Biofilms of *Candida* spp. from the ocular conjunctiva of horses with reduced azole susceptibility: a complicating factor for the treatment of keratomycosis?

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Abstract

Objectives This study aimed to assess the biofilm-forming ability of *Candida* spp. from the ocular conjunctiva of horses and to investigate the antifungal susceptibility of these biofilms.

Procedures Initially, the biofilm-forming ability of 15 strains was assessed by crystal violet staining, which reveals the fungal biomass adhered to the polystyrene plates, and scanning electron microscopy. Then, the minimum inhibitory concentrations (MICs) of amphotericin B, fluconazole, itraconazole, and caspofungin were initially determined against strains in planktonic form. Afterward, antifungal susceptibility of mature biofilms was evaluated by exposing them to $10 \times \text{MIC}$ and $50 \times \text{MIC}$ of the tested drugs, followed by the assessment of their metabolic activity, using the oxidoreduction indicator XTT. Results were analyzed through ANOVA and Tukey's post-test, and *P*-values below 5% led to significant conclusions.

Results Eight strains produced biofilms and were classified as strong (1/15), moderate (3/15) and weak (4/15) producers, according to the amount of crystal violet retained by the adhered fungal biomass. Biofilm metabolic activity of one *C. tropicalis* did not decrease after exposure to the tested antifungals, while biofilm metabolic activity of five strains was reduced by amphotericin B, but not the other drugs. One *C. parapsilosis sensu stricto* and one *C. glabrata* showed significant reduction in biofilm metabolic activity after exposure to fluconazole, itraconazole, and caspofungin, but not amphotericin B.

Conclusions The results demonstrate that *Candida* from the ocular conjunctiva of horses can pose as a risk to animal health as they are capable of forming biofilms, which are commonly involved in fungal keratitis.

Key Words: antifungal susceptibility, biofilm, *Candida* spp, horses, microbiota, yeasts

INTRODUCTION

Fungi are normally present in places where horses inhabit and are part of their corneal and conjunctival microbiota.¹ The conjunctival microbiota of horses is variable, depending on the animal's age, season of the year, geographic location, and presence of bacteria and fungi, including *Candida* spp., in the environment where animals are raised.

The prevalence of these yeasts varies from 3 to 20%^{2–4} and they can act as ocular pathogens, when there are adequate conditions in the host.^{1,2,5,6} It is known that the presence of fungi in the conjunctival microbiota, including *Candida* spp., is a risk factor for the development of keratomycosis.^{1,4} Moreover, *Candida* spp. have been reported as the third most common etiological agent of equine keratomycosis.⁷

The pathogenicity of *Candida* species is mediated by several virulence factors, including the ability to evade host defenses, production of hydrolytic enzymes, and the ability to adhere to the host tissues, especially mucous membranes, and medical devices, such as central venous catheters or prostheses, where these yeasts can form biofilms.⁸ Biofilms are defined as well-structured microbial communities that are adhered to a surface, and embedded in an extracellular polymeric matrix.⁹ The advantages of living within a biofilm include protection against the environment, resistance to physical and chemical stresses, metabolic cooperation, and joint regulation of gene expression for the associated microorganisms.¹⁰ It is important to highlight that biofilm growth is often involved in the development of *Candida* keratitis,¹¹ and these biofilms are commonly resistant to antifungal agents, thus, becoming inherently difficult to treat.¹²

Therefore, the aim of this study was to assess the biofilm-forming ability of *Candida* spp. from the ocular conjunctiva of horses and to investigate the antifungal susceptibility of these biofilms.

MATERIALS AND METHODS

Microorganisms

Fifteen strains of *Candida* (four *C. tropicalis*, three *C. parapsilosis sensu stricto*, two *C. glabrata*, one *C. rugosa*, one *C. famata*, one *C. ciferrii*, one *C. guilliermondii*, one *C. catenulata*, and one *C. krusei*) obtained from the ocular conjunctiva of clinically healthy horses from the state of Ceará, Northeastern Brazil, were used in this study. The strains belong to the culture collection of the Specialized Medical Mycology Center (CEMM) of the Federal University of Ceará, where they are stored in saline solution at 4 °C. The strains were recovered by seeding onto potato agar. Then, they were subcultured on a chromogenic medium to assure the purity of the stocks, followed by phenotypical identification based on morphological and biochemical features. This study was approved by the ethics committee of the State University of Ceará (UECE), protocol number 12776861-0.

Biofilm formation

The 15 studied strains were analyzed for their ability to form biofilm, through the method described by Chatzimoschou *et al.*,¹³ with some modifications. Initially, the strains were grown on Sabouraud agar for 48 h at 30 °C. Then, they were transferred to Sabouraud broth and incubated under agitation (150 rpm) for 24 h. After this period, the tubes containing the isolates were centrifuged (300 rpm, 10 min) and the supernatant was discarded. The resulting pellets were washed with sterile PBS, twice, followed by another centrifugation step. Then, the fungal pellets were resuspended in RPMI medium and the inoculum turbidity was adjusted to 0.5 on McFarland scale, which contains approximately 1 to 5×10^6 cfu/mL. Then,

100-μL aliquots of each *Candida* inoculum were incubated in the wells of a 96-well flat-bottom polystyrene plate, in triplicate, and kept under agitation for 48 h (150 rpm) at 37 °C. Yeast-free negative control wells were included in the analysis, which contained only RPMI medium. *C. albicans* ATCC 10231 is a well-known biofilm producer and was used as positive control for biofilm production. After incubation, the supernatant from the wells was carefully aspirated and washed three times with PBS-Tween. The wells were washed with 100 μL of 100% methanol. After drying, 100 μL of a 0.3% crystal violet solution was added to each well and incubated for 20 min. Afterward, crystal violet was removed and the wells were washed twice with 200 μL of sterile-distilled water. Then, 150 μL of a 33% acetic acid solution was added to each stained well and allowed to act for 30 s. Immediately after this period, the volume from each well was transferred to the well of another 96-well flat-bottom polystyrene plate and read with a spectrophotometer at 590 nm. Crystal violet dyes microbial structures and extracellular polymeric substances adhered to the 96-well polystyrene plates, and it is used to quantify biofilm biomass. Higher absorbance values at 590 nm indicate greater crystal violet retention, hence, thicker and denser biofilm biomass.¹⁴

The cut-off value (ODc) for biofilm formation was defined as three standard deviations above the mean absorbance of the yeast-free negative control.¹⁴ The strains were, then, classified as nonbiofilm producers, when $OD_{590\text{ nm}} \leq ODc$, weak biofilm producers, when $ODc < OD_{590\text{ nm}} \leq 2 \times ODc$, moderate biofilm producers, when $2 \times ODc < OD_{590\text{ nm}} \leq 4 \times ODc$, or strong biofilm producers, when $OD_{590\text{ nm}} > 4 \times ODc$.¹⁴ The ODc value obtained in these analyses was 0.477.

Scanning electron microscopic study of *Candida* spp. biofilms

The biofilms were analyzed by scanning electron microscopy (SEM) according to Wang *et al.*,¹⁵ with modifications. Biofilms were formed directly on sterile glass slides covered with poly-L-lysine, using the method described above. After incubation, biofilm supernatants were removed and the slides were washed twice with cacodylate buffer (0.15 M). Biofilms were then covered with glutaraldehyde (2.5% in cacodylate buffer 0.15 M) and incubated at 4 °C overnight. After incubation, the biofilms were washed twice with cacodylate buffer for 5 min and the slides were dehydrated in an ascending ethanol series (50, 70, 80, 95 and 100%) twice, for 10 min each time. The slides were dried at room temperature and covered with hexamethyldisilazane (HMDS) during 30 min, after which the HMDS was removed and the slides were dried overnight, coated with 10 nm of gold (Emitech Q150T), and observed with an FEI Inspect S50 scanning electron microscope, in high vacuum mode at 15 kV. The images were processed with the Photoshop software (Adobe Systems, San José, Calif.).

Determination of the antifungal minimum inhibitory concentration against planktonic *Candida* spp. cells

The minimum inhibitory concentration (MIC) for the antifungal drugs against the strains of *Candida* in free-flowing planktonic growth was determined by the broth microdilution method, as standardized by the Clinical and Laboratory Standards Institute.¹⁶ Amphotericin B (Sigma, USA), itraconazole (Janssen Pharmaceutica, Belgium), fluconazole (Pfizer, Brazil) and caspofungin (Sigma, USA) were prepared according to CLSI guidelines.¹⁶ Amphotericin B and itraconazole were initially diluted with DMSO, while fluconazole and caspofungin were diluted with water.¹⁶ Even though amphotericin B and caspofungin are not commonly used to treat fungal infections in animals, they were tested as representatives of the polyene and echinocandin class of antifungal drugs, respectively. Additionally, fluconazole and itraconazole were included as representatives of the azole antifungals because they are the most commonly used antifungal drugs in veterinary practice. The tested concentration ranges were 0.125–64 µg/mL for fluconazole and 0.03125–16 µg/mL for amphotericin B, itraconazole, and caspofungin. Each test was performed in duplicate. The plates were incubated at 35 °C for 48 h. Yeast growth was visually evaluated, and the MICs for the azole derivatives and caspofungin were determined as the lowest concentration able of reducing 50% of yeast growth, when compared to drug-free growth control, while the MICs for amphotericin B were determined as the lowest concentration capable of inhibiting 100% of fungal growth. MIC values ≥ 64 and ≥ 1 µg/mL indicated *in vitro* resistance to fluconazole and itraconazole, respectively. For the species *C. parapsilosis* and *C. tropicalis*, MICs ≥ 8 µg/mL for fluconazole indicated resistance (CLSI, 2012). For caspofungin, MIC values ≥ 0.5 µg/mL against *C. glabrata*, ≥ 1 µg/mL against *C. tropicalis*, and *C. krusei* and ≥ 8 µg/mL against *C. parapsilosis* and *C. guilliermondii* indicated resistance.¹⁷ For the other *Candida* species, caspofungin MIC values >2 µg/mL indicated that the strain is not susceptible to this drug.¹⁶

Antifungal susceptibility of mature *Candida* biofilms

The method described by Ramage *et al.*,¹⁸ with modifications, was used to assess the inhibitory effect of the antifungal drugs on the biofilms formed by the tested strains. Biofilms were produced as described above. Afterward, the medium was aspirated and the nonadhered cells were removed by three washes with sterile PBS. The residual PBS was completely removed and the plate was dried with paper towel, before adding 100 µL of the drugs amphotericin B, fluconazole, itraconazole, and caspofungin, at two concentrations, 10xMIC and 50xMIC, based on the MICs obtained against each strain in planktonic form. After the addition of 100 µL of each drug, the plates were further incubated at 35 °C, for 48 h.

The viability of the mature biofilms was assessed by the XTT assay, according to Sidrim *et al.*¹⁹ XTT is an oxidoreduction indicator that is reduced by mitochondrial dehydrogenases of metabolically active cells to the water-soluble formazan product. This product is spectrophotometrically measured, thus, allowing the quantification of cell metabolism and biofilm metabolic activity. This is essential for biofilm susceptibility assays, as the viability of these fungal structures cannot be visually assessed, as described for the antifungal susceptibility assays of planktonic fungal growth.¹⁸ The XTT stock solutions (1 mg/mL) were previously prepared, filtered, and stored at –20 °C until use. Menadione (Sigma) (0.4 mM in acetone), prepared at the moment of use, was employed as an electron-coupling agent. Aliquots of 50 µL of sterile PBS, 75 µL of XTT solution, and 6 µL of menadione solution were added to each of the wells containing biofilms. The plates were incubated at 35 °C for 5 h in the dark. After this period, XTT solution was transferred to a new flat-bottom 96-well plate and spectrophotometrically read, at 492 nm. All tests were run in triplicate, and drug-free growth control and yeast-free sterility control were included.

Statistical methods

Regarding the biofilm assay, all tests were performed in triplicate and the results of the absorbance of the XTT assay were evaluated by ANOVA and Tukey's multiple comparisons post-test. *P*-values <0.05 were considered to be statistically significant.

RESULTS

Biofilm formation was evaluated for all 15 strains, of which eight (3/4 *C. tropicalis*; 1/3 *C. parapsilosis sensu stricto*; 2/2 *C. glabrata*; 1/1 *C. guilliermondii* and 1/1 *C. catenulata*) were able to form biofilms (Table 1). One strain was classified as a strong biofilm producer ($OD_{590\text{ nm}} > 1.908$, Fig. 1a), three as moderate producers ($0.954 < OD_{590\text{ nm}} \leq 1.908$, Fig. 1b), and four as weak producers ($0.477 < OD_{590\text{ nm}} \leq 0.954$, Fig. 1c), as reported in Table 1. As shown in the SEM analysis, *C. tropicalis* and *C. guilliermondii* biofilms are formed by layers of blastoconidia and pseudohyphae, and *C. glabrata* biofilms are formed by layers of blastoconidia only (Fig. 1).

Regarding antifungal susceptibility, the antifungal MICs against *Candida* strains in planktonic form were visually determined, through broth microdilution. The obtained MICs varied from 0.03125 to 1 µg/mL for amphotericin B, from 0.03125 to >16 µg/mL for itraconazole, from 0.25 to >64 µg/mL for fluconazole, and from 0.03125 to 1 µg/mL for caspofungin (Table 2). Two strains of *C. tropicalis* and one of *C. catenulata* were resistant to itraconazole (MIC ≥ 1 µg/mL), while only one strain of *C. parapsilosis sensu stricto* was resistant to fluconazole (MIC ≥ 8 µg/mL). One strain of *C. glabrata* was resistant

Table 1. Biofilm production of *Candida* species recovered from the ocular conjunctiva of horses and antifungal susceptibility of the strains in planktonic form

Strains	<i>Candida</i> species	Biofilm production	Minimum inhibitory concentration ($\mu\text{g/mL}$)			
			Amphotericin B	Fluconazole	Itraconazole	Caspofungin
01	<i>C. tropicalis</i>	Absent	1	1	0.25	0.03125
02	<i>C. tropicalis</i>	Moderate	1	1	1*	0.03125
03	<i>C. tropicalis</i>	Strong	1	0.5	4*	0.0625
04	<i>C. tropicalis</i>	Moderate	1	1	0.5	0.125
05	<i>C. parapsilosis</i>	Absent	0.25	16*	0.0625	0.03125
06	<i>C. parapsilosis</i>	Weak	0.25	0.5	0.03125	1
07	<i>C. parapsilosis</i>	Absent	0.03125	2	0.03125	0.03125
08	<i>C. glabrata</i>	Weak	1	64*	16*	0.03125
09	<i>C. glabrata</i>	Moderate	1	2	1*	0.5*
10	<i>C. rugosa</i>	Absent	0.5	4	0.5	0.0625
11	<i>C. famata</i>	Absent	1	4	0.25	0.0625
12	<i>C. ciferrii</i>	Absent	0.5	2	0.0625	0.0625
13	<i>C. guilliermondii</i>	Weak	1	0.25	0.0625	0.03125
14	<i>C. catenulata</i>	Weak	0.5	4	1*	0.25
15	<i>C. krusei</i>	Absent	1	0.25	0.03125	1*

Antifungal minimum inhibitory concentrations against planktonic *Candida* spp. were determined through broth microdilution (CLSI, document M27-A3). *Antifungal resistance (fluconazole: $\geq 8 \mu\text{g/mL}$ against *C. parapsilosis* and *C. tropicalis*, $\geq 64 \mu\text{g/mL}$ against *C. glabrata*; itraconazole: $\geq 1 \mu\text{g/mL}$; caspofungin: $\geq 8 \mu\text{g/mL}$ against *C. parapsilosis* and *C. guilliermondii*, $\geq 1 \mu\text{g/mL}$ against *C. tropicalis* and *C. krusei*, $\geq 0.5 \mu\text{g/mL}$ against *C. glabrata*).

Figure 1. Mature biofilms of *Candida* spp. (a) *C. tropicalis* with strong biofilm production ($\text{OD}_{590 \text{ nm}} > 1.908$). Notice the multilayer arrangement of blastoconidia and pseudohyphae; (b) *C. glabrata* with moderate biofilm production ($0.954 < \text{OD}_{590 \text{ nm}} \leq 1.908$). Notice the clusters of small round blastoconidia, in a monolayer arrangement, and the absence of pseudohyphae, with empty areas on the coverslide; (c) *C. guilliermondii* with weak biofilm production ($0.477 < \text{OD}_{590 \text{ nm}} \leq 0.954$). Notice clusters of blastoconidia and pseudohyphae, in a monolayer arrangement, with empty areas on the coverslide.

to both fluconazole and itraconazole, with MICs $\geq 64 \mu\text{g/mL}$ and $1 \mu\text{g/mL}$, respectively, and another one was simultaneously resistant to itraconazole (MIC = $1 \mu\text{g/mL}$) and caspofungin (MIC = $0.5 \mu\text{g/mL}$). Resistance to caspofungin was also observed in one strain of *C. krusei* (MIC = $1 \mu\text{g/mL}$). The antifungal susceptibility results are shown in Table 1.

In order to assess biofilm antifungal susceptibility, the eight biofilm-producing strains were used, and mature biofilms were exposed to two antifungal concentrations, 10xMIC and 50xMIC, based on the MICs obtained against each strain in planktonic form. Considering that the effect of antifungal drugs on biofilms cannot be

visually read, as described for broth microdilution, the biofilm metabolic activity, after exposure to antifungals, was measured through XTT assay, in order to assess biofilm viability. Thus, the biofilm metabolic activity of one strain of *C. tropicalis* did not reduce after exposure to any of the tested antifungal drugs. Five strains (two *C. tropicalis*, one *C. glabrata*, one *C. guilliermondii*, and one *C. catenulata*) presented significant reduction in biofilm metabolic activity, when compared to the growth control ($P < 0.05$), only after exposure to amphotericin B at 50xMIC. The other tested drugs (azoles and caspofungin) presented no inhibitory effects on the biofilm of these strains. Finally, two strains (one *C. parapsilosis sensu stricto*

Table 2. Effect of antifungal drugs on the metabolic activity of mature biofilms of *Candida* spp. from the ocular conjunctiva of horses

Strains	Candida Species	Growth Control	Amphotericin B			Fluconazole			Itraconazole			Caspofungin		
			10 × MIC	50 × MIC	50 × MIC	10 × MIC	50 × MIC	50 × MIC	10 × MIC	50 × MIC	50 × MIC	10 × MIC	50 × MIC	50 × MIC
2	<i>C. tropicalis</i>	1.1 ± 0.019	1.1 ± 0.035	0.94 ± 0.054	0.99 ± 0.025	1.1 ± 0.033	0.99 ± 0.025	1.1 ± 0.015	1.0 ± 0.015	1.1 ± 0.018	0.99 ± 0.067	1.0 ± 0.062	1.0 ± 0.062	1.0 ± 0.062
3	<i>C. tropicalis</i>	1.3 ± 0.013	0.99 ± 0.067	0.68 ± 0.41*	1.2 ± 0.024	1.2 ± 0.054	1.2 ± 0.024	1.1 ± 0.017	1.1 ± 0.017	1.1 ± 0.041	1.2 ± 0.036	1.1 ± 0.053	1.1 ± 0.053	1.1 ± 0.053
4	<i>C. tropicalis</i>	1.1 ± 0.011	1.1 ± 0.12	0.88 ± 0.046*	1.1 ± 0.028	1.2 ± 0.0015	1.1 ± 0.028	1.2 ± 0.028	1.2 ± 0.028	1.1 ± 0.048	1.0 ± 0.028	1.0 ± 0.06	1.0 ± 0.06	1.0 ± 0.06
6	<i>C. parapsilosis</i>	0.89 ± 0.19	0.97 ± 0.063	0.95 ± 0.024	0.31 ± 0.008*	0.4 ± 0.064*	0.31 ± 0.008*	0.63 ± 0.098*	0.63 ± 0.098*	0.49 ± 0.017*	0.21 ± 0.023*	0.43 ± 0.036*	0.43 ± 0.036*	0.43 ± 0.036*
8	<i>C. glabrata</i>	1.1 ± 0.059	0.97 ± 0.082	0.85 ± 0.015	0.19 ± 0.028*	0.34 ± 0.13*	0.19 ± 0.028*	1.1 ± 0.023	1.1 ± 0.023	0.75 ± 0.15*	0.73 ± 0.16*	0.57 ± 0.05*	0.57 ± 0.05*	0.57 ± 0.05*
9	<i>C. glabrata</i>	1.1 ± 0.051	1.1 ± 0.039	0.14 ± 0.058*	1.0 ± 0.23	1.2 ± 0.047	1.0 ± 0.23	1.1 ± 0.033	1.1 ± 0.033	1.2 ± 0.022	0.96 ± 0.17	1.1 ± 0.013	1.1 ± 0.013	1.1 ± 0.013
13	<i>C. guilliermondii</i>	1.2 ± 0.002	1.1 ± 0.058	0.97 ± 0.11*	1.1 ± 0.035	1.2 ± 0.051	1.1 ± 0.035	1.1 ± 0.033	1.1 ± 0.033	1.2 ± 0.019	1.2 ± 0.051	1.1 ± 0.027	1.1 ± 0.027	1.1 ± 0.027
14	<i>C. catenulata</i>	1.1 ± 0.007	1.0 ± 0.2	0.82 ± 0.066*	1.1 ± 0.026	1.1 ± 0.015	1.1 ± 0.026	1.1 ± 0.018	1.1 ± 0.018	1.1 ± 0.017	1.2 ± 0.013	1.1 ± 0.029	1.1 ± 0.029	1.1 ± 0.029

Results are shown as the mean ± standard deviation of the absorbance values obtained through XTT metabolic assay at a wavelength of 492 nm. Absorbance values are directly correlated to biofilm metabolic activity. *Statistically significant difference when compared to the values obtained for drug-free growth control.

and one *C. glabrata*) did not show reduction in biofilm metabolic activity after exposure to amphotericin B at either of the tested concentrations. However, biofilm metabolic activity of the *C. parapsilosis sensu stricto* strain significantly declined ($P < 0.05$) in the presence of fluconazole, itraconazole, and caspofungin, at both tested concentrations. As for the *C. glabrata* strain, a significant reduction in biofilm metabolic activity was observed at both tested concentrations of fluconazole ($P < 0.05$) and caspofungin ($P < 0.05$), and only at 50xMIC of itraconazole ($P < 0.05$) (Table 2).

DISCUSSION

The tested species of the *Candida* genus have been described as components of the microbiota of several animal species^{3,20–25} and pathogenic agents causing keratitis in horses.^{4–7} Studies on the conjunctival microbiota of healthy horses are scarce, but they are important because the fungal composition may be influenced by geographic region.²⁶ Some authors have reported the isolation of *Candida* spp., but the species were not determined.^{2,3,27} The strains used in this research were recovered from healthy horses, providing data on the yeast composition of the conjunctival microbiota.

Fungal infections are a common cause of corneal diseases in horses, accounting for up to one-third of all inflammatory keratopathies in this animal species.²⁸ Keratomycosis is a sight-threatening, frequently painful condition that can result in vision or eye globe loss, often requiring an aggressive and long-lasting treatment.^{4,26,29} The high incidence of corneal infections in horses is mainly associated with the large size of the eye globe with large corneal surface area; the prominent lateral position of the eye; low resting temperature of the cornea; and the presence of fungi in the conjunctival microbiota and in the environment where the animals are kept.^{1,4} Keratomycosis is a common infection in equines because the tear film of these animals has weak immunoprotective properties.^{4,27}

In addition to these anatomical and physiological predisposing features of the equine eye, some specific conditions also contribute for the development of keratomycosis in these animals. For instance, corneal epithelial injuries or alterations of the tear film allow the entrance of opportunistic fungi from the conjunctival microbiota or the environment into the corneal tissue.^{4,28} These fungi may colonize the cornea, penetrate its stroma, and migrate between its collagen layers.³⁰ Fungal invasion triggers an inflammatory response and protease overproduction, leading to corneal stromal defects, which may progress to corneal perforation.²⁸ The most common fungi associated with cases of equine keratomycosis are *Aspergillus* spp., *Fusarium* spp., and *Candida* spp.^{7,29}

The pathogenicity of *Candida* species is mediated by several virulence factors, including biofilm formation on

biotic and abiotic surfaces,⁸ which is associated with the development of antimicrobial resistance.¹² In veterinary medicine, the presence of microbial biofilms has also been associated with antimicrobial resistance and clinical relapses. The importance of biofilm infections in animals still needs to be addressed, but evidences show that several bacterial infections are associated with biofilm growth, including pneumonia, liver abscesses, enteritis, otitis, mastitis, and wound infections,⁹ especially in horses.³¹ On the other hand, little is known on the biofilm-forming ability of fungal pathogens from animals and its association with the development of diseases. For instance, it has been shown that veterinary strains of *Malassezia pachydermatis*,³² *C. parapsilosis sensu lato*,³³ and *C. tropicalis*³⁴ are capable of producing biofilms *in vitro*.

In our study, 53.3% of the strains from the ocular conjunctiva of horses showed the ability to form biofilms, as evidenced by the crystal violet technique, which quantifies microbial biofilm-forming ability through the amount of dye retained by the yeast cells and extracellular polymeric substances adhered to the walls of a polystyrene plate.^{13,14} Two *C. tropicalis* strains were characterized as moderate producers and one as a strong producer. Some studies have shown that *C. tropicalis* has a remarkable biofilm-forming ability,^{8,34} and Cordeiro *et al.*,³⁴ for instance, have shown that *C. tropicalis* isolated from animals has a noteworthy *in vitro* biofilm production. Additionally, two strains of *C. glabrata* were able to produce biofilm, and were classified as weak and moderate producers, corroborating the findings of Silva *et al.*,⁸ who reported a weaker biofilm-forming ability for this species.

Several studies have reported that biofilms formed by *Candida* spp. are resistant to antifungal drugs commonly used in clinical practice, especially azole derivatives.^{35,36} Our results corroborate these observations, once *Candida* biofilms were able to tolerate higher concentrations of antifungal drugs, when compared to their planktonic counterparts, as demonstrated by the XTT assay, which detects biofilm metabolic activity, hence, allowing the assessment of biofilm viability and its susceptibility to antifungal drugs.^{18,19} In the present study, only one strain of *C. parapsilosis sensu stricto* and one *C. glabrata* were inhibited by itraconazole and fluconazole, while the other tested strains tolerated both azole drugs, even at 50xMIC. Similarly, Brillhante *et al.*³⁷ demonstrated that *C. tropicalis* biofilms are not affected by very high concentrations of azole drugs. Some hypotheses have been proposed to explain the reduced susceptibility of *Candida* biofilms to azole drugs, including the reduced metabolism of biofilm cells, decreased drug penetration through the biofilm matrix, and increased expression of specific genes by biofilm cells, especially those that encode efflux pumps.^{38,39}

Regarding the inhibitory activity of amphotericin B, five of the eight tested biofilm-producing strains presented a reduction in biofilm metabolic activity, after exposure to this drug, corroborating the findings of Brucker *et al.*⁴⁰

and Brillhante *et al.*,³⁷ who reported that amphotericin B has a consistent activity against biofilms of *Candida* spp. Unlike azole derivatives, whose inhibitory activity depends on the metabolism of fungal cells, more specifically on ergosterol synthesis, amphotericin B exerts its activity through hydrophobic interactions with preformed ergosterol molecules on cell membrane, disrupting membrane function.⁴¹ Therefore, amphotericin B does not need metabolically active cells to exert its inhibitory effect, which may explain its better activity against *Candida* biofilms, when compared to azole drugs.

Caspofungin plays an important role in the treatment of infections caused by different azole-resistant fungi.⁴² Moreover, it has been described to have a remarkable activity against *C. albicans*³⁶ and *C. tropicalis*³⁷ biofilms. However, in our study, this drug only significantly reduced the biofilm metabolic activity of one *C. parapsilosis sensu stricto* and one *C. glabrata*.

Our results demonstrate that the isolates from the conjunctival microbiota of horses were able to produce biofilms, which presented reduced susceptibility to the four tested antifungal drugs. This fact highlights the potential involvement of *Candida* spp. from the conjunctival microbiota of horses in keratomycoses, as these infections are often associated with biofilm growth on and within corneal tissue.¹¹ Therefore, even though the tested strains were recovered from healthy horses, it is important to highlight that *Candida* species are opportunistic pathogens that may become pathogenic, under certain circumstances,^{4,28} emphasizing the importance of assessing their biofilm-producing ability and antifungal susceptibility. Finally, further studies comparing *Candida* spp. from healthy and diseased horses with keratomycosis may be beneficial to elucidate other pathophysiological aspects of *Candida* corneal infections.

In summary, the data from this study show that the *Candida* species isolated from the ocular conjunctiva of horses can pose a risk to animals' health, as they are capable of forming biofilms, which are commonly involved in fungal keratitis.

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CONFLICT OF INTEREST

None.

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