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Candida tropicalis biofilm's matrix—involvement on its resistance to amphotericin B



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ABSTRACT

Candida tropicalis has emerged as one of the most prevalent fungal pathogens, and its ability to form biofilms has been considered one of the most important virulence factors, since they represent high tolerance to antifungal agents. However, the mechanisms of biofilm resistance to antifungal agents remain poorly understood. Thus, the main goal of this study was to infer about the ability of amphotericin B (AMB) to control and combat *C. tropicalis* biofilms. Additionally, it was also intended to determine the influence of matrix components in biofilm resistance. AMB was unable to totally prevent biofilm formation and to eradicate *C. tropicalis* preformed biofilms. Moreover, AMB led to a significant increase of the biofilm production due to an augment of the total protein and carbohydrate contents of the matrix. The *C. tropicalis* biofilm matrix assumes an important role on its resistance to AMB.

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1. Introduction

Candidiasis is the most prevalent opportunistic fungal infection on humans and, as such, a major public health problem (Silva et al., 2012). In the recent decades, candidiasis have been associated to *Candida tropicalis*, and it has been reported as an important causative agent of the bloodstream and urinary tract infections in hospitalized patients, causing high mortality and morbidity (Negri et al., 2010, 2012; Silva et al., 2012).

During the last decades, the levels of candidiasis due to *C. tropicalis* have been increasing, and it could be attributed to the greater use of antifungal agents nowadays and over long periods of time, which enabled an increase of resistance of *C. tropicalis* to certain antifungal agents, specifically to amphotericin B (AMB) (Muñoz et al., 2011; Negri et al., 2012). In clinical practice, AMB, belonging to the polyene group, is 1 of the antifungal agents most commonly used to treat candidiasis (Silva et al., 2012; Williams et al., 2011).

Biofilms have been considered the most prevalent growth form of microorganisms and 1 of the virulence factors that mostly contribute to the pathogenicity of *Candida* species (Negri et al., 2012; Silva et al., 2011, 2012). Moreover, from the clinical perspective, the most important feature of *Candida* biofilms is its role in increasing tolerance to conventional antifungal therapy (Silva et al., 2012).

However, despite that there is some knowledge about the effect of AMB against *C. tropicalis* planktonic cells (Negri et al., 2010; Silva et al., 2012), there is a lack of information concerning its role on *C. tropicalis* biofilms. Thus, the main aim of this work was to infer about

the AMB ability to control and combat *C. tropicalis* biofilms and to determine the influence of matrix in the biofilm resistance.

2. Materials and methods

2.1. Organisms and growth conditions

A total of 3 different *C. tropicalis* strains were used during this work: 1 oral strain (AG1), from the biofilm group of the Centre of Biological Engineering, originally isolated from Clinic Dentistry, Congregados, Braga, Portugal; 1 urinary tract strain (519468), recovered from patients of the Hospital of S. Marcos, Braga, Portugal; and 1 vaginal strain (12), obtained from the archive collection of the University of Maringá, Brazil. The identification of all isolates was confirmed using CHROMagar *Candida* medium (CHROMagar, Paris, France) and by polymerase chain reaction (PCR)-based sequencing using specific primers (ITS1 and ITS4) against the 5.8S subunit gene reference. Genomic deoxyribonucleic acid (DNA) was extracted following previously described procedures (Williams et al., 1995). The PCR products were sequenced using the ABI-PRISM Big Dye terminator cycle sequencing kit (Perkin Elmer; Applied Biosystems, Warrington, UK). For all experiments, *C. tropicalis* strains were subcultured on Sabouraud dextrose agar medium (SDA; Merck, Darmstadt, Germany) for 48 h at 37 °C.

2.2. Minimum inhibitory concentrations determination

MICs of *C. tropicalis* strains were determined for the antifungal agent AMB (Pfizer, NY, USA), using the microdilution method, in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Pfaller et al., 2008). The AMB concentrations tested were 0.5, 1, 1.5, 2, 2.5, 4, and 6 mg/L.

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To confirm the MICs values, cell suspensions were recovered to a new well microplate, and serial dilutions were plated in SDA at 37 °C. After 24 h of incubation, the total number of colony-forming units (CFUs) was determined, and the results were presented as Log₁₀ CFUs per milliliter (Log₁₀ CFUs/mL).

Assessments of the response profiles with different antifungal agents were performed in triplicate and on 3 different occasions.

2.3. Biofilm formation

An inoculum of each yeast strain, obtained from SDA plates, was resuspended in 30 mL of Sabouraud dextrose broth medium (SDB; Merck, Darmstadt, Germany) and incubated for 16–18 h at 37 °C, under agitation at 120 rpm. Then, cells were harvested by centrifugation at 5000g for 10 min at 4 °C and washed twice with phosphate-buffered saline (PBS), pH 7.0, 0.1 mol/L. The pellets were then resuspended in PBS. Prior to being used in each experiment, the cellular density adjusted to 2×10^5 cells/mL, in Roswell Park Memorial Institute 1640 medium (RPMI; Sigma, St. Louis, MO, USA) supplemented with 2% of glucose, using a Neubauer counting chamber.

2.3.1. Influence of AMB in biofilm formation

In order to study the effect AMB on *C. tropicalis* biofilm formation, different concentrations of the agent (0.5, 1, and 2 mg/L) were prepared in RPMI medium and added at the beginning of the biofilm process formation. AMB solutions were prepared with twice the concentration tested in RPMI medium, from stock solutions of 1000 mg/L in dimethyl sulfoxide (Sigma). Cells were prepared as described above, and each well of a 96-well microplate (Orange Scientific, Braine-l' Alleud, Belgium) was filled with 100 µL of the antifungal agent and 100 µL of the standardized cell suspension. Controls were also performed with *Candida* cells without antifungal agents. The microplates were incubated for 48 h at 37 °C and 120 rpm. The experiments were repeated in triplicate in 3 different occasions.

2.3.2. Influence of AMB on preformed biofilms

In order to test the AMB ability to destroy preformed biofilms, *C. tropicalis* biofilms were formed during 24 h at 37 °C and 120 rpm, in RPMI medium. For that, 200 µL of each *Candida* suspension, containing 1×10^5 cells/mL in RPMI medium supplemented with 2% of glucose, was added to the respective 96-well microplates. After the biofilm formation, the entire medium was aspirated, and different concentrations of AMB (0.5, 1, 2, and 10 mg/L) prepared in RPMI medium were added to the specific wells. The microplates were incubated for additional 48 h at 37 °C and 120 rpm. Controls were also performed with *Candida* cells without antifungal agents. The experiments were repeated in triplicate on 3 different occasions.

2.4. Biofilm analyses

2.4.1. Biofilm total biomass

Biofilm total biomass was quantified by crystal violet (CV) staining (Silva et al., 2009). For that, the medium was totally aspirated, and the nonadherent cells were removed by washing the biofilms once with 200 µL of PBS. The biofilm was fixed with 200 µL of methanol (100% v/v), which was removed after 15 min of contact. The microplates were allowed to dry at room temperature, and 200 µL of CV (1% v/v) was added to each well and incubated for additional 5 min. The wells were then gently washed twice with 200 µL of sterile ultrapure water, and 200 µL of acetic acid (33% v/v) was added to release and dissolve the absorbed stain. The absorbance of the obtained solution was read in triplicate in a microplate reader at 570 nm. The results were presented as absorbance per unit of area (Abs_{570nm}/cm²).

2.4.2. Biofilm cultivable cells

The number of cultivable cells in biofilms was determined by the enumeration of CFUs. For that, the medium was aspirated, and the

biofilms were washed once with 200 µL of PBS to remove nonadherent cells. Then, the biofilm was scraped from the 96-well microplates, and the suspensions were vigorously vortexed for 2 min to disaggregate cells from the matrix. In addition, in order to confirm the complete removal of the biofilms from the wells, after biofilm scraping, a CV staining was performed. Serial 10-fold dilutions in PBS were plated in SDA and incubated at 37 °C for 24 h. The results were presented as the total of CFUs per unit of area (log₁₀ CFUs/cm²).

2.4.3. Biofilm structure

The structure of preformed biofilms was examined by scanning electron microscopy (SEM). For the conditions tested (absence or presence of 2 mg/L of AMB), biofilms were formed into 24-well microplates (Orange Scientific, Braine-l' Alleud, Belgium) with an inoculum of 1 mL of yeast cell suspension containing 1×10^5 cells/mL in RPMI medium, as described previously.

The samples were dehydrated with increasing percentages of ethanol (using 70% ethanol for 10 min, 95% ethanol for 10 min, and 100% ethanol for 20 min) and air dried for additional 20 min. Samples were kept in a desiccator until the base of the wells was mounted onto aluminum stubs, sputter coated with gold, and observed with an S-360 SEM.

2.5. Biofilm matrix composition analysis

2.5.1. Matrix extraction method

Biofilms for analysis of matrix compounds were formed in 24-well microplates, in order to obtain a higher amount of biomass. As such, an inoculum of 1 mL of yeast cell suspension (1×10^5 cells/mL in RPMI medium) was added to each well, and biofilms were formed as described previously for preformed biofilms. After 24 h, the medium was aspirated, and 2 mg/L of AMB was added to the specific wells and incubated for more 48 h at 37 °C and 120 rpm. Controls were also performed with *Candida* cells growing in the absence of AMB. After 48 h, the biofilms were washed once with 1 mL of ultrapure water to remove nonadherent cells, and the biofilms were scraped from the wells and resuspended in 500 µL of ultrapure water. The suspensions were vortexed for 2 min and filtered through a 0.20-µm filter to evaluate the dry weight of the biofilm. The remaining suspension was sonicated for 30 s at 30 W, vortexed for 2 min, and centrifuged at 5000g for 10 min at 4 °C, in order to recover only the matrix, which was stored at –20 °C before analysis. The experiments were performed in triplicate and in 3 independent assays.

2.5.2. Proteins and carbohydrates quantification

The proteins' content of the biofilm matrix of *C. tropicalis* strains was measured using the bicinchoninic acid kit (Thermo Fisher Scientific, Rockford, IL, USA), using bovine serum albumin as standard. The absorbance was determined in a microplate reader at 562 nm.

The total carbohydrate content of the biofilm matrix of *C. tropicalis* strains was estimated according to the procedure of DuBois et al. (1956), using glucose as standard. The absorbance was read in a 96-well microplate reader at 490 nm.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software for Windows. The results were compared using a 2-way analysis of variance with the Bonferroni post hoc test. All tests were performed with a 95% confidence level.

Table 1
C. tropicalis MICs obtained with AMB

<i>C. tropicalis</i> strains	MICs (mg/L)
AG1	4–6
12	4–6
519468	2.5–4

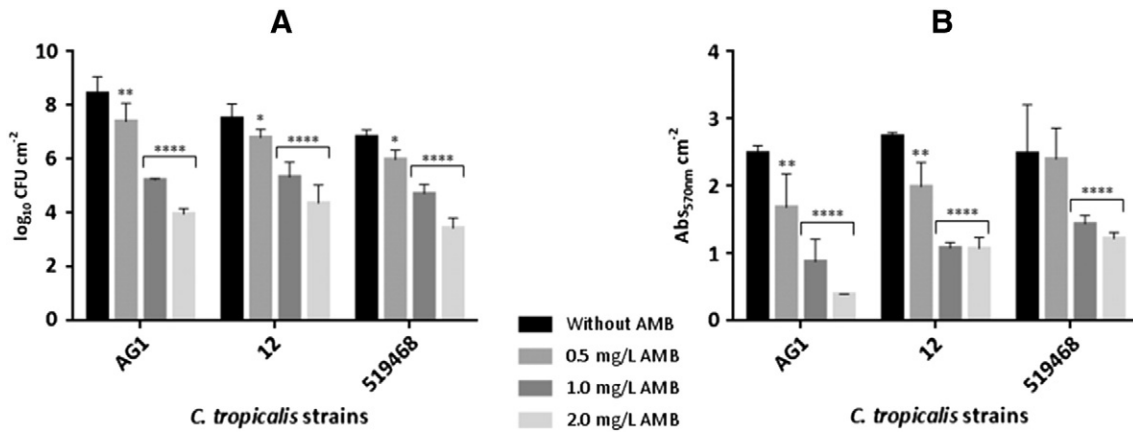


Fig. 1. Effect of AMB on *C. tropicalis* biofilm formation after 48 h. (A) Mean values of the logarithm of CFUs normalized by unit of area (\log_{10} CFU/cm²). (B) Mean values of the absorbance at 570 nm normalized by unit of area (Abs_{570nm}/cm²). Error bars indicate the SD. *, **, and **** correspond to $P < 0.05$, $P < 0.01$, and $P < 0.0001$, respectively.

3. Results

3.1. Effect of the AMB on *C. tropicalis* planktonic cells

The results revealed that all *C. tropicalis* strains were resistant to AMB according to the MIC breakpoint of CLSI (>2 mg/L) (Table 1). The *C. tropicalis* strain that showed the lowest value of MIC (2.5–4 mg/L) was the clinical isolate from the urinary tract, *C. tropicalis* 519468.

3.2. Amphotericin B effect on *C. tropicalis* biofilms

3.2.1. Influence of AMB on biofilm formation

The results revealed significant reductions in the number of viable cells of the 3 strains (Fig. 1A) and in total biomass values (Fig. 1B) in the presence of the higher concentrations of AMB (1 and 2 mg/L), compared to the control condition ($P < 0.0001$). Moreover, it was also verified that there is a significant reduction on biofilm formation (in terms of total biomass and number of cultivable cells) in the presence of the lowest concentration of AMB (0.5 mg/L), for the 3 *C. tropicalis* strains studied (Fig. 1A and B, respectively), compared to biofilms formed in the absence of the AMB ($P < 0.05$ or $P < 0.01$), except in the case of the total biomass for *C. tropicalis* 519468 (Fig. 1B).

3.2.2. Amphotericin B effect against preformed biofilms

It can be observed that the effect of AMB against *C. tropicalis* preformed biofilms (Fig. 2) is not so notorious as the effect on biofilm formation (Fig. 1). In fact, only the highest concentration of AMB tested

(10 mg/L) was capable of causing a significant reduction in the number of viable cells (Fig. 2A) but only for *C. tropicalis* 12 ($P < 0.01$) and *C. tropicalis* 519468 ($P < 0.05$) biofilms. It is important to address that the treatment of preformed biofilms with AMB resulted in an increase of the total biomass values (Fig. 2B), specifically for the lowest concentrations of AMB (0.5, 1, and 2 mg/L) in the case of *C. tropicalis* AG1, 12, and 519468, compared to the control ($P < 0.01$ only for 0.5 and 1 mg/L of AMB, $P < 0.0001$ and $P < 0.001$, respectively).

3.3. Effect of AMB on *C. tropicalis* biofilm structure and matrix composition

SEM analysis was performed to examine the effect of the AMB on the structure of *C. tropicalis* preformed biofilms. As such, each biofilm was treated with AMB (2 mg/L) (Fig. 3, II) and compared with the respective untreated biofilm (Fig. 3, I). It was evident that *C. tropicalis* biofilms are formed by different yeast morphologies in a strain-dependent manner (Fig. 3, I). *C. tropicalis* AG1 biofilm is constituted by higher quantity of filamentous forms (Fig. 3A, I) presenting a more continuous layer, while *C. tropicalis* 12 (Fig. 3B, I) and *C. tropicalis* 519468 (Fig. 3C, I) presented biofilms with lower quantity of filamentous forms.

Interestingly, it was observed that *C. tropicalis* biofilms, when treated with AMB (Fig. 3, II), presented a more compact and continuous structure with the cells more interlinked. In fact, after treatment of the biofilms with AMB, they appear with more compact aspect and with all cells embedded into the extracellular matrix (ECM) (Fig. 3, I). Moreover, in the presence of AMB, *C. tropicalis* biofilms presented a reduction in the number of filamentous forms (Fig. 3, II).

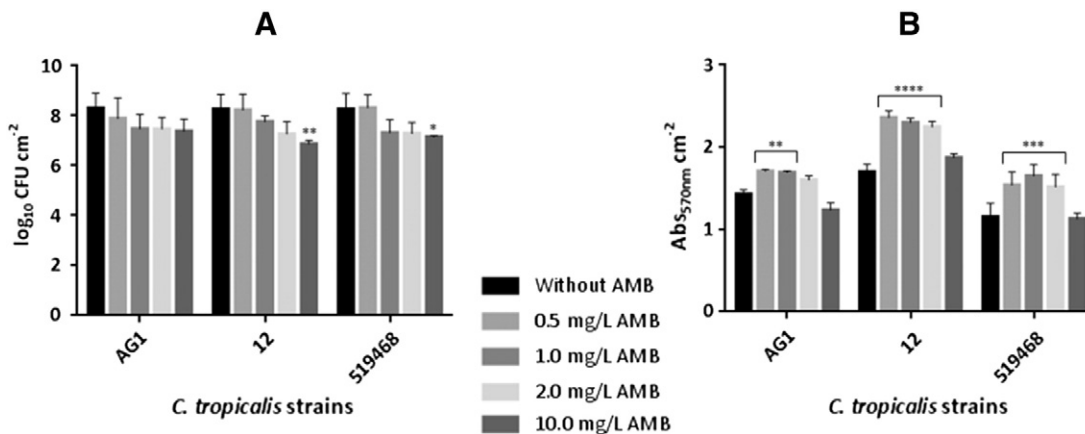


Fig. 2. Effect of AMB against 24 h preformed *C. tropicalis* biofilms during additional 48 h. (A) Mean values of the logarithm of CFUs normalized by unit of area (\log_{10} CFU/cm²). (B) Mean values of the absorbance at 570 nm normalized by unit of area (Abs_{570nm}/cm²). Error bars indicate the SD. *, **, ***, and **** correspond to $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively.

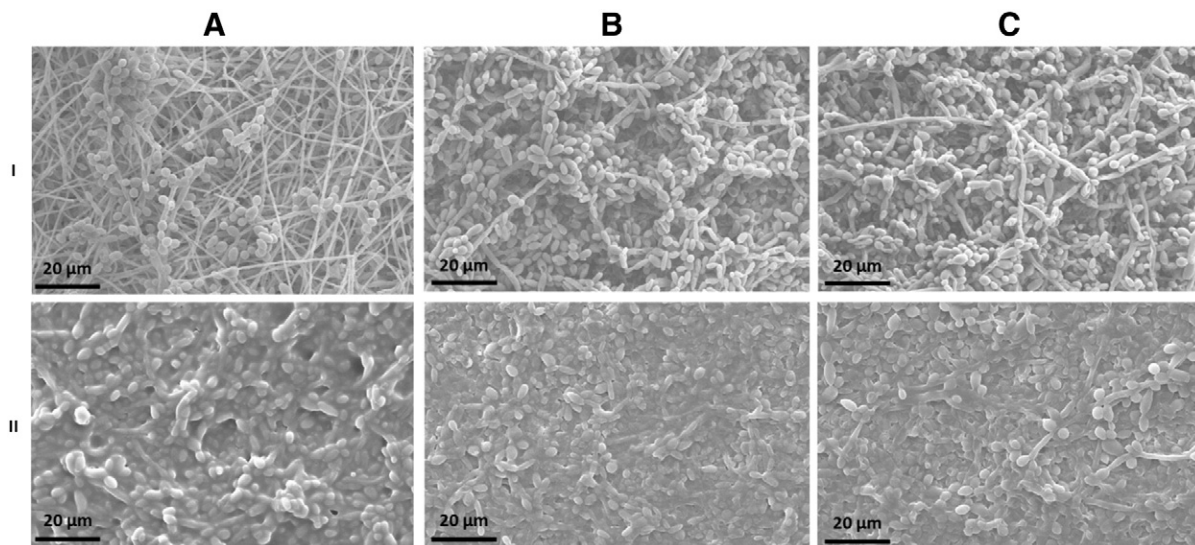


Fig. 3. Scanning electron microscopy images of 24 h preformed *C. tropicalis* biofilms. AG1 (A), *C. tropicalis* 12 (B), and *C. tropicalis* 519468 (C) biofilms grown in RPMI medium for additional 48 h (I) in the absence of amphotericin B and (II) in the presence of 2 mg/L of the AMB. The bar in the images corresponds to 20 µm. Magnification $\times 1000$.

In order to infer about the role of AMB in the *C. tropicalis* biofilm matrix production and composition, the total protein and carbohydrate content were also determined (Table 2).

Concerning the total protein content, the results showed a significant increase in the biofilm matrices treated with 2 mg/L of AMB for the 3 *C. tropicalis* strains tested ($P < 0.0001$ for *C. tropicalis* AG1 and *C. tropicalis* 12 and $P < 0.001$ for *C. tropicalis* 519468). It should be noted that the *C. tropicalis* 519468 biofilm's matrices presented approximately half of the protein composition, compared to *C. tropicalis* AG1 and *C. tropicalis* 12, in the absence and presence of AMB.

Regarding the results obtained with respect to the analysis of the carbohydrate content, an increase after biofilm treatment with 2 mg/L of AMB, in the case of the *C. tropicalis* AG1 and *C. tropicalis* 519468, was also observed, in comparison to the absence of AMB ($P < 0.01$). In contrast, *C. tropicalis* 12 biofilm matrices showed a significant reduction in the carbohydrates values ($P < 0.001$). Moreover, likewise to protein content, *C. tropicalis* 519468 showed the lower carbohydrate content in the absence and presence of AMB, in comparison to the other 2 *C. tropicalis* strains tested.

4. Discussion

C. tropicalis has been reported to be 1 of the *Candida* species that is most likely to cause bloodstream and urinary tract infections in hospitalized patients and also with high potential for dissemination, mobility, and mortality (Kothavade et al., 2010; Negri et al., 2010, 2012; Silva

et al., 2011, 2012). This is due to several virulence factors exhibited by *C. tropicalis*, namely, the biofilm formation ability, since the biofilms confer significant resistance to antifungal therapy and host immune responses (Negri et al., 2012; Silva et al., 2009, 2011, 2012; Williams et al., 2011). Additionally, it is known that *C. tropicalis* isolates have the ability to form compact biofilms. Thus, the main aim of this study was to infer about the AMB ability to control and combat *C. tropicalis* biofilms and to determine the role of the matrix compounds in the *C. tropicalis* biofilm resistance to AMB.

In the clinical perspective, the most important characteristic of *Candida* species is their ability to form biofilms and their role in the increased tolerance to conventional antifungal therapy, since they are much more resistant to antifungal agents than planktonic cells (Donlan and Costerton, 2002; Fonseca et al., 2014; Galán-Ladero et al., 2013; Shanmughapriya et al., 2014; Silva et al., 2012). As expected, all strains were able to form biofilms and exhibited resistance to AMB but in a strain-dependent manner (Table 1). Additionally, the strain with lower MIC value is also the strain that presented the lowest ability to form biofilm. These results appear to be in agreement with previous studies involving *C. tropicalis* biofilms (Galán-Ladero et al., 2013; Shanmughapriya et al., 2014).

Moreover, in general, *C. tropicalis* biofilms are more resistant to AMB, when the biofilms were formed before the addition of this antifungal agent. In fact, some studies reported that *C. tropicalis* planktonic cells were susceptible to AMB, although their biofilms exhibited resistance, normally due to the synthesis of large amounts of matrix (Al-Fattani and Douglas, 2006; Bizerra et al., 2008; Kothavade et al., 2010; Negri et al., 2012; Silva et al., 2009). This fact was also described by other authors for *Candida albicans* and *Candida glabrata* biofilms (Fonseca et al., 2014; Kumamoto, 2002; Silva et al., 2012). As it is well known, mature biofilms are in cells organized into structured communities embedded within an ECM (Negri et al., 2012; Silva et al., 2009, 2011, 2012). The matrix production has been reported as a *Candida* response to stress conditions, namely, to the presence of antifungal agents (Al-fattani and Douglas, 2004; Silva et al., 2011, 2012). From the analysis of the results obtained (Fig. 2B), it is possible to assume that biofilm matrix may have a role in the biofilm resistance to this agent. Notwithstanding the inherent destructive nature of the preparation of samples to be observed in the SEM, with the possible loss of some cells and matrix during the dehydration process of this technique, it is possible to observe that *C. tropicalis* biofilms consist of a dense network of cells with a variety of cell morphologies (Fig. 3, I), presenting a mixture of yeast cells and evident filamentous morphologies, in agreement with what was already

Table 2

Effect of the AMB in the *C. tropicalis* preformed biofilm matrix composition in terms of protein and carbohydrate quantities.

<i>C. tropicalis</i> strains	Concentration of AMB (mg/L)	Protein quantity (mg protein/g biofilm)	Carbohydrate quantity (mg carbohydrate/g biofilm)
AG1	0	17.519 ± 1.963	285.365 ± 0.273
	2	22.383 ± 0.607****	333.963 ± 0.287**
12	0	14.568 ± 1.220	363.476 ± 1.184
	2	23.220 ± 0.845****	294.998 ± 31.520***
519468	0	6.304 ± 0.085	133.174 ± 21.349
	2	10.212 ± 1.009***	190.519 ± 0.262**

Mean ± SD values of the proteins (mg protein/g dry weight biofilm) and carbohydrates (mg carbohydrate/g dry weight biofilm).

** $P < 0.01$, when the presence of AMB was compared to its absence.

*** $P < 0.001$, when the presence of AMB was compared to its absence.

**** $P < 0.0001$, when the presence of AMB was compared to its absence.

described (Donlan and Costerton, 2002; Silva et al., 2009, 2011). These results are in accordance with the values of total biomass obtained (Fig. 2B) and also highlight the important role of the biofilm matrix in the *Candida* resistance to AMB.

Generally, the biofilm matrix compounds include (in addition to water): carbohydrates, proteins, hexosamines, and phosphorus (Baillie and Douglas, 2000; Silva et al., 2009, 2012). Our results point out to the fact that AMB, besides inducing changes in the structure of *C. tropicalis* biofilms (Fig. 3), can also cause alterations in their matrix composition (Table 2). It can be speculated that AMB might then trigger the protein and carbohydrate production, resulting in a well-structured and dense biofilm. Thus, it is possible to assume that the matrix increase, in particular of protein and carbohydrate contents, may contribute to the *C. tropicalis* biofilm resistance to AMB.

5. Conclusions

In conclusion, this work demonstrated that AMB is unable to totally prevent biofilm formation and to eradicate *C. tropicalis* preformed biofilms. The significant increase of the protein and carbohydrate contents in the biofilms matrices treated with AMB seems to be the main explanation to the increase of *C. tropicalis* biofilm resistance. More in vitro (medical devices) and in vivo models can be used in the future to corroborate these results.

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