Cell dispersion during biofilm formation by *Scedosporium apiospermum, Scedosporium aurantiacum, Scedosporium minutisporum* and *Lomentospora prolificans*

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ABSTRACT

Dispersion is an essential step in the lifecycle of biofilms, since it enables the dissemination of microbial cells and, consequently, the potential colonization of new sites. Filamentous fungi belonging to the *Scedosporium/Lomentospora* genera are opportunistic human pathogens able to form multidrug-resistant biofilms on surfaces of different chemical compositions, environments and nutritional conditions. Despite the rising understanding of how biofilms are formed by *Scedosporium/Lomentospora* species, the cell dispersal step has not yet been explored. In the present study, the cell dispersion was investigated during biofilm formation by *S. apiospermum, S. minutisporum, S. aurantiacum* and *L. prolificans* cells. The results revealed that conidia were the major type of dispersed cells, which were detected throughout biofilm development (from 24 to 72 h). Dispersion was not influenced by increased glucose concentration (the main source for energetic metabolism) neither the presence of voriconazole (the most common antifungal used to treat scedosporiosis); however, the presence of mucin (a component of mucous, present in the lungs of cystic fibrosis patients, who are usually affected by these filamentous fungi) triggered cell dispersion. Contrarily, a poor nutritional environment (e.g., phosphate-buffered saline) inhibited this step. Overall, our study reveals new insights into the biofilm development of *Scedosporium/Lomentospora* species.

Introduction

Biofilms are microbial communities attached to a surface, encased in a self-produced polymeric substance known as the extracellular matrix. Several molecules make up this structure, including peptides, (glyco) proteins, (glyco)lipids, (oligo/poly)saccharides, extracellular DNA (eDNA), minerals and water (Flemming and Wingender, 2010; Flemming et al., 2016). The mature biofilm provides protection for microbial cells from the environmental aggressions faced by free planktonic cells, in part due to its distinct architecture (e.g., high population density, microbial cells in different metabolic/growth phases, effective cooperation between biofilm-forming cells, expression of genes associated with drug resistance and the unique composition of the extracellular matrix) (Flemming et al., 2016). In the proposed classical model for filamentous fungi, biofilm formation initiates with conidial cell adhesion to a biotic/abiotic surface, which triggers the germination program with consequent and time-dependent hyphal formation, followed by the production of a rich and complex extracellular matrix and, finally, cell dispersion (Harding et al., 2009).

Cell dispersion is a crucial step in the biofilm lifecycle since it allows the seeding of new communities in different locations and, in the context of infection, the spread of the pathogen inside the host body, which opens the possibility of the establishment of a chronic disease (Uppuluri et al., 2010). Dispersion is triggered by different signals and changes in environmental conditions, such as variations in nutrient availability (which involves not only the types but also concentrations of essential nutrients), pH, oxygenation and stressors (Uppuluri et al., 2010; Rumbaugh and Sauer, 2020). In addition, cells derived from biofilms are phenotypically distinct from planktonic cells, presenting an increased virulence arsenal, resistance to distinct chemical compounds and the

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ability to adhere and form new biofilm structures (Uppuluri et al., 2010; Chua et al., 2014; Uppuluri and Lopez-Ribot, 2016).

Scedosporium and *Lomentospora* species are fungal pathogens that mainly affect immunocompromised individuals (e.g., organ transplant recipients, chronic granulomatous disease and hematological malignancies cases, among others) and cystic fibrosis patients. However, these fungi can also cause illness in immunocompetent patients through traumatic inoculation of conidial cells and/or hyphal fragments mainly in lower and upper members (Cortez et al., 2008; Ramirez-Garcia et al., 2018; Mello et al., 2019). *Scedosporium/Lomentospora* species are able to form robust biofilms on several abiotic (polystyrene, glass, catheters with different chemical compositions) and biotic (e.g., over the monolayer of lung epithelial cells) substrates, presenting a highly resistant profile to all classes of antifungal drugs (e.g., azoles, echinocandins and polyenes) currently available for use in clinical settings (Mello et al., 2016, 2018; 2020b; 2022; Rollin-Pinheiro et al., 2017). The resistance profile of mature biofilms formed by *Scedosporium/Lomentospora* species is mainly due to the production of the complex extracellular matrix (rich in polysaccharides, (glyco)proteins and eDNA), the expression of efflux pumps and the elaborated antioxidant responses (Mello et al., 2022). Despite an increase in understanding of *Scedosporium* and *Lomentospora* biofilm formation over recent years, little is known about cell dispersion during the biofilm lifecycle. In the present work, we started to explore this event by analyzing cell dispersion from biofilms formed by *S. apiospermum, S. minutisporum, S. aurantiacum* and *L. prolificans* over a 72-h period over a polystyrene surface. In order to evaluate whether changes on culture conditions were able to modulate biofilm dispersal, fungal cells were incubated under different nutritional conditions as well as in the presence of the antifungal drug voriconazole.

Materials and methods

Microorganisms and growth conditions

Scedosporium apiospermum (strain RKI07_0416) was kindly provided by Dr. Bodo Wanke (Evandro Chagas Hospital, Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro, Brazil), and *S. minutisporum* (strain FMR 4072), *S. aurantiacum* (strain FMR8630) and *L. prolificans* (strain FMR 3569) were kindly given by Dr. Josep Guarro (Microbiology Unit, Medical School and Institute of Advanced Studies, Reus, Spain). Fungal cells were maintained in Sabouraud (2% glucose, 1% peptone and 0.5% yeast extract) liquid culture medium for 7 days at room temperature with orbital shaking (200 rpm). Conidia were obtained by growing each fungus at room temperature in Petri dishes containing potato dextrose agar (PDA; Difco Laboratories, USA). Subsequently, conidial cells were obtained by washing the plate surfaces with phosphate-buffered saline (PBS; 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 150 mM NaCl, pH 7.2) followed by filtration through a 40-mm nylon cell strainer (BD Falcon, EUA) in order to remove hyphal fragments (Mello et al., 2016). The conidial cells were counted in a Neubauer chamber.

Biofilm formation

Fungal suspensions (200 μL containing 10^6 conidial cells), obtained as described above, were placed in Sabouraud medium in flat-bottom 96-well polystyrene microtiter plates and then incubated at 37°C with 5% CO_2 for 72 h as previously described (Mello et al., 2016). In parallel, medium-only blanks were set up. Afterwards, the supernatant from each well was discarded and the plate wells were washed twice with PBS in order to remove non-adherent fungal cells (Mello et al., 2016).

Dispersion kinetics

Biofilms formed after 24, 48 and 72 h of incubation, as earlier detailed, were washed and incubated for a further 24-h interval in fresh Sabouraud medium at 37°C with 5% CO_2. Subsequently, the supernatants were collected and dispersed fungal cells (released from biofilm structures) were quantified in a Neubauer chamber. In parallel, these cells were visualized with the aid of an inverted microscope (Zeiss, Germany) (Uppuluri et al., 2010).

Dispersion under incubation in different nutritional conditions

The 24-h biofilms were washed and incubated for further 24 h at 37°C in an atmosphere of 5% CO_2 under the following growth conditions: fresh Sabouraud medium (containing approximately 110 mM glucose); Sabouraud medium supplemented with different glucose concentrations (5, 50 and 500 mM); and PBS (a nutrient-poor medium). Subsequently, the supernatants were collected and the dispersed cells were quantified in a Neubauer chamber (Uppuluri et al., 2010).

Dispersion under incubation in a medium mimicking cystic fibrosis environment

The 24-h biofilms were washed and incubated for further 24 h at 37°C in an atmosphere of 5% CO_2 in synthetic cystic fibrosis sputum medium (SCFM) supplemented or not with 1% mucin (Sigma-Aldrich, USA), in order to mimic the cystic fibrosis (CF) sputum. Subsequently, the supernatants were collected and the dispersed cells were quantified in a Neubauer chamber (Co et al., 2018).

Dispersion under incubation with voriconazole

The 24-h biofilms were washed and incubated for further 24 h at 37°C in an atmosphere of 5% CO_2 in RPMI 1640 medium supplemented or not with 4 or 16 μg/mL voriconazole (Sigma-Aldrich, USA), the major antifungal drug employed in the treatment of scedosporiosis (Tortorano et al., 2014). Subsequently, the supernatants were collected and the dispersed cells were quantified in a Neubauer chamber (Uppuluri et al., 2011).

Statistics

All experiments were performed in triplicate, in three independent experimental sets. Data were expressed as mean ± standard deviation (SD). The results were evaluated by 2way ANOVA followed by Tukey's or Sidak's multiple comparison test using Graphpad Prism 8 computer software (GraphPad Software, Inc., La Jolla, CA, USA). In all analyses, *p*-values of 0.05 or less were considered statistically significant.

Results

Kinetics of cell dispersion in Scedosporium/Lomentospora biofilms

To start exploring the cell dispersion step in *Scedosporium* and *Lomentospora* species, biofilms formed at daily intervals (from 24 to 72 h) were analyzed. Dispersion was observed throughout the maturation course of biofilms, presenting a greater number of dispersed cells in the initial stage (24 h) and a concomitant decrease along the maturation period (72 h) (Fig. 1). In accordance with these results, 24 h-formed biofilms were selected for subsequent analyzes. The dispersed cells from *S. apiospermum, S. minutisporum, S. aurantiacum* and *L. prolificans* biofilms were mainly composed of conidia, whereas germinated conidia and hyphae of different sizes were observed in substantially smaller proportions (Fig. 2).

Modulation of biofilm cell dispersion: effects of the nutritional conditions

To assess whether nutrient availability could modulate the cell dispersion event in *Scedosporium/Lomentospora* biofilms, Sabouraud medium (a rich nutritional culture medium containing approximately 110 mM glucose) supplemented or not with distinct glucose
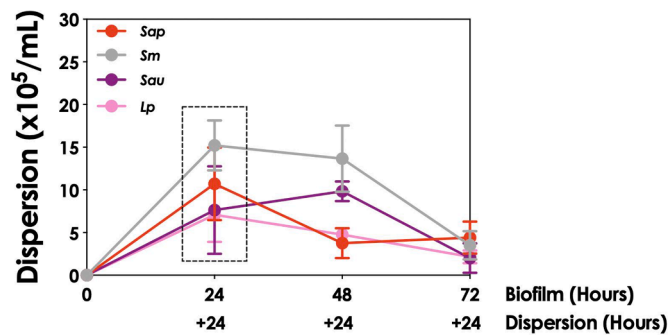
Fig. 1. Quantification of dispersed cells from *S. apiospermum, S. minutisporum, S. aurantiacum* and *L. prolificans* biofilms along 72 h. The biofilms of *S. apiospermum* (*Sap*), *S. minutisporum* (*Sm*), *S. aurantiacum* (*Sau*) and *L. prolificans* (*Lp*) were formed for 24, 48 and 72 h at 37 °C on polystyrene surface. After each time interval, biofilms were washed and fresh Sabouraud medium was added to the systems, which were then incubated for additional 24 h at 37 °C in an atmosphere of 5% CO_2. Subsequently, the supernatants were collected and dispersed cells were quantified in a Neubauer chamber. The dashed box indicates the dispersion time that was selected for further experiments. The results were expressed as the mean ± the standard deviation of three independent experiments.

concentrations (5, 50, and 500 mM) as well as PBS were tested. For *S. apiospermum, S. minutisporum, S. aurantiacum* and *L. prolificans*, fluctuations in the glucose concentration did not interfere with the cell dispersion in 24 h-formed biofilms (Fig. 3A). On the contrary, the incubation under a nutritionally poor environment (PBS) significantly decreased the cell dispersion in 2- to 6-fold compared to the incubation in Sabouraud medium (Fig. 3B).
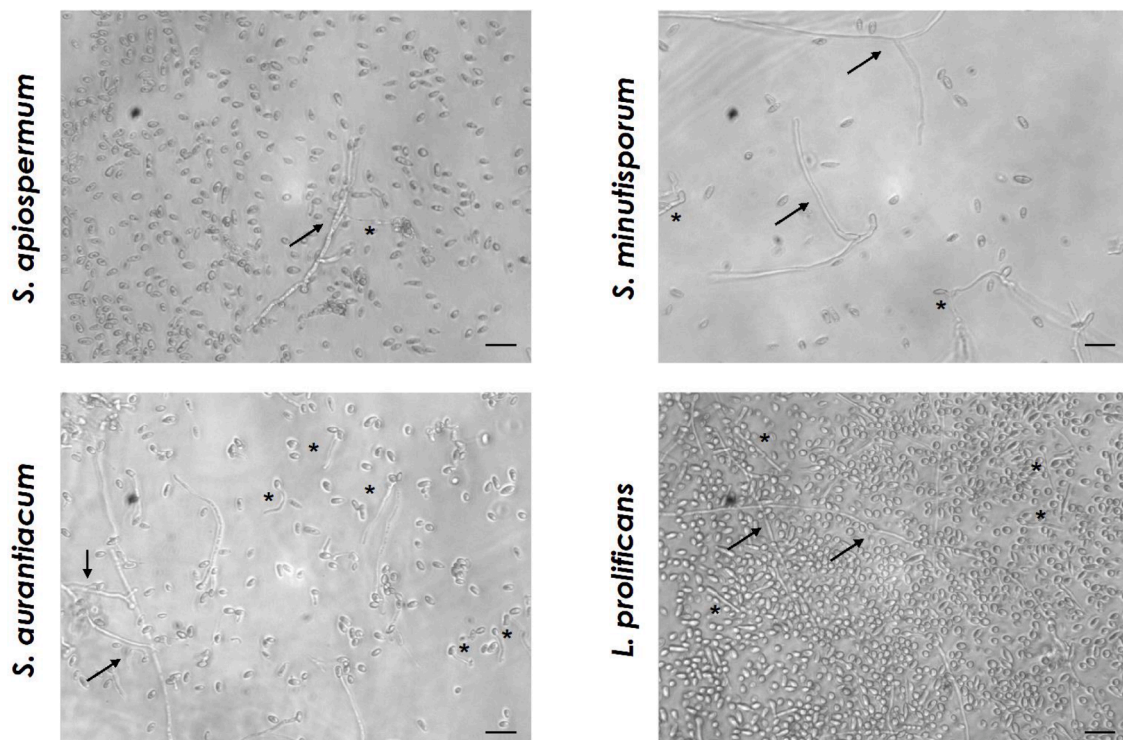
Modulation of biofilm cell dispersion: effects of cystic fibrosis airway mimic environment
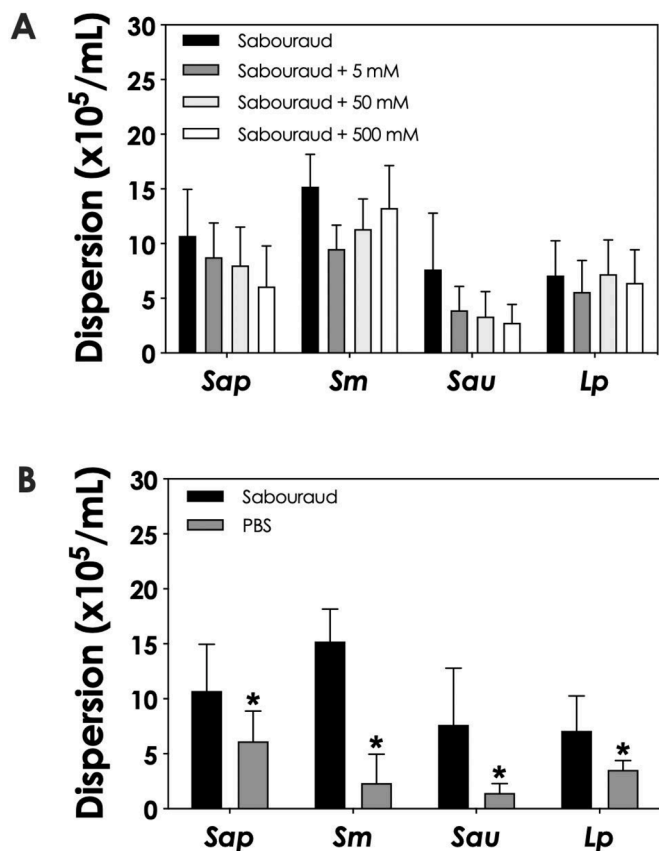
To assess how biofilm cells disperse in an environment that mimics the cystic fibrosis airway conditions, SCFM and SCFM supplemented with mucin were selected. The results evidenced that the dispersion event occurred in both culture media. However, a significant increase in the dispersion was observed when fungal cells were incubated in SCFM + mucin compared to SCFM alone by approximately 4-, 2.5- and 2.7-times in *S. apiospermum, S. aurantiacum* and *L. prolificans*, respectively (Fig. 4).

Modulation of biofilm cell dispersion: effects of the antifungal voriconazole

Voriconazole, the drug of choice for the treatment of scedosporiosis, was used in order to evaluate the dispersion of cells under the antifungal drug pressure. The results showed that the incubation of biofilms in the presence of voriconazole at different concentrations (4 and 16 μg/mL) was not able to block and/or decrease the dispersion of cells from *Scedosporium/Lomentospora* biofilms (Fig. 5).
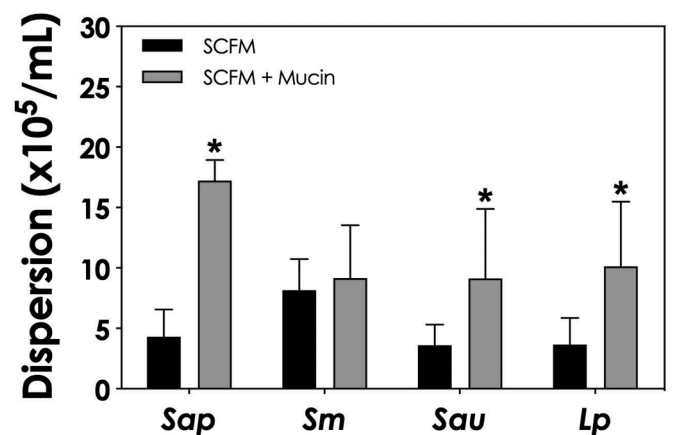
Discussion and conclusion

Biofilm formation passes through several stages of development, beginning with cell adhesion to a surface, microcolony formation, cell proliferation, maturation, extracellular matrix production and dispersion (Harding et al., 2009). At each step of this process, microbial cells display phenotypes and properties that are distinct from planktonic counterparts. For example, dispersed cells from *Candida albicans* biofilms are more adherent, and form germ tubes and biofilm more quickly than planktonic cells; in addition, the biofilm-released cells are more virulent in a murine model of in vivo infection (Uppuluri et al., 2010). Cell dispersion is one of the crucial steps in biofilm development, as it

Fig. 2. Representative images of dispersed cells from *S. apiospermum, S. minutisporum, S. aurantiacum* and *L. prolificans* biofilms. Biofilms were formed along 24 h at 37 °C in an atmosphere of 5% CO_2 over a polystyrene surface. Then, biofilms were washed, fresh Sabouraud medium was added and the systems were incubated for additional 24 h at 37 °C with 5% CO_2. Subsequently, the supernatants were collected and bright-field microscopy photos were obtained from dispersed cells. Asterisks indicate germinated conidia and arrows evidence the hyphae. The bars represent 15 μm.

A

B
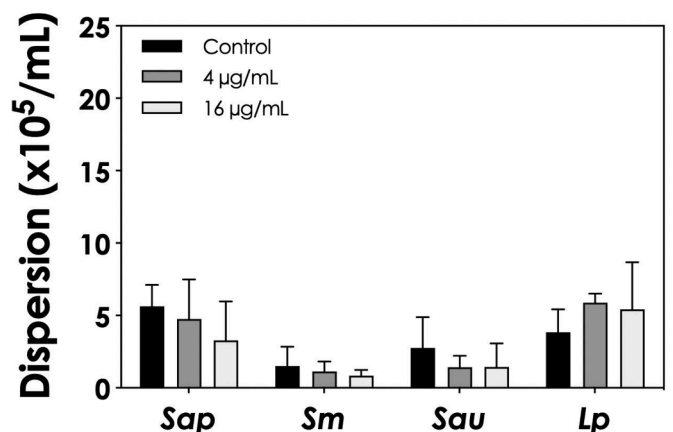

Fig. 3. Quantification of dispersed cells from *S. apiospermum, S. minutisporum, S. aurantiacum* and *L. prolificans* biofilms under different nutritional conditions. The biofilms of *S. apiospermum* (*Sap*), *S. minutisporum* (*Sm*), *S. aurantiacum* (*Sau*) and *L. prolificans* (*Lp*) were formed for 24 h at 37 °C in an atmosphere of 5% CO_2 over a polystyrene surface. After this time, the biofilms were washed and (A) Sabouraud medium containing different glucose concentrations or (B) PBS were added to the systems, which were then incubated for additional 24 h at 37 °C with 5% CO_2. Subsequently, the supernatants were collected and dispersed cells were quantified in a Neubauer chamber. The results were expressed as the mean ± the standard deviation of three independent experiments. Asterisks represent significant differences (*$P < 0.05$, 2way ANOVA, Sidak's multiple comparison test) between the number of cells dispersed in Sabouraud and PBS.

Fig. 4. Quantification of dispersed cells from *S. apiospermum, S. minutisporum, S. aurantiacum* and *L. prolificans* biofilms in environments that mimic the lung of cystic fibrosis patients. *S. apiospermum* (*Sap*), *S. minutisporum* (*Sm*), *S. aurantiacum* (*Sau*) and *L. prolificans* (*Lp*) biofilms were formed for 24 h at 37 °C in an atmosphere of 5% CO_2 over a polystyrene surface. Then, the biofilms were washed and fresh SCFM medium supplemented or not with mucin was added to the systems, which were incubated for further 24 h at 37 °C with 5% CO_2. Supernatants were collected and dispersed cells were quantified in a Neubauer chamber. The results were expressed as the mean ± the standard deviation of three independent experiments. Asterisks represent the significant difference (*$P < 0.05$, 2way ANOVA, Sidak's multiple comparison test) between the number of cells dispersed in the presence and in the absence of mucin.

Fig. 5. Quantification of dispersed cells from *S. apiospermum, S. minutisporum, S. aurantiacum* and *L. prolificans* biofilms in the presence of voriconazole. The biofilms of *S. apiospermum* (*Sap*), *S. minutisporum* (*Sm*), *S. aurantiacum* (*Sau*) and *L. prolificans* (*Lp*) were formed for 24 h at 37 °C in an atmosphere of 5% CO_2 over a polystyrene surface. After, the biofilms were washed and RPMI medium containing or not 4 or 16 µg/mL voriconazole were added to the systems, which were then incubated for additional 24 h at 37 °C with 5% CO_2. Subsequently, the supernatants were collected and the scattered cells were quantified in a Neubauer chamber. The results were expressed as the mean ± the standard deviation of three independent experiments.

allows the generation of new communities in different locations, in addition to the dissemination of the pathogen during the infection that increases the number of potential colonized/infected host sites (Uppuluri et al., 2010). Despite being a fundamental step, the dispersion event is still a latent area of studies in fungal biofilms, with the few available works focused only on the dispersion of *C. albicans* biofilms (Uppuluri et al., 2010; 2011; 2018; Pentland et al., 2021). In order to contribute to this scarce area of knowledge, in the present work, our group evaluated the cell dispersion profile of *S. apiospermum, S. minutisporum, S. aurantiacum* and *L. prolificans* biofilms.

Unlike the model previously proposed for filamentous fungi, in which dispersion occurs only after biofilm maturation (Harding et al., 2009), we observed that cell dispersion occurs continuously during the development of the biofilms in *Scedosporium/Lomentospora* species and it is predominantly composed of conidial cells. The highest levels of dispersion were observed in the initial stages (24 h) of biofilm formation and these levels decreased concomitantly with biofilm maturation (48 h and 72 h). Most of the cells dispersed at 24 h should be from the initial inoculum, composed of conidia that either did not adhere nor germinate due to the high cell density, even after washing the plate wells. Similarly, in *C. albicans*, regardless of the culture medium used, the highest levels of dispersion also occur in the early stages (5–12 h), decaying after

biofilm maturation (20–24 h) (Uppuluri et al., 2010). These results may have an important implication in the context of *Scedosporium/Lomentospora* infections, since the spread of biofilm cells can occur continuously in the environment and the inhalation of conidia dispersed in the air is the main form of transmission of these filamentous fungal pathogens (Cortez et al., 2008; Ramirez-Garcia et al., 2018; Mello et al., 2019).

The dispersion of cells from biofilms is triggered by several factors, including environmental, nutritional and gene regulation changes (Sauer et al., 2004). In this context, we evaluated the dispersion of

Scedosporium/Lomentospora biofilms under cultivation in various growth conditions. Increasing concentrations of glucose (from 5 to 500 mM) did not modulate fungal dispersion, while an environment without available nutrients (e.g., PBS), significantly decreased dispersion when compared to the nutrient-rich medium (Sabouraud). As previously published by our research group, different glucose concentrations did not modulate biofilm formation dynamics in *Scedosporium/Lomentospora* species, reiterating the idea that these fungal species probably use other components of the culture medium, for example peptone, as a carbon source to sustain their basic metabolism (Mello et al., 2020a). The modulation of dispersion as a function of nutritional status is a survival strategy, in which cells propagate when a favorable environment is detected, while a cohesive biofilm is formed in a nutrient-poor environment (Uppuluri et al., 2010). In this way, the dispersion of *C. albicans* biofilms is about 50 times lower in PBS than in high glucose concentrations. In that study, the increase in glucose concentration enabled more cells to disperse into the environment (Uppuluri et al., 2010).

In addition to the concentration of nutrients, we observed that mucin also affected cell dispersion of *Scedosporium/Lomentospora* biofilms. In the presence of soluble mucin, dispersion increased by 2- to 4-fold compared to in the absence of this glycoprotein. Mucins are *O*-linked glycoproteins produced and secreted by the epithelial mucosa, being one of the major molecules that confer high viscosity to the mucus of patients with cystic fibrosis (Palmer et al., 2007). Our findings are consistent with those observed in *Pseudomonas aeruginosa*, a gram-negative bacterium usually found in the lung of cystic fibrosis patients, in which mucin is able to induce the separation of cells from biofilm and to disperse them into the environment (Co et al., 2018). The dispersion of biofilms caused by mucin molecules may be due to: (i) breaking of biofilm matrix caused by highly glycosylated mucins, which compete for binding sites on the cell surface and in the extracellular matrix and/or (ii) suppression of cell aggregation through retention in planktonic state (Caldara et al., 2012; Rendueles et al., 2013; Co et al., 2018). In the context of an infectious process, these results could indicate a greater probability of dissemination of microbial cells in patients with cystic fibrosis.

We also evaluated cell dispersion in *Scedosporium/Lomentospora* biofilms under antifungal treatment. Voriconazole was not able to perturb the mature biofilm dynamics in these filamentous fungi, as judged by the measurement of the biofilm metabolic activity after drug treatment (Mello et al., 2016; 2020b; Rollin-Pinheiro et al., 2017). In the same way, the number of dispersed cells was not affected by the two voriconazole concentrations (4 and 16 μg/mL) used in our study. Likewise, fluconazole had a minor effect on *C. albicans* dispersion levels (Uppuluri et al., 2011). *Scedosporium/Lomentospora* infections, like mycetoma and fungal ball, present biofilm-like characteristics (Mowat et al., 2009; Mello et al., 2016), and the result found herein could indicate the reason for so many failures in scedosporiosis treatment, even when the drug of choice is correctly used (Ramirez-Garcia et al., 2018).

In conclusion, we have demonstrated for the first time that cell dispersion is a process dependent on the disposition of nutrients, and it occurs uninterruptedly during the biofilm development of selected species within the *Scedosporium* and *Lomentospora* genera. Whether this observation holds when a greater number of strains are tested is not known. Moreover, we found that the antifungal drug of choice for the treatment of scedosporiosis, voriconazole, had no effect on the process of cell dispersion at the concentrations tested in this work. Taken together, the results reported herein provide new insights on cell dispersion from *Scedosporium/Lomentospora* biofilms, a step that should have essential roles in the dissemination of these emerging and multidrug-resistant fungal pathogens both in the environment and in the vertebrate host.

Author contribution

All authors have contributed to discuss experimental design, discussing the data and manuscript writing. TPM and ICB performed the experiments.

CRediT authorship contribution statement

Thaís P. Mello: Conceptualization, Methodology, Writing – original draft. **Iuri C. Barcellos:** Methodology. **Marta H. Branquinha:** Supervision, Writing – review & editing. **André L.S. Santos:** Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Andre Luis Souza dos Santos reports financial support and equipment, drugs, or supplies were provided by Carlos Chagas Filho Foundation for Research Support of Rio de Janeiro State. Andre Luis Souza dos Santos reports financial support and equipment, drugs, or supplies were provided by CAPES. Andre Luis Souza dos Santos reports financial support and equipment, drugs, or supplies were provided by CNPQ. Thais Pereira de Mello reports financial support and equipment, drugs, or supplies were provided by Carlos Chagas Filho Foundation for Research Support of Rio de Janeiro State. Iuri C. Barcellos reports financial support was provided by Carlos Chagas Filho Foundation for Research Support of Rio de Janeiro State. Marta Helena Branquinha reports financial support and equipment, drugs, or supplies were provided by Carlos Chagas Filho Foundation for Research Support of Rio de Janeiro State. Marta Helena Branquinha reports financial support and equipment, drugs, or supplies were provided by CAPES. Marta Helena Branquinha reports financial support and equipment, drugs, or supplies were provided by CNPQ.

Data availability

Data will be made available on request.

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