Bacterial and Fungal Pathogenesis

Elastin increases biofilm and extracellular matrix production of *Aspergillus fumigatus*

Ildnay de Souza Lima Brandão[a], *Heloiza Maria da Silva Oliveira-Moraes*[b], *Cristina Maria de Souza Motta*[c], *Neiva Tinti de Oliveira*[c], *Oliane Maria Correia Magalhães*[a,*]

[a] *Universidade Federal de Pernambuco, Centro de Ciências Biológicas, Departamento de Micologia, Cidade Universitária, PE, Brazil*
[b] *Universidade Federal de Pernambuco, Centro de Ciências Biológicas Rua Departamento de Micologia, Cidade Universitária, PE, Brazil*
[c] *Universidade Federal de Pernambuco, Centro de Ciências Biológicas, Departamento de Micologia, Pernambuco, PE, Brazil*

ARTICLE INFO

ABSTRACT

Aspergillus fumigatus is an opportunistic saprobe fungus that accounts for 90% of cases of pulmonary aspergillosis in immunosuppressed patients and is known for its angiotropism. When it reaches the respiratory tract, *A. fumigatus* interacts with structural components and blood vessels of the lungs, such as elastin. To understand the effect of this structural component, we examined the effect of elastin on the production and development of the biofilm of *A. fumigatus*. In RPMI containing 10 mg/mL of elastin, a significant increase (absorbance $p < 0.0001$; dry weight $p < 0.0001$) in the production of biofilm was observed in comparison to when RPMI was used alone, reaching a maximum growth of 18.8 mg (dry weight) of biofilm in 72 h. In addition, elastin stimulates the production ($p = 0.0042$) of extracellular matrix (ECM) and decreases ($p = 0.005$) the hydrophobicity during the development of the biofilm. These results suggest that elastin plays an important role in the growth of *A. fumigatus* and that it participates in the formation of thick biofilm.

Introduction

Aspergillus fumigatus is an opportunistic saprobe fungus that accounts for 90% of pulmonary aspergillosis cases in immunosuppressed patients. This disease can exhibit various clinical forms, mainly consisting of allergic bronchopulmonary aspergillosis, aspergilloma, and invasive aspergillosis (IA), which are important causes of morbidity and mortality ranging from 70 to 90%.[1,2]

In aspergilloma and IA, *A. fumigatus* behaves as a multicellular community surrounded by an extracellular matrix (ECM), which is characteristic of a biofilm[3,4] and may explain, together with histological evidence, the resistance to antifungal agents when these clinical forms are treated.[5,6]

The development of this fungus within the lungs and the angiotropism[7,8] allow this microorganism to be in direct contact with elastin, one of the main structural components of the

* Corresponding author.
  E-mail: olimicomed@yahoo.com.br (O.M. Magalhães).

lungs and blood vessels, which is fundamental for their physiology. Correlation between elastase production by A. fumigatus and the development of IA has been observed.[9]

It has recently been demonstrated the influence of host factors such as serum components, as fetuin A,[10] and extracellular DNA[11] in the promotion of growth of A. fumigatus biofilm; however, no studies have investigated the influence of lung tissue constituents on the promotion of biofilm development.

In this perspective, the aim of this work was to determine the influence of elastin in the growth and development of the biofilm of A. fumigatus.

Materials and methods

Fungal strain and growth conditions

Based on data obtained from previous analysis of virulence factors such as biofilm and gliotoxin production, and ability to cause pulmonary aspergillosis in mice (unpublished data), we selected two isolates of A. fumigatus, URM5992 (environmental origin) and URM6575 (clinical sample), from the Culture Collection University Recife Mycology (URM) of the Federal University of Pernambuco (Universidade Federal de Pernambuco – UFPE), Recife, Pernambuco (PE), Brazil, were used. The isolates were maintained at 28 °C in malt extract agar.

Growth conditions and inoculum standardization

A. fumigatus isolates were grown on Sabouraud dextrose agar at 37 °C for 72 h. The conidia were collected by washing the surface of the culture with 5 mL of phosphate buffer saline (PBS), pH 7.2, supplemented with 0.025% (v/v) Tween 20. The inoculum was adjusted to 1×10^5 cells in RPMI 1640 (Sigma-Aldrich Corporation, USA) and buffered to pH 7.0 with 0.165 M MOPS (Sigma-Aldrich Corporation, USA) for the production of biofilm in 96-well plates.[12] For quantification of the dry weight, another inoculum was adjusted to 3.75×10^4 cells/cm^2.[10]

Production of A. fumigatus biofilm

A. fumigatus biofilm were produced in flat-bottom 96-well polystyrene plates. Then, 200 μL of the standardized cell suspension of each A. fumigatus isolate was added separately in MOPS-RPMI 1640 (Sigma-Aldrich Corporation, USA) or MOPS-RPMI 1640 containing elastin (RPMI/Elastin) (Sigma-Aldrich Corporation, USA) at concentration of 10 mg/mL for each time (24, 48, and 72 h). Plates were incubated at 37 °C. For each time interval, the culture medium was removed from the wells, and the cells were washed three times with PBS, pH 7.2, to remove all non-adherent cells.[12]

To quantify the dry weight of the biofilm, 3 mL suspensions of each isolate were placed separately in 6-well polystyrene plates with MOPS-RPMI 1640 or RPMI/Elastin (10 mg/mL), incubation times, and temperatures listed above.[10]

Biofilm quantification

Biofilm was quantified using the technique developed by O'Toole and Kolter[13] and subsequently modified by Mowat

et al.[12] The plates were dried, and 100 μL of 0.5% (w/v) crystal violet solution was added for 5 min. The solution was removed by thorough washing under running water. Biofilms were unstained by adding 100 μL of 95% ethanol to each well for 1 min. The ethanol was transferred to another microtiter plate (96-well), and the absorbance was measured at 570 nm (A570) using a VarioskanFlash fluorescence meter with SkanIt™ 2.4.5 RE software (Thermo Fisher Scientific, USA).

Quantification of the biofilm biomass (dry weight)

After the predetermined time, the biofilm was removed by scraping and filtered through paper filters (Miracloth/22 μm, Merck, Germany), which were then dried to a constant weight.[10]

Quantification of the ECM

The biofilm formed in RPMI and RPMI/Elastin (10 mg/mL) for 48 h at 37 °C were stained by the addition of 100 μL of a solution of 25 μg/mL Alexa Fluor 488 (CAAF; Life Technologies, Germany) in PBS, followed by incubation for 45 min at 37 °C and stirring at 250 rpm. The biofilm was washed three times with PBS.[11] The fluorescence intensity was measured using a VarioskanFlash fluorescence meter with SkanIt™ 2.4.5 RE software (Thermo Fisher Scientific, USA) at excitation and emission wavelengths of 485 nm and 520 nm, respectively. CAAF stock solutions of 5 mg/mL were stored at −20 °C and thawed immediately before use.

Quantification of biofilm hydrophobicity

A microsphere adhesion assay with fluorescent orange sulfate-modified latex microspheres (0.806 μm, Sigma-Aldrich Corporation, USA) was used to test biofilm hydrophobicity. The biofilm in RPMI alone and RPMI/Elastin (10 mg/mL) were washed with 0.1 M KNO$_3$, pH 6.5, and then mixed with an equal volume of the microsphere solution (10^9/mL). Subsequently, the mixture was stirred for 30 s and extensively washed with the same solution.[3] The amount of fluorescence emitted resulting from the adherence of the microspheres to the hyphae was measured with a VarioskanFlash fluorescence meter with SkanIt™ 2.4.5 RE software (Thermo Fisher Scientific, USA) at excitation and emission wavelengths of 520 nm and 540 nm, respectively.

Biofilm microscopy

For microscopic analysis, the biofilm was grown on coverslips (22 mm × 22 mm) in RPMI and RPMI/Elastin (10 mg/mL) at 37 °C for 48 h in 6-well polystyrene plates. The coverslips were removed, and the biofilm was analyzed.

To visualize the structure of the biofilm, 100 μL of Calcofluor White® (Sigma-Aldrich Corporation, USA) and excitation/emission filters of 346/433 nm were used to obtain a blue color. The ECM quantification and hydrophobicity assays were conducted as described above for the quantification of fluorescence.

The images were obtained using a Leica DMI 4000B fluorescence microscope and Leica Microsystems LAS AF software (Germany).

Analysis of biofilm structure by scanning electron microscopy (SEM)

The A. *fumigatus* biofilms for electron microscopy were developed as described in the previous biofilm formation section, in 6-well polystyrene plates at 37 °C during 48 h were used for this experiment. For the SEM, the samples were processed as described by González-Ramírez et al.[14] Briefly, the biofilms were washed with PBS and fixed with 2% Glutaraldehyde (Electron Microscopy Sciences®, Washington PA, USA) for 2 h. Then, the biofilms were post-fixed with 1% Osmium Tetroxide (Electron Microscopy Sciences®, Washington PA, USA) for 2 h. The bottoms of the 6- polystyrene plates were cut with a hot punch, and the intact biofilm was obtained. The samples were dehydrated with ethanol at 10, 20, 30, 40, 50, 60, 70, 80 and 90% for 10 min and with absolute alcohol for 20 min. Then, the biofilms were placed into a critical point dryer and were coated with ionized gold for 400 s at 15.0 kV. The SEM images were obtained using a Shimadzu-SS550 microscope with tungsten filament (Kyoto, Japan).

Statistical analysis and data presentation

All data represent the mean and standard deviation of six replicates of each isolate. The graphical representation and statistical analysis of experimental data were performed by analysis of variance (ANOVA) and/or Tukey's test for multiple comparisons using the GraphPad Prism 5 software (GraphPad Software, Inc., California, USA). p values less than 0.05 were considered statistically significant.

Results

Biofilm growth in the presence of elastin

The biofilm formation by A. *fumigatus* (URM5992 and URM6573) was assessed in microplate assay using crystal violet staining and dry weight. The addition of elastin (10 mg/mL) to the medium promoted significant biofilm growth ($p < 0.0001$) in all evaluated time points (Fig. 1A and B). No difference in biofilm production was observed between the isolates ($p = 0.1447$). Regardless of the source, both strains were able to produce biofilm.

As expected, the addition of elastin led to increased biomass after 48 h, when compared to biofilm in RPMI medium alone (9.4 mg), reaching 18.8 mg at 72 h. These data strongly suggest the influence of elastin on A. *fumigatus* biofilm growth and development.

Biofilm stability was assessed by shear mechanical force by serially pipetting the biofilms with PBS during the washing procedure (results not shown). We observed during the first 24 h of growth, the biofilm appeared consistent, but it did not strongly adhere to the plates. After 48 h, the structure was stable and adherent.

Morphologically, bundles of parallel hyphae (Fig. 1E and F) as well as an interlaced arrangement, acting possibly to strengthen and stabilize the structure as a whole, were observed at 48 h. In addition, we observed circular arrangements (Fig. 1E and F), indicating the possible formation of channels for air circulation.

Increased of ECM provided by the elastin

Elastin (10 mg/mL) increased the amount of ECM produced by A. *fumigatus* ($p = 0.0042$) (Fig. 2) when compared to RPMI medium alone. This increase becomes the biofilm of A. *fumigatus* strains to be more cohesive, as shown photomicrographs (Fig. 3A and B). In addition, we observed strong staining by CAAF in amorphous materials around the ends of the hyphae (Fig. 3C and D). These results indicate strongly that pulmonary constituent stimulates A. *fumigatus* to develop evasion mechanism to host defenses.

Modification of hydrophobicity of the mycelium by the elastin

We found different hydrophobic properties in the biofilm of A. *fumigatus*. The mycelium grown in the biofilm in RPMI was more hydrophobic than observed in RPMI supplemented with elastin (10 mg/mL) (Fig. 4movefigure2). The hydrophobicity was assessed in hydrophobicity assay of the mycelium surface with fluorescent orange sulfate-modified latex microspheres (0.806 μm, Sigma-Aldrich Corporation, USA).

In this assay, a large number of microspheres adhered to the biofilm grown in RPMI alone (Fig. 5A and B), while in the presence of elastin, the hydrophobicity was decreased ($p = 0.005$) (Fig. 5C and D). What is interesting in this data is that different than we expected, the hydrophobicity decreased while ECM production increased.

Our finding revealed that the presence of host components, such as elastin, is capable of altering the behavior of A. *fumigatus*, as shown here for hydrophobicity.

Discussion

Biofilms are multicellular communities of microorganisms surrounded by an ECM. Growth in communities confers advantages to fungi, including the ease of colonization of the substrate, protection from environmental aggression, resistance to physical and chemical stress, metabolic cooperation, and regulation of gene expression.[3,6]

Several reports have shown that adding new components to RPMI 1640 medium produces conditions that mimic the host organism during infection.[4,11,15]

As far as we know, this is the first report of biofilm production by A. *fumigatus* on the presence of elastin. In this study, the presence of elastin in the RPMI medium led to increased biomass in 100% after 48 h, when compared to biofilm in RPMI medium alone. The increase biofilm when added elastin to the growth medium, suggests the influence of lung constituents on the promotion of biofilm development of A. *fumigatus*. Recently, has been demonstrated the influence of other host factors, such as serum components, such as fetuin A,[10] and
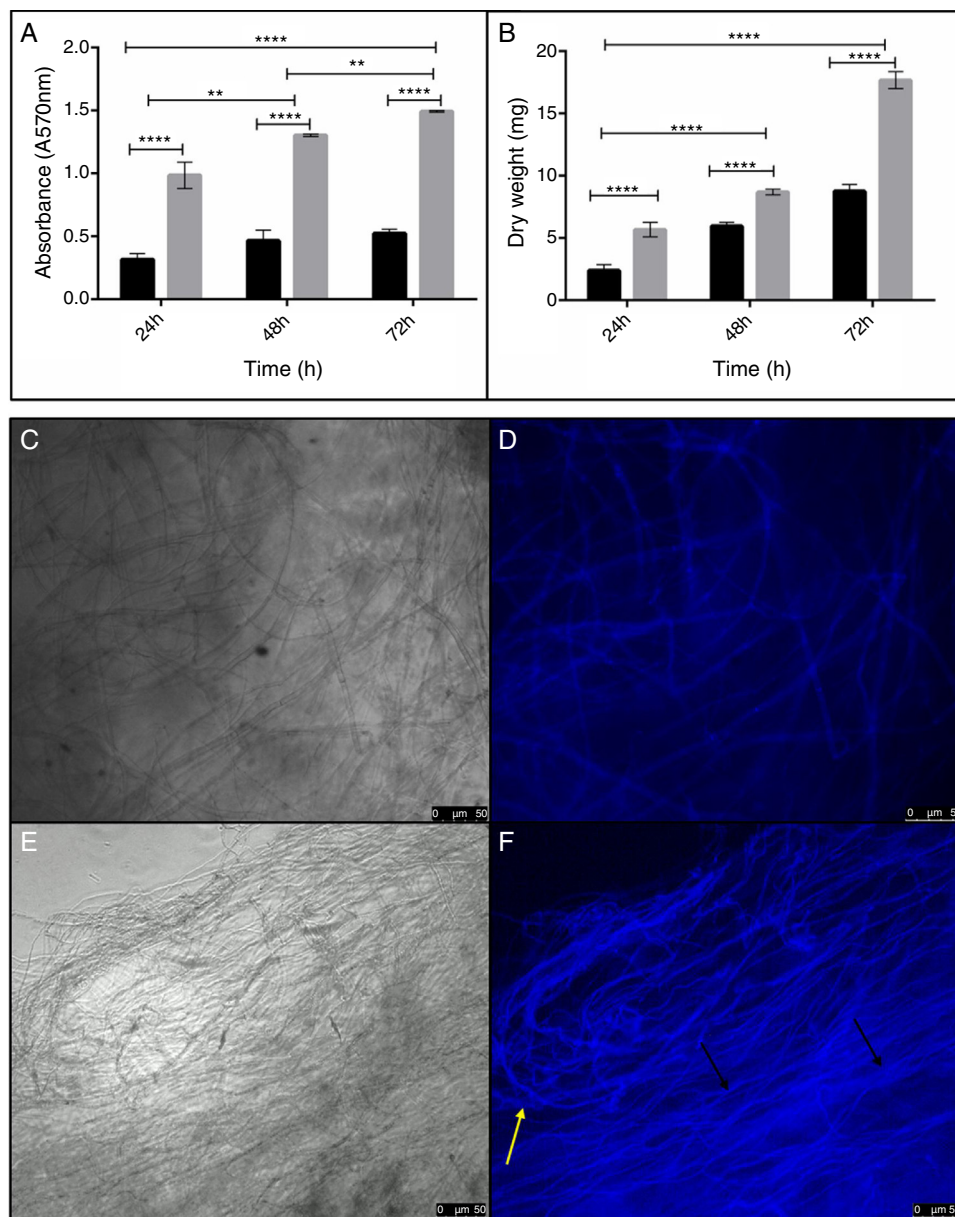
Fig. 1 – Influence of elastin on the growth of A. *fumigatus* biofilm. The graphics represent the mean values with SDs of the biofilm production of both isolates of A. *fumigatus* measured by absorbance with 0.5% crystal violet (A) and (B) the dry weight. A significant increase in the presence of 10 mg/mL of elastin (gray bars) compared to RPMI alone (black bars) was observed for all time periods (**$p < 0.01$; ****$p < 0.0001$; ANOVA); (C) A. *fumigatus* URM6575 biofilm grown in RPMI is shown after 48 h at 37 °C under light in microscopy and (D) stained with Calcofluor White® (E) and in RPMI supplemented with 10 mg/mL of elastin under microscopy and (F) stained with Calcofluor White®. An increased amount of biofilm was produced in the presence of elastin (10 mg/mL). Circular arrangements (yellow arrow) and parallel bundles of hyphae (black arrow) are shown. Scale bar, 50 μm.

extracellular DNA[11] on promoting the growth of A. *fumigatus* biofilm.

No difference was observed in biofilm production between the isolates ($p = 0.1447$). Both isolates of A. *fumigatus* were able to produce biofilm independent of their substrate of origin (clinical or environmental). This result is consistent with those of González-Ramírez et al.[14] who did not observe difference in development and biofilm production between clinical and environmental isolates of A. *fumigatus*.

We obtained maximum dry weight at 72 h of 18.8 mg is more than twice the maximum value (8.3 mg) obtained for Seidler et al.[4] during the same time period of co-culture with bronchial epithelial cells, using the A. *fumigatus* ATCC 9197 strain. While the biofilm of A. *fumigatus* (IFM 49896 strain) demonstrated by Toyotome et al.[10] in presence of serum protein fetuin A reached an average mass of 30 mg. Indicating that other host constituents may be necessary for biofilm development in A. *fumigatus*. In addition, the biofilm
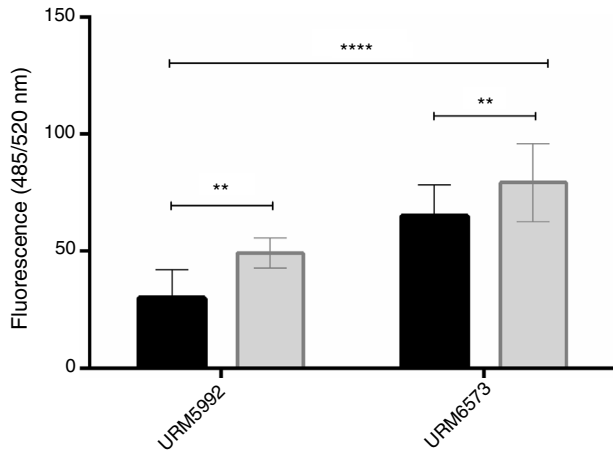
Fig. 2 – Influence of elastin on extracellular matrix (ECM) of *A. fumigatus* biofilm. The means and SDs show that the amount of ECM produced in the presence of elastin (gray bars) was significantly greater ($p = 0.0042$), than that produced in RPMI alone (black bars). The difference between isolates of clinical (URM6573) and environmental (URM5992) origin was highly significant ($p < 0.001$, ANOVA). ** $p < 0.01$; **** $p < 0.0001$.

production variation observed in these studies may be related to the biofilm-forming capacity of each organism, varying according to the isolates used, as known for group A *Streptococcus*.[16]

The observed increase in biofilm production can be attributed to several factors, firstly the elastin be an abundant protein in lung tissue and blood vessel walls, likely serving as an important source of nutrients for biofilm production.[9] Elastase activity must be highlighted among all the factors supposedly related to pathogenicity in this mold, because not only elastin can serve as a source of nitrogen for the fungus, but also its degradation could allow pathogen invasion through host tissues.[17,18] This activity has also been described in other important pulmonary pathogens, such as *Pseudomonas aeruginosa*.[19]

Moreover, the elastin could be able to promote modulation of genes involved in the regulation of biological processes, which may be related to the establishment of deep infections, like has been demonstrated to *Trichophyton rubrum*.[17]

Previous studies evaluating biofilm growth kinetics observed increase for biofilm produced over time,[3,12] which is in good agreement with the results of the present study.

Regarding morphology, the hyphae arrangement in the biofilm of *A. fumigatus* observed is similar to that found by Seidler et al.,[4] where parallel-packed hyphae strengthen the structure in one direction while crossing hyphae further stabilize the structure. In addition, the presence of circular
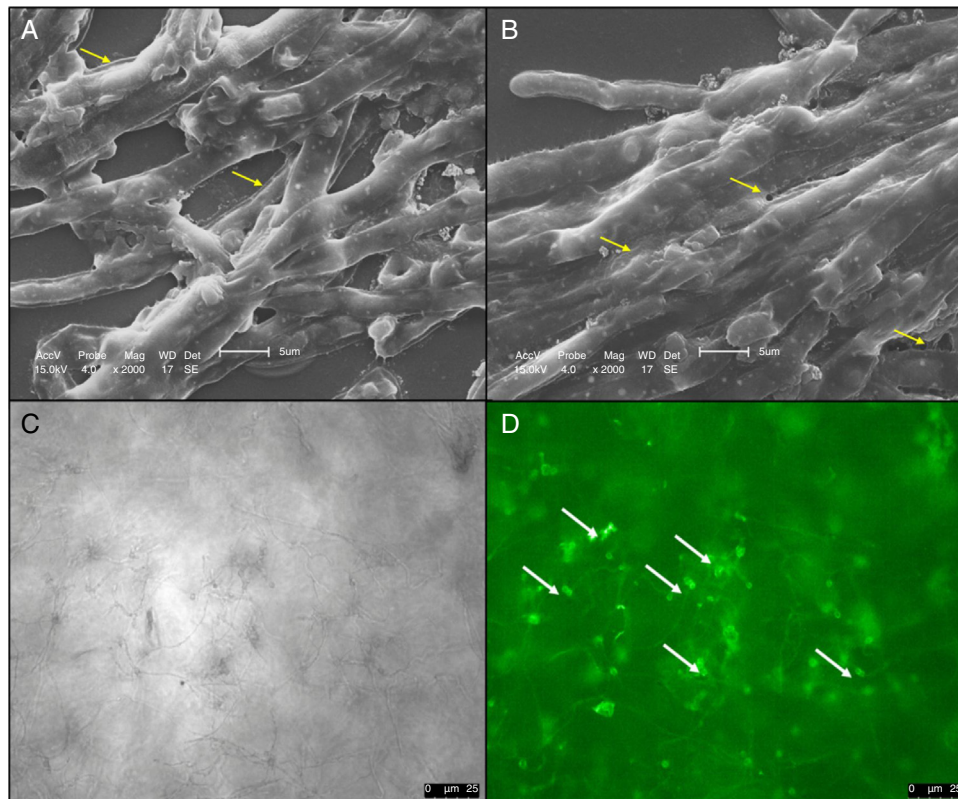

Fig. 3 – Enhanced production extracellular matrix (ECM) of *A. fumigatus* biofilm. Photomicrographs taken by scanning electron microscopy analysis of *A. fumigatus* URM6575 biofilm in RPMI (A) and RPMI with elastin (B) show increased production of ECM in the presence of elastin (yellow arrow). Magnification of 2000×. *A. fumigatus* URM6575 biofilm grown in RPMI supplemented with 10 mg/mL of elastin (C) under light microscopy and stained with concanavalin A-Alexa Fluor 488® (D) show greater amounts of ECM at the ends of the hyphae (white arrow). Scale bar, 50 μm.
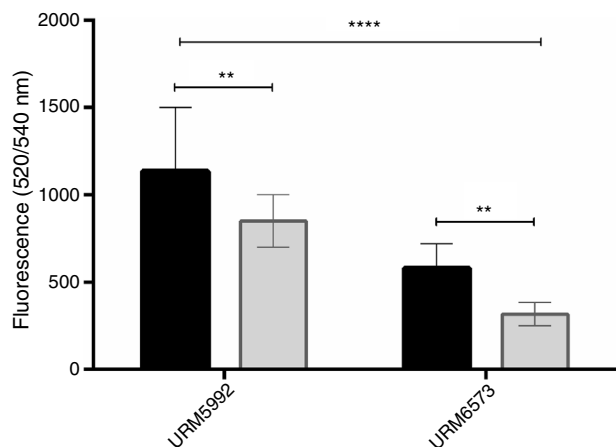
Fig. 4 – Influence of elastin on the hydrophobicity of _A. fumigatus_ biofilm. The hydrophobicity in the presence of elastin (gray bars) was significantly lower ($p = 0.005$) than that in RPMI alone (black bars). The difference between the clinical (URM6573) and environmental (URM5992) isolates was highly significant ($p < 0.0001$, ANOVA). **$p < 0.01$; ****$p < 0.0001$.

arrangements, also observed by Beauvais et al.[3] suggests the possible formation of channels for air circulation, which could be the origin of the oxidation mechanisms responsible for the production of melanin, a known constituent of the ECM.[3,20]

As expected, the increase of biofilm was followed by increase amount of ECM, this result is consistent with those of Seidler et al.[4] who observed the increase in the amount of ECM is related to the greater amount of biofilm.

This matrix is an essential component for the development of biofilm because it glues together the hyphae and fixes them to the surface.[3] In addition, it provides protection against external factors, such as the action of the host immune cells and antifungal agents.[20–22]

In this study, we used CAAF to stain the polysaccharides and SEM to analyze the ECM. In agreement with previous studies,[3,4,10] we found the ECM as a matrix diffused between the hyphae and surrounds them, where it apparently glues together the hyphal threads of the network.[3]

Consistent with early observations,[3,4,11] the ECM was also more evident ending flow tubes, suggesting it not only covers and protects the structure as a whole but also connects the ends of the hyphae to each other.[11]

The presence of carbohydrate, evidenced by CAAF stain of α-mannopyranosyl and α-glucopyranosyl residues of the polysaccharides, suggests its availability in the ECM is a result of need to provide nutrition to the hyphae that make up the biofilm structure, especially those that are most distant from the nutrient source.[12]

Our findings consist of a multicellular complex structure and highly organized, with polysaccharides in the cell wall and surrounding the hyphae in the biofilm like reported by early studies.[3,4,12,22]

This is the first study to observe an increase in ECM production in _A. fumigatus_ biofilm in the presence of a pulmonary and blood vessels constituent, the elastin. This finding

suggests the development of biofilm of _A. fumigatus_ is strongly influenced by factors present in the host because the ECM is also present in aspergilloma and provides greater stability to the hyphae network.[20] In addition, it is hypothesized that ECM plays a significant role in antifungal resistance by adsorbing antifungal drug molecules and preventing their diffusion.[4,23]

A possible explanation for this might be the greater availability of nutrients, which probably led to higher growth with increased production of ECM. Previously was demonstrated the fundamental role of elastase on tissue invasion, suggesting _A. fumigatus_ opens breaches in the pulmonary barriers, secreting these proteases, which are related to pathogenicity this fungus.[24]

Another possible explanation for this is that the increase in ECM production observed in this study may be related to the stimulation of the pulmonary environment, which causes _A. fumigatus_ to develop strategies to survive the host. This matrix contains toxins, such as gliotoxin, which is capable of inducing apoptosis in macrophages, polymorphonuclear leukocytes and dendritic cells, as well as melanin, the pigment that protects the fungus against oxidative stress.[3,15,25,26] Thus, as suggested, the lung environment appears to select the isolates that are best adapted.[27,28]

In terms of hydrophobicity, we used an assay with sulfate-modified latex microspheres, because they have a low density of negative charges, and more than 90% of their surface is available for hydrophobic interactions.[29]

Adhesion is the first step in colonization of a substrate by a fungus, which occurs through various interactions between conidia and the substrate surface.[30] Despite the importance of the adhesion, little is known about this process.[31] Several studies have focused primarily on the fungal cell wall, emphasizing proteins, especially the hydrophobins, which are responsible for the high hydrophobicity of conidia and hyphae walls. These proteins stabilize the adhesion of spores to hydrophobic surfaces, both natural and artificial, possibly generating morphogenetic signals.[30,32].

In _A. fumigatus_ have been demonstrated hydrophobins are responsible for the hydrophobic characteristic of the ECM.[3,33,34] Bruns et al.[15] and Gibbons et al.[35] showed up-regulation of hydrophobins genes of _A. fumigatus_ in biofilm condition, suggesting that several hydrophobins are expressed in the _A. fumigatus_ ECM.

This is the first study to report a change in the hydrophobicity of _A. fumigatus_ biofilm when elastin, a factor present in the host, is added to growth medium. Environmental conditions such as temperature, nutrient supply, and humidity,[36] as well as the culturing conditions, i.e., solid or liquid media and biofilm conditions, can affect hydrophobicity.[3,37,38]

Contrary to expectations, this study finds a significant decrease on hydrophobicity when elastin is added to RPMI medium, even though their presence has caused an increase in the ECM.

This inconsistency may be due to the role played by hydrophobins in completing the life cycle of these fungi, causing the surface to be hydrophobic and resistant to humidity, thereby facilitating the dispersion of spores through the air.[39,40] Thus, after established a community in the lung environment, _A. fumigatus_, most likely, does not need to expend energy for production of dispersing proteins.
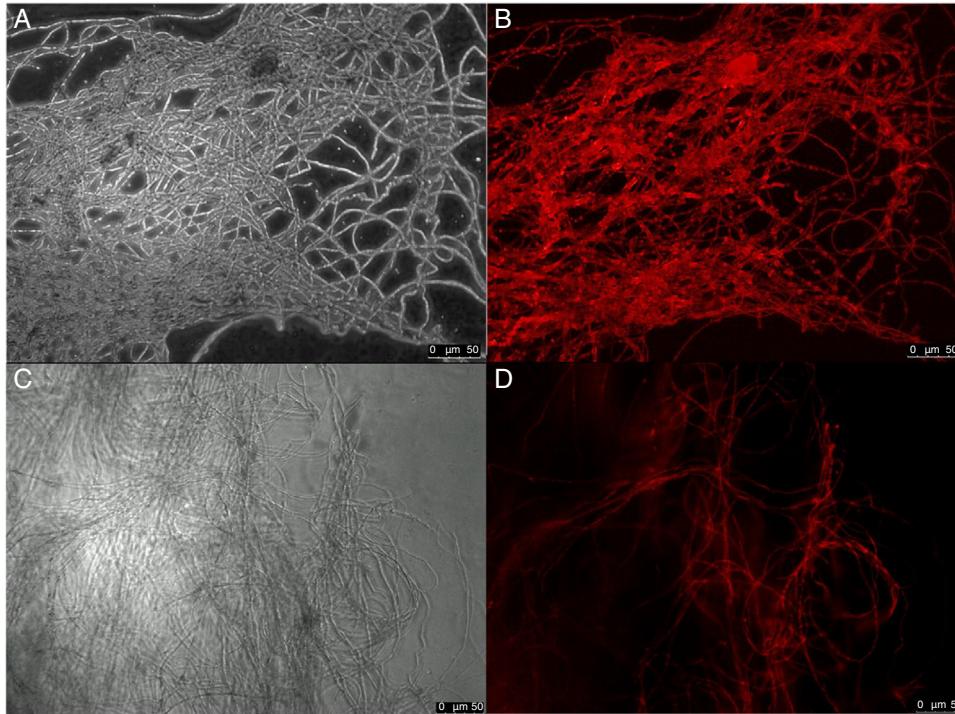
Fig. 5 – **Decreased hydrophobicity of** *A. fumigatus* **biofilm.** *A. fumigatus* **URM5992 (environmental origin) biofilm after 48 h at 37 °C. (A and B) Biofilm Grown in RPMI without elastin under light microscopy and stained with latex beads, respectively. (C and D) biofilm grown in RPMI with elastin – 10 mg/mL under light microscopy and stained with latex beads, respectively. Scale bar, 50 μm.**

However, other roles have been attributed to hydrophobins, as demonstrated by RodAp, which hamper immune recognition; and its absence is associated with a reduction of virulence in this fungus.[41–43] These findings reinforce the need for further studies to elucidate the biological roles of hydrophobins, especially in the context of the host-parasite relationship because several host factors may act together, in addition to elastin, to induce the expression of these proteins *in vivo*.

In conclusion, this study demonstrated that elastin influences biofilm development of *A. fumigatus*. The results showed that the ECM production was strongly increased while the hydrophobicity of the biofilm decreased. The present study confirms previous findings and contributes additional evidence that suggests pulmonary constituents can also influence biofilm development of *A. fumigatus*.

However, additional experiments should be necessary to allow for increased understanding of the role of host constituents for development of *A. fumigatus* biofilm.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

REFERENCES

1. Fernández LK, Charterina SA, Rubio AAG, Sánchez Nistal MA. The different manifestations of pulmonary aspergillosis: multidetector computed tomography findings. *Radiologia*. 2014;56(6):496–504.
2. O'Gorman CM. Airborne *Aspergillus fumigatus* conidia: a risk factor for aspergillosis. *Fungal Biol Rev*. 2011;25:151–157.
3. Beauvais A, Schmidt C, Guadagnini S, et al. An extracellular matrix glues together the aerial-grown hyphae of *Aspergillus fumigatus*. *Cell Microbiol*. 2007;9:1588–1600.
4. Seidler JM, Salvenmoser S, Müller FMC. *Aspergillus fumigatus* forms biofilms with reduced antifungal drug susceptibility on bronchial epithelial cells. *Antimicrob Agents Chemother*. 2008;52(11):4130–4136.
5. Mowat E, Williams C, Jones B, McChlery S, Ramage G. The characteristics of *Aspergillus fumigatus* mycetoma development: is this a biofilm? *Med Mycol*. 2009;47(1):S120–S126.
6. Rajendran R, Mowat E, McCulloch E, et al. Azole resistance of *Aspergillus fumigatus* biofilms is partly associated with efflux pump activity. *Antimicrob Agents Chemother*. 2011;55(5):2092–2097.
7. Filler SG, Sheppard DC. Fungal invasion of normally non-phagocytic host cells. *PLoS Pathog*. 2006;2(12):e129.

682   BRAZILIAN JOURNAL OF MICROBIOLOGY 49 (2018) 675–682

8. Kamai Y, Chiang LY, Lopes Bezerra LM, et al. Interactions of *Aspergillus fumigatus* with vascular endothelial cells. *Med Mycol*. 2006;44(1):S115–S117.

9. Khosravi AR, Mahdavi Omran S, Shokri H, Lotfi A, Moosavi Z. Importance of elastase production in development of invasive aspergillosis. *J Mycol Med*. 2012;22:167–172.

10. Toyotome T, Yamaguchi M, Iwasaki A, et al. Fetuin A, a serum component, promotes growth and biofilm formation by *Aspergillus fumigatus*. *Int J Med Microbiol*. 2012;302(2):108–116.

11. Shopova I, Bruns S, Thywissen A, Kniemeyer O, Brakhage AA, Hillmann F. Extrinsic extracellular DNA leads to biofilm formation and colocalizes with matrix polysaccharides in the human pathogenic fungus *Aspergillus fumigatus*. *Front Microbiol*. 2013;4:141.

12. Mowat E, Butcher J, Lang S, Williams C, Ramage G. Development of a simple model for studying the effects of antifungal agents on multicellular communities of *Aspergillus fumigatus*. *J Med Microbiol*. 2007;56:1205–1212.

13. O'Toole GA, Kolter R. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol*. 1998;28:449–461.

14. González-Ramírez AI, Ramírez-Granillo A, Medina-Canales MG, Rodríguez-Tovar AV, Martínez-Rivera MA. Analysis and description of the stages of *Aspergillus fumigatus* biofilm formation using scanning electron microscopy. *BMC Microbiol*. 2016;16:243–255.

15. Bruns S, Seidler M, Albrecht D, et al. Functional genomic profiling of *Aspergillus fumigatus* biofilm reveals enhanced production of the mycotoxin gliotoxin. *Proteomics*. 2010;10:3097–3107.

16. Fiedler T, Köller T, Kreikemeyer B. Streptococcus pyogenes biofilms – formation, biology, and clinical relevance. *Front Cell Infect Microbiol*. 2015;5:1–11.

17. Bitencourt TA, Macedo C, Franco ME, et al. Transcription profile of *Trichophyton rubrum* conidia grown on keratin reveals the induction of an adhesin-like protein gene with a tandem repeat pattern. *BMC Genomics*. 2016;17:249.

18. Rementería A, López-Molina N, Ludwig A, et al. Genes and molecules involved in *Aspergillus fumigatus* virulence. *Rev Iberoam Micol*. 2005;22(1):1–23.

19. Smith K, Rajendran R, Kerr S, et al. *Aspergillus fumigatus* enhances elastase production in *Pseudomonas aeruginosa* co-cultures. *Med Mycol*. 2015;53(7):645–655.

20. Loussert C, Schmitt C, Prevost MC, et al. In vivo biofilm composition of *Aspergillus fumigatus*. *Cel Microbiol*. 2010;12(3):405–410.

21. Müller CF, Seider M, Beauvais A. *Aspergillus fumigatus* biofilms in the clinical setting. *Med Mycol*. 2011;49:96–100.

22. Rajendran R [Ph.D. thesis] *Adaptive Resistance Mechanisms of Aspergillus fumigatus Biofilms*. Glasgow, United Kingdom: University of Glasgow; 2013:267.

23. Mowat E, Lang S, Williams C, McCulloch E, Jones B, Ramage G. Phase-dependent antifungal activity against *Aspergillus fumigatus* developing multicellular filamentous biofilms. *J Antimicrob Chemother*. 2008;62:1281–1284.

24. Blanco JL, Hontecillas R, Bouza E, et al. Correlation between the elastase activity index and invasiveness of clinical isolates of *Aspergillus fumigatus*. *J Clin Microbiol*. 2002;40:1811–1813.

25. Comera C, Andre K, Laffitte J, Collet X, Galtier P, Maridonneau-Parini I. Gliotoxin from *Aspergillus fumigatus* affects phagocytosis and the organization of the actin cytoskeleton by distinct signalling pathways in human neutrophils. *Microb Infect*. 2007;9:47–54.

26. Schrettl M, Bignell E, Kragl C, et al. Siderophore biosynthesis but not reductive iron assimilation is essential for *Aspergillus fumigatus* virulence. *J Exp Med*. 2004;200:1213–1219.

27. De Valk HA, Meis J, De Pauw BE, Donnelly PJ, Klaassen CHW. Comparison of two highly discriminatory molecular fingerprinting assays for analysis of multiple *Aspergillus fumigatus* isolates from patients with invasive aspergillosis. *J Clin Microbiol*. 2007;45:1415–1419.

28. Guinea J, García de Viedma D, Peláez T, et al. Molecular epidemiology of *Aspergillus fumigatus*: an in-depth genotypic analysis of isolates involved in an outbreak of invasive aspergillosis. *J Clin Microbiol*. 2011;49: 3498–3503.

29. Hazen KC, Hazen BW. A polystyrene microsphere assay for detecting cell surface hydrophobicity within *Candida albicans* populations. *J Microbiol Methods*. 1987;6:289–299.

30. Ramage G, Rajendran R, Gutierrez-Correa M, Jones B, Williams C. *Aspergillus* biofilms: clinical and industrial significance. *FEMS Microbiol Lett*. 2011;324:89–97.

31. Sheppard DC. Molecular mechanism of *Aspergillus fumigatus* adherence to host constituents. *Curr Opin Microbiol*. 2011;14:375–379.

32. Linder MB, Szilvay GR, Nakari-Setala T, Penttila ME. Hydrophobins: the protein-amphiphiles of filamentous fungi. *FEMS Microbiol Rev*. 2005;29:877–896.

33. Dague E, Alsteens D, Latge JP, Dufrene YF. High-resolution cell surface dynamics of germinating *Aspergillus fumigatus* conidia. *Biophys J*. 2008;94:656–660.

34. Paris S, Debeaupuis JP, Crameri R, et al. Conidial hydrophobins of *Aspergillus fumigatus*. *Appl Environ Microbiol*. 2003;69:1581–1588.

35. Gibbons JG, Beauvais A, Beau R, McGary KL, Latgé JP, Rokas A. Global transcriptome changes underlying colony growth in the opportunistic human pathogen *Aspergillus fumigatus*. *Eukaryot Cell*. 2012;11(1):68.

36. Valdivia RH, Heitman J. Endosymbiosis: the evil within. *Curr Biol*. 2007;17:408–410.

37. Epstein AK, Pokroy B, Seminara A, Aizenberg J. Bacterial biofilm shows persistent resistance to liquid wetting and gas penetration. *PNAS*. 2011;108:995–1000.

38. Siqueira V, Lima N. Surface hydrophobicity of culture and water biofilm of *Penicillium* spp. *Curr Microbiol*. 2012;64: 93–99.

39. Bayry J, Aimanianda V, Guijarro JI, Sunde M, Latgé JP. Hydrophobins – unique fungal proteins. *PLoS Pathog*. 2012;8(5):e1002700.

40. Kwon-Chung KJ, Sugui JA. *Aspergillus fumigatus* – what makes the species a ubiquitous human fungal pathogen? *PLoS Pathog*. 2013;9(12):e1003743.

41. Aimanianda V, Bayry J, Bozza S, et al. Surface hydrophobin prevents immune recognition of airborne fungal spores. *Nature*. 2009;460:1117–1121.

42. Bruns S, Kniemeyer O, Hasenberg M, et al. Production of extracellular traps against *Aspergillus fumigatus* in vitro and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA. *PLoS Pathog*. 2010;6:1000873.

43. Dagenais TR, Giles SS, Aimanianda V, Latgé JP, Hull CM, Keller NP. *Aspergillus fumigatus* LaeA-mediated phagocytosis is associated with a decreased hydrophobin layer. *Infect Immun*. 2010;78:823–829.