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Report

Characterization of a biofilm formed by *Fusarium oxysporum* on the human nails

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Abstract

Background *Fusarium* spp. has been considered as an onychomycosis agent, but little is known about the etiopathogenesis of fusarial onychomycosis; thus, the objective of this study was to characterize the fungal-nail interaction and the consequences of the nail infection process by *Fusarium oxysporum* using the human nail, in an ex vivo model.

Methods The kinetic of biofilm production and infection by *F. oxysporum* using the nail as the only nutritional source were evaluated by scanning electron microscopy, number of culturable cells, metabolic activity, characterization of extracellular matrix, spectroscopy and histopathology analyses.

Results After evaluating the biofilm kinetic over 7 days using different parameters and techniques, it was possible to characterize the *Fusarium*-nail interaction.

Conclusions This study is a part of a big project aiming to clarify the fusarial pathogenesis and contributes to proving *F. oxysporum* is able to adapt, grow, develop, and form a biofilm on healthy human nails, which are crucial steps for the invasion process.

Introduction

The main agents of onychomycosis are a homogeneous group of keratinophilic fungi known as dermatophytes. However, the ability of nondermatophyte fungi to cause nail fungal infections has also been described.[1,2] In this context, among others, the genus *Fusarium* has come to be a cause of onychomycosis, especially in immunocompromised patients or in the presence of already existing nail trauma.[3–6] In addition, the incidence of fusarial onychomycosis has increased, affecting even healthy individuals.[7–12] In our medical mycology laboratory, this rate increased from 7.5 to 13.3% in the last 15 years.[1]

It is important to emphasize that besides the common damages caused by onychomycosis,[13] those provoked by *Fusarium* are usually accompanied by paronychia.[3,12,14] Moreover, the vulnerability of the sick nail becoming an entrance portal for disseminated infections has been reported.[3,12,14] In terms of

etiopathogenesis, there is strong evidence for *Fusarium* spp. involved in biofilms causing onychomycosis.[15]

Our research group has conducted studies involving *Fusarium*-nail relationships. We were the pioneer to prove *Fusarium oxysporum* was able to cause onychomycosis, including in immunocompetent individuals, and associate it with painful lesions and paronychia.[7–12] Next, we showed, for the first time, this fungus was able to use the nail as a unique nutritional source, to penetrate nail layers unassisted, in addition to being able to infect and invade across the healthy human nail through both dorsal and ventral nail surfaces.[16] Additionally, our findings reinforced the evidence that biofilms are related to the fungal nail infection, since we produced a biofilm during the fungal growth in an ex vivo model on human nail. This set of findings allowed us to confirm that *F. oxysporum* is able to be a primary agent of onychomycosis. However, even knowing this fungus penetrates into the nail tissue, the pathogenesis of fusarial onychomycosis and possible association with biofilm have still not been characterized.[16,17]

Considering the lack of knowledge on the pathogenesis of onychomycosis caused by *F. oxysporum*, and the need for elucidation of virulence factors behind a nail infection, the objectives of the present study were to further develop the previous study, by daily monitoring the formation of the supposed biofilm and characterize it, aiming to better understand how this *Fusarium*-nail interaction happens over time.

Methods

Fungal strain

This study was conducted with a *F. oxysporum* strain isolated from a patient suffering from onychomycosis. The fungus was identified and confirmed by classic methods[18] and molecular studies.[19] This isolate was deposited in the Microbial Collections of Paraná Network-TAX online, at the Federal University of Paraná under registration number (CMRP2925) and GenBank (MG692504.1). Prior to the experimental trials, the isolate was reactivated, which confirmed its purity and identification. In each experiment, the isolates were grown on Sabouraud Dextrose Agar (SDA; Difco™, Detroit, MI, USA) for 7 days at 25°C.

Nail infection and biofilm formation

Nails were infected following a modified version of the previously described method.[17] The procedure was approved by the Ethical Committee on Human Experimentation, under the number 615.643/2014. A set of nail fragments were collected from healthy female volunteers and sterilized by autoclaving at 121°C, for 20 minutes. Afterwards, 3 μl of a *Fusarium* conidial suspension (containing 1.2×10^7 conidia/ml, in sterile saline 0.85% [SS] and calibrated by a count in a Neubauer chamber) were carefully pipetted on the ventral side of the nail surface. The infected nails were incubated for 7 days at 35°C, in a humid chamber to ensure biofilm formation. All experiments were carried out in triplicate, and they were repeated six times on different days.

Scanning electron microscopy analyses

During the 7-day incubation period, *F. oxysporum*-infected human nail fragments were observed by scanning electron microscopy (SEM) based on the approach described previously by Galletti *et al.*, 2017. For SEM analysis, after each incubation period, the nails were washed with SS, dehydrated with alcohol (using 50% ethanol for 10 minutes, 70% for 10 minutes, 80% for 10 minutes, 95% for 10 minutes, and 100% for 20 minutes) and air dried. Prior to observation, the nails were cut out and mounted onto aluminium stubs, sputter-coated with gold, and observed with an FEI Quanta™ 250 scanning electron microscope (Leo, MA, USA).

Number of culturable cells determination from biofilm

Total number of culturable cells on the infected human nail with *F. oxysporum* was determined every day over the 7-day period. Three nail fragments were deposited in a microtube with 1 ml of SS, and total biomass was vortexed vigorously for 5 minutes and subjected to 35% sonication for 10 seconds. Serial dilutions in SS were subcultured onto SDA and incubated for 48 hours at 25°C to determine CFU/ml. Determination of CFUs was performed in triplicate and repeated three times on different days. Results were presented as log of CFU/ml.

Metabolic activity assay determination

Tetrazolium salt 2,3-(2-methoxy-4-nitro-5-sulphophenyl)-5-([phenylamino] carbonyl)-2H tetrazolium hydroxide reduction assay (XTT; Sigma-Aldrich, MO, USA) was used to determine *in situ* biofilm mitochondrial activity.[19] Three nail fragments were used to determine the metabolic activity of the *Fusarium* cells at each incubation time point over seven days. This assay was performed in triplicate on two different days.

Characterization of biofilm

Total biomass was recovered, followed by separation of the extracellular matrix (ECM) of the biofilm using mechanical filtration methods with a 0.22 μm membrane (Kasvi, São José dos Pinhais, PR, Brazil). Thereafter, filtrate containing the ECM was analyzed to quantify the total extracellular deoxyribonucleic acid (eDNA), extracellular ribonucleic acid (eRNA), protein and polysaccharide content,[20] with some modifications. Quantities of the first three components were determined by measuring the optical density (OD) with the Nanodrop 2000™ spectrophotometer (Nanodrop 2000 UV-Vis Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). OD values were measured at the 260/230 nm ratio to estimate the concentration of total polysaccharides. Diluent (SS) was used as a negative control. A Bradford protein test was used to measure the

extracts total protein concentration, with bovine serum albumin as standard.[21]

Spectroscopy analysis by FTIR-ATR

To evaluate the fungal-nail relationship in an *F. oxysporum* infection, readings were taken from both surfaces of the nail, ventral and dorsal, according to Galletti *et al.* 2016,[16] at days 1, 4, and 7 of infection. Measurements were performed using a Fourier transform infrared (FTIR) spectrometer coupled to a diamond crystal ATR accessory. Spectral range was 400–4,000 cm^{-1} with 128 scans and a resolution of 4 cm^{-1}. Data acquisition was performed via software on a computer connected to the spectrometer, with ATR and background correction.

Histopathology

One fragment of each experiment, including the contaminated nails, in days 1, 4, and 7 of infection, and the negative control group (equal volume of saline, without fungus) were prepared and analyzed by histopathology.

Results

The interaction between *F. oxysporum* and a sterile healthy human nail, where the nail was the only organic source of nutrients in an *ex vivo* infection model, was monitored for 7 days. Microscopic analysis, by SEM, of the samples revealed a morphological change from conidia (fungal form put in contact) to hyphae, over a 7-day period. A panoramic magnification (×1,000) allowed for the evaluation of morphological alterations in the fungal structures. On the first day, after 24 hours of infection (Fig. 1a), fungi in the conidial form were observed, exactly as they were inoculated. On day 2, the image indicates a transition from conidia to hyphae (Fig. 1b), while the next 3 days (3–5) appear to be clear of the formation of hyphae, its development and consolidating, besides the increase in number of cells (Fig. 1c–e). On the sixth day (Fig. 1f), it was possible to visualize, on the surface of the nail, a large quantity of well-organized hyphae in a structure exhibiting ECM, therefore suggesting a biofilm formation.

To better characterize the ECM and to confirm the ability of *F. oxysporum* to form biofilm and infect the nail surface using it as a unique nutritional source, the total eDNA, eRNA, polysaccharide, and protein contents were quantified over time (Fig. 2). Both eDNA and eRNA decreased from the first to the second day. Subsequently, they increased until the sixth day, where eRNA continued to decline until the seventh day and eDNA decreased (Fig. 2a). According to Figure 2b, the total polysaccharide content remained stable in the ECM, having only a small decrease from the fifth to the seventh day that tended to

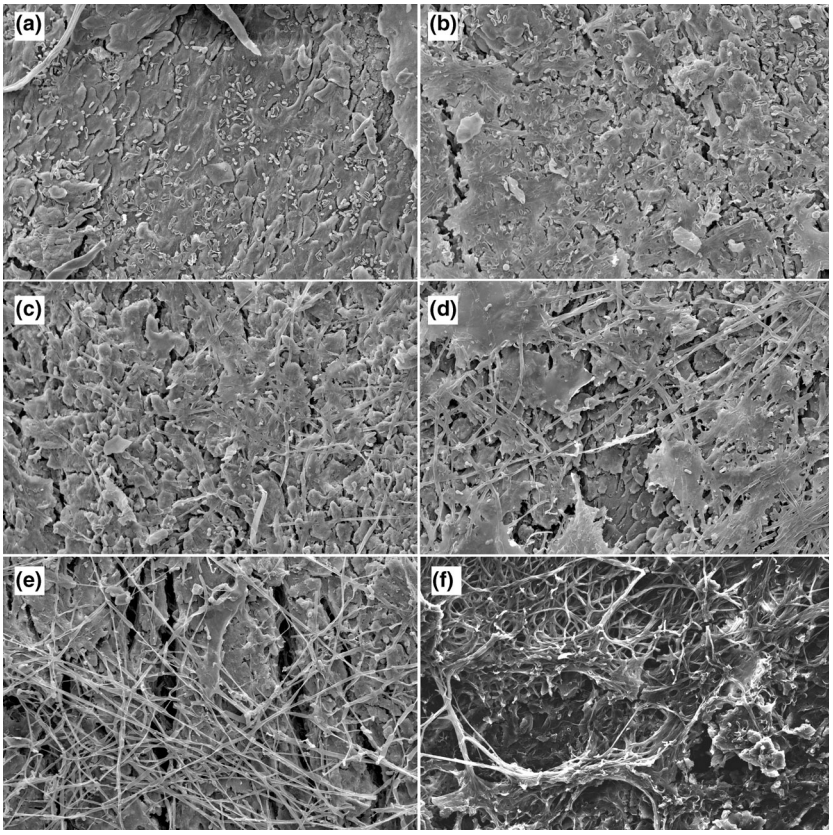

Figure 1 Morphological evaluation by SEM to characterize the evolution of growth of *Fusarium oxysporum* on human nail causing the development of infection in the period of 1 (a), 2 (b), 3 (c), 4 (d), 5 (e), and 6 (f) days. ×1,000 magnification

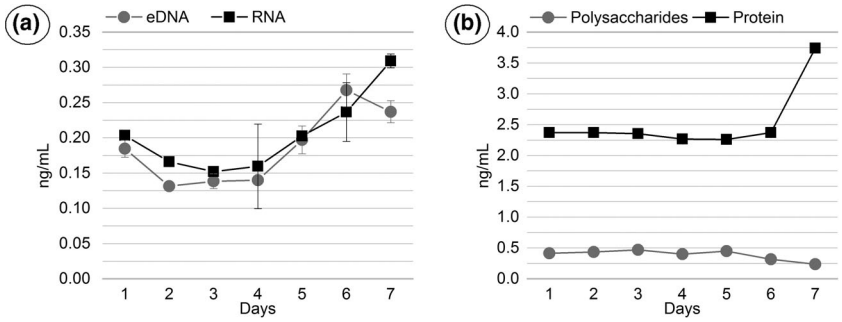

Figure 2 Concerning the total nucleic acids (eDNA and eRNA) (a), polysaccharides and protein (b) (ng/ml) contents on the growth of the *Fusarium oxysporum* on the nail at different times of incubation (1–7 days)

be inversely related to the quantity of total proteins, which in turn had a significant increase on day 7.

Figure 3 presents a semiquantitative evaluation regarding the infection dynamics by *F. oxysporum* over time, according to two indicative parameters of growth. Based on the analysis of the number of viable cells, a progressive increase of fungal growth on the nail surface over time was verified. Generally, the number of culturable cells (Fig. 3a) increased at all time points assayed. However, the evolution of fungal growth on the surface of the nail expressed as CFU showed that the stationary phase was reached between 3 and 5 days, while the maximum growth yield in the cultures occurred in 7 days. In contrast, the metabolic activity, measured by the *in situ* mitochondrial metabolic activity using the absorbance values from the XTT reduction assay, showed a significant increase in absorbance values at day 4, while these values declined and stabilized for the other evaluated days (Fig. 3b). Altogether, these data suggest that day 4 was crucial for the mechanism of fungal growth and its adaptation to the nail. While the number of viable cells reached a certain level of stability but continued to increase, the metabolic activity was highly stimulated and returned to baseline levels.

Associated to morphological evaluation using SEM and ECM components, standard over the period days 1, 4, and 7 were considered key and were used as markers for in-depth studies. Figure 4 details the morphological aspects observed on these days. Compared to the first day (Fig. 1a), a greater magnification exhibited in Figure 1b (×5,000) allowed for the observation that in 4 days, there was already a consolidated structure with a very well interlaced hyphae providing an environment auspicious to the development of ECM. Meanwhile, on day 7, it was possible to observe the presence of ECM

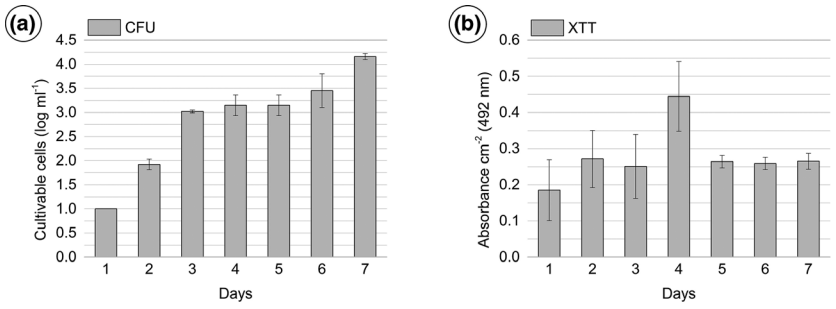

Figure 3 Viable cells by log of colony forming units per milliliter (CFU/ml) (a) and absorbance values by XTT reduction (b) on the growth of the *Fusarium oxysporum* on the nail at different times of incubation (1–7 days)

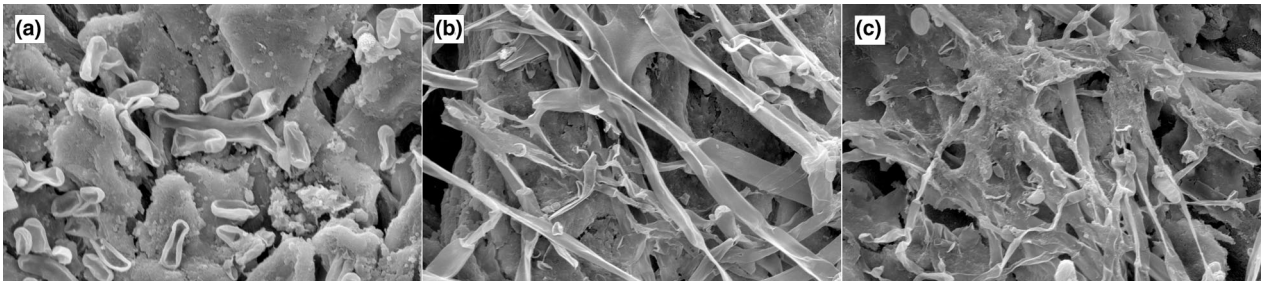

Figure 4 SEM showing the initial surface binding at 1 day of infection (a); this is followed by filament formation with 4 days (b) and finally completely adaptation in 7 days (c) after infection with *Fusarium oxysporum* in human nail. ×5,000 magnification

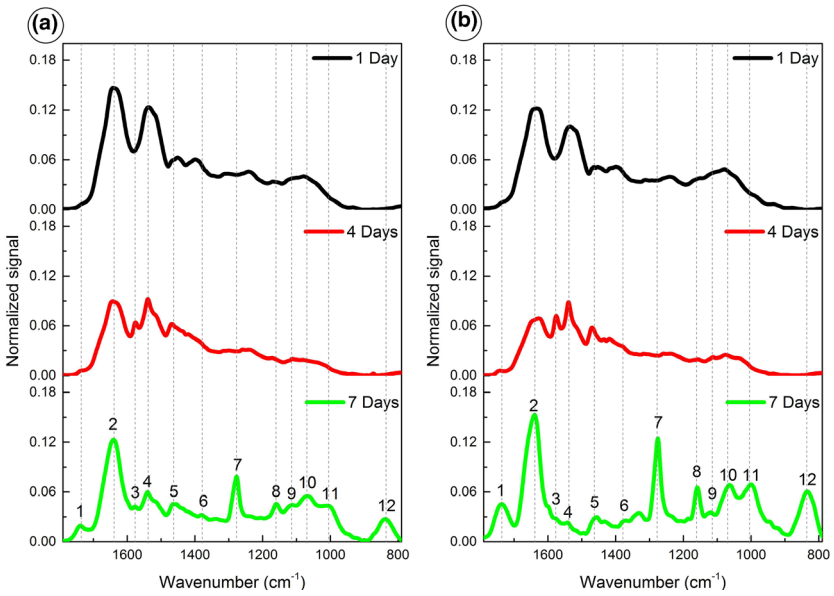

Figure 5 FTIR-ATR analysis of *Fusarium oxysporum* growth on nail, using it as unique nutritional source at 1, 4 and 7 days. (a) Ventral surface; (b) dorsal surface

(white arrow) associated with hyphae and communication channels (black arrow).

Figure 5 shows the spectra obtained by FTIR-ATR, at days 1, 4, and 7 of infection, the main peaks identifying the main organic compounds produced during this process, which are indicated by numbers and listed in Table 1. The evolution of fungal growth on the nail surface was evidenced mainly by the peaks 1, 7, 10, 11, and 12, which are related to lipids and proteins, should be noted. Attention was drawn to peak 1, which signal began on the fourth day and became evident on the seventh day; this peak was detected in the fat region (1,734–1,754 cm^{-1}), a range referring to different forms of biomolecules, such as lipids, phospholipids and triglycerides. The chemical compounds regarding each main peak are presented on Table 1.

Table 1 Summary of the peaks observed in the FTIR-ATR spectra, together with the attributions of the organic compounds

Peak no.	Peak (cm^{-1})	Suggested assignment
1	1,737	C=O, lipids
2	1,641	Protein, Amide I
3	1,577	NADH
4	1,540	Amide carbonyl group vibrations and aromatic hydrogens
5	1,460	CH_2/CH_3, lipids
6	1,380	dCH_3, lipids
7	1,276	Lipids, Amide III
8	1,150	Glycogen
9	1,112	Saccharide band
10	1,068	Proline
11	1,008	Phenylalanine
12	838	Deformative vibrations of amine groups

Spectra peak assignments.[22]

To further examine the findings of fungal growth (CFU) and metabolic activity (XTT), histopathology tests were performed on the days considered to be key (days 1, 4, and 7) in the process of *F. oxysporum* infecting the human nail. After exhaustive histological preparations made in three different days, results indicate that on days 1 and 4, no fungal structures were observed invading the nail tissue (Fig. 6b,c), which is similar to the negative control, the uninfected nail (Fig. 6a). Surprisingly, the presence of short hyphae invading the infected ventral surface was observed on day 7 only (Fig. 6d).

Discussion

In a previous study, we evaluated the *F. oxysporum*-nail interaction in a single day, the seventh day after the fungal inoculation. It was reported that the process of infection occurred with the fungi using the nail as the only nutritional resource.[16] In the present study, we evaluated the kinetics of this interaction process, day by day, over a 7-day period. A clear evolution was observed by ultra-morphological, biochemical and metabolic aspects. It was observed that days 1, 4, and 7 presented increased, indicating critical times in the fungal-nail relationship.

Microscopic analysis revealed a fungal transition from the conidia to the hyphae form and consequently the establishment of a biofilm on the sixth day (Fig. 1). Notably, a large quantity of well-organized hyphae in F1 can be seen in a structure exhibiting ECM, consistent with images illustrating the biofilm of *Candida albicans* formed on the human nail.[22] These findings reinforce the results observed in our previous study[16] and, furthermore, they shed light on what occurs on the early days of this fungus/nail interaction.

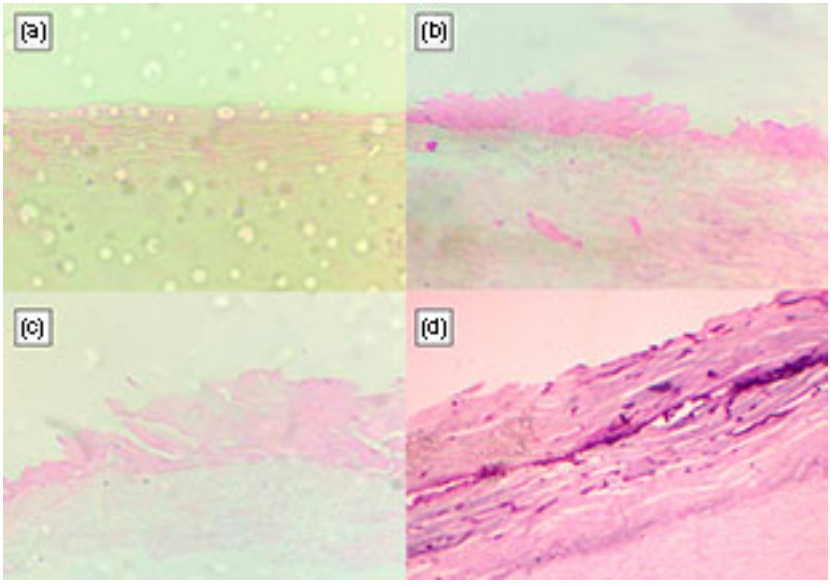

Figure 6 Light microscopy histological sections showing the invasion of *Fusarium oxysporum* in nail. The 3-μl inoculum containing 1.2 × 107 conidia/ml was added to the ventral surface and incubated 1 (b), 4 (c) and 7 (d) days at 35°C in a humid chamber, negative control (a). PAS staining, ×400 magnification

Additionally, we provided a quantification and biochemical characterization of the ECM components, by their importance into a biofilm related to onychomycosis. Among others, it protects the fungi from the host's immune response, of the antifungal drugs, and especially of the physical and chemical removal.[23] Regarding nucleic acids (eDNA and eRNA), both increased on day 4, and the increase was progressive until the end of the evaluated period. However, from day 6 to day 7, there was an important differentiation between them. While eDNA decreased, there was a significant increase in eRNA, which was proportionally related to the level of proteins expressed (Fig. 2b). This fact is possibly associated with the signaling to infect the nail tissue itself, since protein synthesis is directly dependent on RNA. On the other hand, there was a decrease in eDNA from day 6, probably associated with a biofilm autolysis process, as described for *Aspergillus fumigatus*.[24] The drop in eDNA may also be associated with DNAse production, since this enzyme provides mechanisms for regulating effective strategies for the management of infections from biofilm.[25]

The polysaccharides decreased on the seventh day when there was an increase in protein levels (Fig. 2b). Biochemical profile commonly found in mature biofilm.[26,27]

The amount of CFU from biofilm increasing over time suggests that *F. oxysporum* was well adapted, as it has the capacity to nourish, grow, and multiply on the human nail. Between the third and fifth days, there was a plateau in the CFU growth (Fig. 3a), despite the metabolic activity significantly increased on the fourth day (Fig. 3b). Altogether, these data reflect the possible changes and adjustments in cell composition and fungal metabolism, which are necessary for adaptation and survival solely from the available components of the nail. This phase showed a temporary imbalance until a dynamic stable process was re-established. It has been reported that to guarantee homeostasis, the cells permit the synthesis and degradation of several molecules important for specialized functions in this interaction system.[28,29] Thus, the increase in the number of hyphae, as shown in Figure 4b, justifies this intense metabolic activity of the fungus observed on the fourth day. The process used by fungus to form hyphae, without the addition of external nutrients, involves metabolic reactions with decreasing molecules, such as NADH, which seems to have been reflected in the XTT analysis (Fig. 3b) showing again that the fourth day is fundamentally important in *Fusarium*-nail interaction.

It is characteristic of heterotrophs to obtain carbon and nitrogen from their own microenvironment in the form of complex organic molecules, which are vital for the formation of compounds necessary for the organism to be fully functional. The ability of *F. oxysporum* to use the nail as the only source of nutrients has already been reported.[16,17] However, this is the first time that the kinetics of this process is described. Moreover, in a physical spectra determined by FTIR-ATR, we determined the organic compounds involved. Protein presence was revealed by an Amide I peak (Fig. 5, peak 2), suggesting that catabolism initially occurred, based on the decrease in protein concentration (from the first to the fourth day), followed by a rebound to previous levels (from the fourth to the seventh day). Protein reduction probably occurred because the resultant components were initially used for nutrition and later would have been important in other functions, such as the production of the ECM and invasion of the nail. Furthermore, it is possible to see that amino acid production increased over the study period, peaks 10–12. Thus, by aggregating the ECM formation, the morphological consolidation and with the peaks from the FTIR-ATR spectra, it is possible to infer that increased protein production is crucial in the *Fusarium*-nail interaction. In fact, for

dermatophyte, increased production of proteases has been reported, which are used to degrade keratin and thus facilitate the invasion of the nail.[30,31] This is not yet proven in *Fusarium* spp., but the advance in proteins observed on the seventh day deserves to be further investigated, since it is possibly related to the capacity of invasion of the fungus in the nail. This time coincides with the infection of the deeper layers of the nail tissue by *F. oxysporum*[16] and has been demonstrated in other fungi; this process requires the action of kinases.[31]

Despite the total carbohydrates rates remaining stable until the fourth day, we observed that the amount of simple sugars decreases (Fig. 2b) while glycogen increases (Fig. 5, peak 8). The consumption of electron carriers (NADH) required for this reaction was also demonstrated on day 7 (Fig. 5, peak 3). These reports are compatible with biofilm formation, which was well consolidated on the seventh day. Since carbohydrates are necessary for the construction of several biomolecules and important for the increase of biomass during the biofilm development phase.[28,29,32] Those authors have also shown the variance in metabolic activity according to the stage of biofilm development, that, in the present study, is supported by the results of XTT (Fig. 3b). This would explain differences in the planktonic cell, such as the amount of ergosterol, when it is organized as a biofilm.[33–35]

In relation to lipids, peaks 1, 5, 6, and 7 (Fig. 5) suggested that this component increased over time, in agreement with the literature.[33] Presumably this fact could be related to the adaptation of the fungus to the nail, such as adhesion to its surface and interaction through plasma membrane.[29,33] Besides, it is important to emphasize the importance of lipids in the production of ECM, since it represents 15% of its biofilm composition.[29,36]

The present data orchestrate and reinforce the idea that has been constructed regarding *F. oxysporum* being able to develop heterotrophically on the nail, forming a biofilm. Moreover, the very prominent presence of the biofilm in the nail on the seventh day, where it was possible to discern the presence of biofilm with ECM (Fig. 4c), reinforced the results previously reported.[16] The histopathology analysis surprisingly revealed that until day 4 (Fig. 6b), the fungus had not yet invaded the nail, possibly because it was still adapting to the environment on the surface of the nail tissue. Subsequently, the fungus developed a biofilm and ECM[37] and invaded the layers of nails. Evident nail invasion can be observed just on the seventh day, coincidentally when a large CFU number was observed. Thus, our results substantiate and concretize the theory that onychomycosis is attributed to the organization of fungi (*F. oxysporum*) associated with the biofilms form.

The biological characteristics of the human nail surface make it difficult to treat the microorganisms invasion.[13] Thus, the fungus strategy would be to adhere to the human nail, forming a biofilm facilitating the invasion. This situation is compatible with the infection and invasion of *F. oxysporum* in a clinical fusarial onychomycosis.[16,17,38] It has been accepted that onychomycosis is the result of a well-structured fungal biofilm, which justifies the difficulty of treatment, chronicity, recurrence, and progression of infection.[22,38] This can be explained by the biofilm giving a greater advantage of survival protection and nutrition due to the presence of ECM. Another advantage of the biofilm is the dispersion of daughter cells more virulent than the planktonic counterparts that had the initial contact with the surface.[23,26]

Conclusion

In the present study, we formally established the kinetics of biofilm formation during *F. oxysporum* growth on the healthy human nails, which was the single nutritional source. Our findings show that this fungus forms a well-established structure on the surface of the nail, which subsequently transforms into a biofilm. These are important steps to the nail tissue invasion and consequently a nail infection development. Thus, we can hypothesize that the fusarial nail infection only occurs after the establishment of a mature biofilm on the surface of the nail.

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