI /BBU ratio determined by Griess reagents. (C) FRAP/BBU. (D) CAT/BBU. Mø with RPMI 1640 medium was negative control. Error bars represent the standard deviations of the means of three independent experiments, each in triplicate. $^{*}P \leq 0.05$ and $^{**}P \leq 0.005$ denote statistical significance with untreated biofilms (control).

with the modulation in the formation or maintenance of biofilm.

In this work we describe two experimental model of co-culture *C. albicans* biofilm-Mø *in vitro* and analyzed the profile of stress metabolites (ROS and RNI) and antioxidant system (enzymatic and nonenzymatic) developed in them. In addition, studies by CSLM were realized to evaluate the effect of Mø on the architecture and thickness of *C. albicans* biofilms. These two examples *in vitro* of Mø-biofilms interactions could simulate the first steps of infection with biofilm growth and colonization in presence of immune cells (model 1) and the effect of Mø on a mature biofilms formed, for example, on a medical device (model 2). Our results shown that biofilms formed in presence of Mø previously adhered to surfaces were significantly less thick than those formed in their absence (model 1; Fig. 2A). In contrast, we found that *C. albicans* mature biofilms increased the biomass with the presence of Mø (model 2; Fig. 6A). To examine if the phagocytic activity of the Mϕ could be involved in this effect, these cells were pretreated with chloroquine, a phagocytosis inhibitor, and posteriorly exposed to the biofilms. The biofilm formed in co-cultures of Mφ pretreated with chloroquine was thicker than that formed in presence of Mφ untreated. However, this comparison is more significant for strain 1 ($^{\#\#}P \leq .005$) than strain 2 ($^{\#}P \leq .05$). The BBU observed in biofilms alone and in that incubated with Mø pretreated with chloroquine was similar. This diminution in the biofilm formation observed in the treatment with chloroquine is consistent with the antimicrobial effect attributed to this drug by different authors. In this sense, monocytes treated with chloroquine have increased antifungal activity against *Cryptococcus neoformans* by alkalization of the phagolysosome by this drug. In *C. albicans*, the treatment of mature biofilms with

fluconazole and voriconazole in combination with chloroquine resulted in a significative inhibition of biofilm formation in comparison to the treatment with the antifungals alone. A state of iron deprivation induced by chloroquine has been postulated as the responsible of this phenomenon but the exact mechanism remains to be elucidated. In our study, the possible mechanisms involved in the biofilm formation in presence of Mφ pretreated with chloroquine must be investigated in more details. However, a mechanism mediated by ROS and RNI must be not discarded since this treatment results in an alteration of the oxidative stress in relation to the incubation with Mφ untreated.

Our findings show that the interactions between Mø and *C. albicans* differ depending of the state of maturation of the biofilms (early vs. mature). In a similar way to our results, previous studies findings the immunoprotective effect of bacterial and fungal biofilms and reported that the formation of biofilm was influenced in presence of neutrophils, or adherent peripheral blood mononuclear cells (PBMC) and this activity is mediated by a soluble factor present in biofilm-PBMC co-culture supernatant.[33,34,39]

Heterogeneous architecture is characteristic of fungal biofilms, where in the base there are principally yeast cells and on the top hyphal forms.[5–7] CLSM analyses confirmed these findings and showed that early biofilms formed by *C. albicans* contained profuse hyphal elements over Mø and were significantly less thick than those *C. albicans* biofilms grown alone (model 1; Fig. 2B). The ability to switch between yeast and filamentous forms is central to pathogenesis, as hyphal forms adhere and colonize. However, different characteristics were observed in model 2 (Fig. 6B, C, D). Our analyses showed mature *C. albicans* biofilms that thicker at the presence of Mø. This biofilm was formed with yeast cells, blastopores-rich and less hyphae; at the presence of very few Mø visible only on the top layers of biofilm of the mature *C. albicans* biofilms and they were not found on the basal or middle layers. The thicknesses of biofilms formed from *C. albicans* biofilm formed alone were similar to those obtained from Mø treated with chloroquine, demonstrating that the biofilm-enhancing activity was present only in co-culture with Mø. However, the link between differentially expressed cytokines or other soluble factors and the biofilm-enhancing activity of the immune cells–biofilm culture supernatant is not known.[33,34]

Reactive oxygen species production has been demonstrated to be involved in the biofilm genesis and plays a very important role at the regulation of this biological process.[12–19,37–39] To examine if metabolites of oxidative stress (ROS and RNI) were involved in the biofilm-enhancing activity of Mø in this interaction, we isolated supernatants from the Mø-biofilm co-culture growing. ROS levels found at the early biofilms in contact with Mø previously attached to the surface had significantly higher oxidative stress metabolic activity than those observed in biofilms control (Fig. 4A). In contrast, the production of these oxidative species in Mø-mature biofilms cultures was lower than those produced in biofilms with the absence of Mø (Fig. 7A). As our results demonstrate, the oxidative profile developed at early stages of biofilms formation was vastly different than that induced in the mature biofilms. Nitric oxide is part of the nonspecific host defense that increase during an acute infection as the vulvovaginal candidiasis.[17,18,26,38,40] Previously, our group has demonstrated that RNI modulates biofilm formation.[12–15] In similar form to that observed with ROS, when the early biofilm was formed over Mø, the levels of RNI were higher than those observed in control biofilm (Fig. 4B). However, the RNI levels produced in the cultures Mø-mature biofilms were lower than those observed in biofilms control (Fig. 7B).

According with previous studies in our laboratory have demonstrated that the induction of a state of oxidative stress, generated by modifications of environmental conditions or by the presence of antibiotics or natural extracts, results in the alteration of the biofilm formation by different microorganisms.[12–15,31] For example, the treatment of *Candida tropicalis* biofilms with extracts obtained from *Heterophyllaea pustulata* Hook f. (Rubiaceae) resulted in the reduction of biofilms formation through a mechanism mainly mediated by oxidative stress triggered under photodynamic light action.[14] In addition, the formation of biofilms by *Staphylococcus aureus* under unfavorable conditions was associated with an increase of ROS and RNI production, and a decreased of the extracellular matrix.[12] In concordance, other authors also indicate a relationship between biofilm formation and oxidative stress.[33,39]

Microorganisms such as *C. albicans* have developed different strategies to respond to oxidative stress generated as a by-product of aerobic respiration and thus maintain the redox homeostasis within the cell. In this sense, these pathogens use enzymatic (SODs, CATs, and peroxidases) and nonenzymatic mechanisms to resist oxidative stress and ensure survival within the host.[31,32] The levels of FRAP and SOD in Mø-early biofilm co-cultures were higher than those found in biofilms control (Fig. 5). The current employed methodologies in this study are not able to discriminate between Mø and yeast OSR activities; this is a limitation in this study. However, in model 2, the FRAP levels were decreased significantly compared with those founded in biofilms in absence of Mø and there was no activation of the SOD and CAT enzymes (Fig. 7C–D).

The characterization of the oxidative stress profile and antioxidant defences may lead to the identification

of the pathways that regulate the interactions of *C. albicans* biofilms with host immune cells and may help to develop new strategies to treat infections related to biofilms.[14,15,41–44] In model 1, the misbalance between high oxidative plus nitrosative stress and the poor responses of ORS, enzymatic and nonenzymatic, may contribute to the reduction of BBU of biofilms. On the other hand, we observed that Mø-mature biofilms co-culture (model 2) enhanced biofilm formation, while metabolites of oxidative stress decreased at very low levels. According with our results, Xie et al. found that mature biofilms were resistant to leukocyte killing and did not trigger increased of ROS, even though neutrophils retained their viability and functional activation potential.[40]

In conclusion, our study demonstrated the ability of Mø to influence formation of *C. albicans* biofilm, associated with the prooxidant-antioxidant balance present in biofilms-Mø co-culture. The interactions between biofilms of *C. albicans* and Mø are unique for this mode of growth and differ between early or mature *C. albicans* biofilms. These results contribute to a better understanding of the interaction between biofilms and cells of the immune system, which may help to clarify the relevance of biofilms as virulence factor in the immunopathogenesis of *C. albicans* infection.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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