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Mixed biofilms formed by *C. albicans* and non-*albicans* species: a study of microbial interactions

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Abstract: Most *Candida* infections are related to microbial biofilms often formed by the association of different species. The objective of this study was to evaluate the interactions between *Candida albicans* and non-*albicans* species in biofilms formed *in vitro*. The non-*albicans* species studied were: *Candida tropicalis*, *Candida glabrata* and *Candida krusei*. Single and mixed biofilms (formed by clinical isolates of *C. albicans* and non-*albicans* species) were developed from standardized suspensions of each strain (10^7 cells/mL), on flat-bottom 96-well microtiter plates for 48 hour. These biofilms were analyzed by counting colony-forming units (CFU/mL) in *Candida* HiChrome agar and by determining cell viability, using the XTT 2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide colorimetric assay. The results for both the CFU/mL count and the XTT colorimetric assay showed that all the species studied were capable of forming high levels of *in vitro* biofilm. The number of CFU/mL and the metabolic activity of *C. albicans* were reduced in mixed biofilms with non-*albicans* species, as compared with a single *C. albicans* biofilm. Among the species tested, *C. krusei* exerted the highest inhibitory action against *C. albicans*. In conclusion, *C. albicans* established antagonistic interactions with non-*albicans* *Candida* species in mixed biofilms.

Keywords: *Candida albicans*; *Candida glabrata*; *Candida tropicalis*; Biofilms.

Introduction

Yeasts of the *Candida* genus are opportunistic pathogens frequently encountered in humans, and can be isolated from 60% of the oral cavities of healthy adults.[1] *Candida* species are responsible for the increasing frequency of infections in immunocompromised patient groups, such as HIV-infected individuals and those being treated with chemotherapy or broad-spectrum antibiotics.[2] *C. albicans* is considered the most prevalent and pathogenic species of this gender, and is responsible for most of the superficial and systemic fungal infections.[1] *Candida albicans* is the fourth leading cause of bloodstream infections, and the third most commonly isolated organism from intravascular catheters; moreover, it is associated with the highest incidence of mortality.[3]

Nevertheless, other *Candida* species have also emerged as clinically important opportunistic pathogens, such as *Candida glabrata*, *Candida tropicalis*

and *Candida krusei*.[4] For many years, *C. glabrata* was considered a relatively non-pathogenic saprophyte of the normal flora of healthy humans. However, *C. glabrata* can disseminate rapidly throughout the body, and infection caused by this species is associated with a high mortality rate.[5] *C. tropicalis* is another non-*albicans* species considered an important opportunistic pathogen. It is most frequently isolated from candidiasis, mainly in patients confined to intensive care units, and has been associated with fluconazole resistance.[6] *C. krusei* is an emerging pathogen, described as a systemic pathogen in patients with compromised host resistance, such as those with acquired immunodeficiency syndrome (AIDS).[7]

A major attribute of the *Candida* genus virulence is its ability to form surface-attached microbial communities known as biofilms.[8,9] In general, biofilm development evolves according to four sequential steps: first, adhesion of microorganisms to a surface; second, discrete colony formation and organization of cells; third, secretion of extracellular polysaccharides (EPS) and maturation into a three-dimensional structure; and, fourth, dissemination of progeny biofilm cells.[10] The extracellular matrix of the biofilm can protect the cells from phagocytosis, maintain the integrity of the biofilm, and limit the diffusion of substances.[11] It has been estimated that over 60% of microbial infections are involved with biofilm.[1] The high resistance of biofilms to antimicrobials (compared to their planktonic counterparts) is of major clinical relevance.[12]

Biofilm infections may be caused by a single microbial species or by a mixture of different species.[13] The complex structure of biofilms allows stratification into spatially organized populations of mixed-species communities.[14] Microorganisms have evolved complex mechanisms to promote their survival, defending themselves not only against adverse environmental and nutritional conditions, but also against competing organisms.[15] Polymicrobial biofilms are found in nearly every niche in the human body.[16] These mixtures of species make the therapeutic management of infectious diseases extremely difficult.[12] The heterogeneity of species within mixed biofilms has made it difficult to assess the relevance and contribution of each individual species to pathogenesis and disease.[3]

Bacteria are commonly associated with *Candida* in the polymicrobial biofilms found in medical devices.[8] Harriot and Noverr[17] studied the interactions in polymicrobial biofilms formed by *C. albicans* and *Staphylococcus aureus* in an *in vitro* model, and observed a synergistic relationship between the two species. On the other hand, Tampakakis et al.[18] analyzed an *in vivo* interaction between the species *C. albicans* and the Gram-negative *Salmonella typhimurium*. After observing that *C. albicans* filamentation was inhibited by the bacteria, they concluded that it was an antagonistic interaction.

However, most existing studies on mixed biofilm development involving *Candida albicans* have focused on bacterial-fungal infections, whereas there are only few that deal with interactions among fungi in mixed biofilms. Kirkpatrick et al.[4] and Thein et al.[19] found that *C. albicans* established a competitive interaction with *C. dubliniensis* and *C. krusei*, respectively, in mixed biofilms formed *in vitro*. However, Pereira-Cenci et al.[20] did not find competitive interactions in dual-species biofilms of *C. albicans* and *C. glabrata* grown on different dental materials. Recently, our laboratory developed an *in vitro* and *in vivo* study[21] to evaluate the interactions of *C. albicans* (ATCC 18804) with *C. krusei* (ATCC 6258) and *C. glabrata* (ATCC 9030). We demonstrated that *C. albicans* was able to establish competitive interactions with non-*albicans* species during biofilm formation and infection development processes in animal models.

Thus, the purpose of this study was to investigate the interactions between *Candida albicans* and non-*albicans* species in mixed biofilms formed *in vitro,* using clinical strains of *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. krusei*, isolated from oropharyngeal candidiasis lesions of HIV-positive patients.

Methodology

Microorganisms

This study was approved by the Human Research Ethics Committee of the Institute of Science and Technology of São José dos Campos, *Universidade Estadual Paulista* - UNESP (017/2011-PH/CEP).

Four clinical isolates were used in this study, including species of *Candida albicans, Candida glabrata, Candida tropicalis,* and *Candida krusei*. These strains were provided by the Microbiology Laboratory of

the Department of Biosciences and Oral Diagnosis (UNESP, São José dos Campos, SP, Brazil), after isolation from oropharyngeal candidiasis lesions of HIV-positive patients.[22]

Preparation of *Candida* spp. suspension

Standard suspensions containing 10^7 cells/mL were prepared for each strain. The strains were seeded on Sabouraud dextrose agar (Difco, Detroit, USA) and incubated at 37°C for 24 hour. Next, a loopful of growth was inoculated in Yeast Nitrogen Base (YNB) broth (Difco, Detroit, USA) supplemented with 100 mM glucose (Vetec, Rio de Janeiro, Brazil) and incubated at 37°C for 16 hour. The broth with the microbial growth was centrifuged at 2000 x g for 10 min (MPW Med. Instruments, Warsaw, Poland), after which the growth was washed twice with 5 mL of sterile physiological solution (PBS) (Laborclin, Pinhais, Brazil), resuspended in YNB medium supplemented with 100 mL glucose, and standardized to 10^7 cells/mL in a Neubauer counting chamber (Laboroptik GmbH, Bad Homburg, Germany).

In vitro formation of simple and mixed biofilms

The biofilm formation of *Candida* was performed as described by Seneviratne et al.,[10] with some modifications. *Candida albicans* biofilm was formed by adding 200 µL from a standardized suspension (10^7 cells/mL) to each well of flat-bottom 96-well microtiter plates (Corning Corporation, Corning, USA). The mixed biofilms (composed by *C. albicans* and non-*albicans* species) were formed by adding 100 µL aliquots of standardized suspensions of each species to each well of a flat-bottom 96-well microtiter plate. The plates were incubated for 1.5 h (adhesion phase) at 37°C at 75 rpm in an orbital shaker (Quimis, Diadema, Brazil). After the adhesion phase, the cell suspensions were aspirated, and each well was washed twice with 200 µL of phosphate buffered saline (PBS) to remove any planktonic cells. Then, 200 µL of YNB with 100 mM glucose was added to each washed well, and the plates were incubated for 48 h at 37°C at 75 rpm in an orbital shaker. The growth medium was replenished daily.

Analysis of biofilm by counting colony forming units (CFU/mL)

After formation of the biofilms, each well was washed twice with 200 µL of PBS to remove loosely adhered cells. Then, 200 µL of PBS was added to each well, and the biofilm cells were scraped off of the well using a sterile toothpick. A 100 µL aliquot was transferred to a Falcon tube (J Prolab, São José dos Pinhais, Brazil) containing 6 mL of sterile physiological solution, and an ultrasonic homogenizer (Sonoplus HD 2200, output power of 50 W) (Bandelin Electronic, Berlin, Germany) was used for 30 s to disrupt the biofilms. The homogenized solution was used for serial dilutions of the biofilm suspension, and 100 µL aliquots of each dilution were seeded on plates containing *Candida* HiChrome agar (Himedia, Mumbai, India) and incubated for 48 h at 37°C. After incubation, the CFU/mL values were determined. *Candida* species from the mixed biofilms were differentiated by the color of the colonies on *Candida* HiChrome agar: light green for *C. albicans*, blue for *C. tropicalis*, cream for *C. glabrata* and purple for *C. krusei*.

Analysis of biofilm cell viability using the XTT colorimetric assay

The biofilms formed were evaluated by a metabolic assay based on the reduction of XTT, a tetrazolium salt (Sigma–Aldrich, São Paulo, Brazil). The methodology described by Jin et al.[2] was used for the XTT assay. In brief, XTT salt was dissolved in PBS at a final concentration of 1 mg/mL. The solution was filter-sterilized using a 0.22 µm-pore-size filter (MFS, Dublin, Ireland) and stored frozen at -80°C. Immediately before each assay, a menadione (Sigma–Aldrich, São Paulo, Brazil) solution was prepared at a final concentration of 0.4 mM and filter-sterilized. The XTT solution was thawed prior to each assay, and mixed with the menadione solution at a ratio of 20:1 (v:v).

Each well was washed four times with 200 µL of PBS to remove any non-adherent cells. Next, 158 µL of PBS, 40 µL of XTT and 2 µL of menadione were added to each of the pre-washed wells. The plates were incubated in the dark at 37°C for 3 hour. Afterwards, 100 µL of the solution was transferred to a new well, and any colorimetric change in the solution was measured

using a microtiter plate reader (Tp Reader; Thermo Plate, Shenzhen, China) at 490 nm.

Statistical analysis

Ten assays were carried out per experimental group. For analysis purposes, CFU/mL values were transformed into logarithm values (log_{10}). The CFU/mL count in monotypic and heterotypic biofilms was submitted to the Student ttest for each species studied. The results obtained from the XTT assay were evaluated by analysis of variance (ANOVA) and the Tukey test. A significance level of 5% ($p < 0.05$) was adopted (GraphPad Software - Inc., La Jolla, USA).

Results

The mean and standard deviation values of the CFU/mL, obtained from the experiments described above, are shown in Figures 1, 2 and 3. All species were able to form *in vitro* biofilms, attaining 6.24 to 6.75 CFU/mL (log) in the single biofilms.

In the study of microbial interactions, *C. albicans* showed a significantly higher number of CFU/mL in the single biofilm as compared with the mixed biofilm, as regards the non-*albicans Candida* species. However, the most significant difference between the single and the mixed biofilm was observed for the interactions of *C. albicans* and *C. krusei* ($p < 0.0001$). In the single biofilm, *C. albicans* grew to 6.24 log (CFU/mL), but the result was 0.40 log when it was grown in the mixed biofilm with *C. krusei*. These data indicate that the presence of *C. krusei* in the biofilm inhibited the growth of *C. albicans*.

The results from the cell viability XTT colorimetric assay are shown in Figure 4. The metabolic activity observed in the single biofilms formed by *C. albicans*,

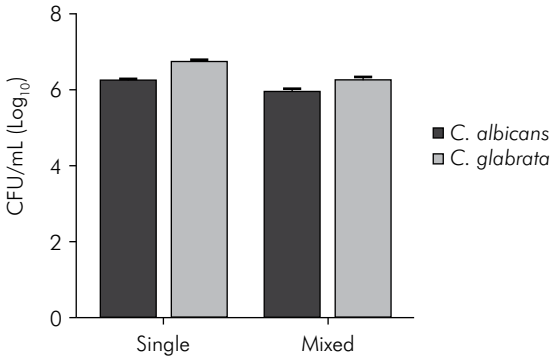

Figure 2. Mean values and standard deviation (n = 10) of CFU/mL (log_{10}) of *C. albicans* and *C. glabrata*, organized in single and mixed biofilms. Student t-test. *C. albicans* counts: statistically significant difference between biofilm formed by *C. albicans* and biofilm formed by *C. albicans* + *C. glabrata* ($p < 0.0001$). *C. glabrata* counts: statistically significant difference between biofilm formed by *C. glabrata* and biofilm formed by *C. albicans* + *C. glabrata* ($p < 0.0001$).

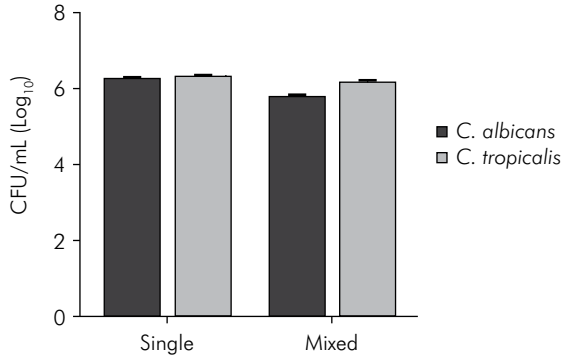

Figure 1. Mean values and standard deviation (n = 10) of CFU/mL (log) of *C. albicans* and *C. tropicalis*, organized in single and mixed biofilms. Student t-test. *C. albicans* counts: statistically significant difference between biofilm formed by *C. albicans* and biofilm formed by *C. albicans* + *C. tropicalis* ($p < 0.0001$). *C. tropicalis* counts: statistically significant difference between biofilm formed by *C. tropicalis* and biofilm formed by *C. albicans* + *C. tropicalis* ($p = 0.0023$).

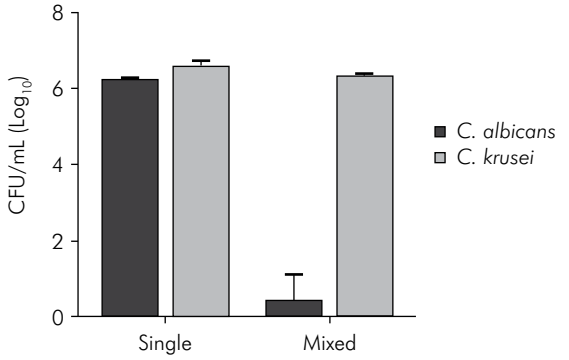

Figure 3. Mean values and standard deviation (n = 10) of CFU/mL (log_{10}) of *C. albicans* and *C. krusei*, organized in single and mixed biofilms. Student t-test. *C. albicans* counts: statistically significant difference between biofilm formed by *C. albicans* and biofilm formed by *C. albicans* + *C. krusei* ($p < 0.0001$). *C. krusei* counts: statistically significant difference between biofilm formed by *C. krusei* and biofilm formed by *C. albicans* + *C. krusei* ($p < 0.0001$).

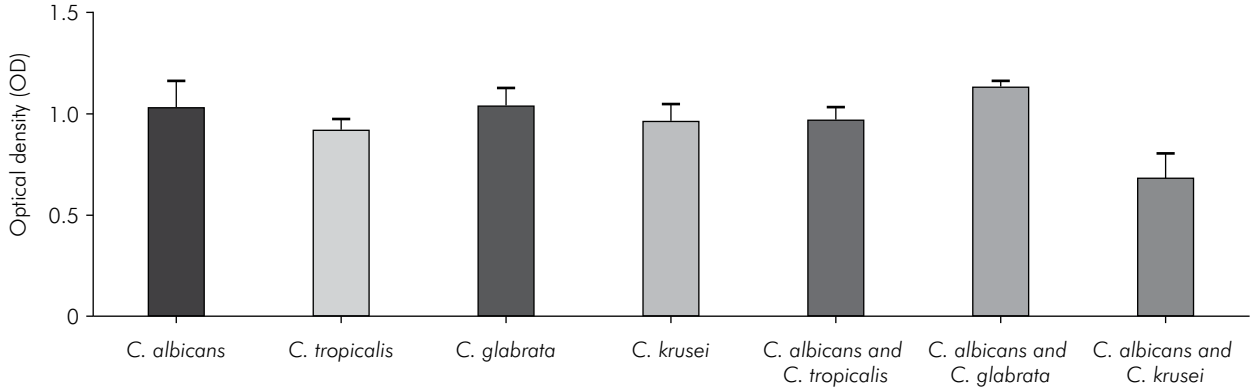

Figure 4. Mean values and standard deviation (n = 10) of the metabolic activity (XTT analysis) for single biofilms formed by *C. albicans*, *C. tropicalis*, *C. glabrata* or *C. Krusei*, and for mixed biofilms formed by the interactions of *C. albicans*-*C. tropicalis*, *C. albicans*-*C. glabrata* or *C. albicans*-*C. krusei*. Analysis of variance (ANOVA): statistically significant differences among the groups ($p < 0.0001$). Tukey test: *C. albicans vs. C. tropicalis* ($p = 0.0982$), *C. albicans vs. C. glabrata* ($p = 0.9999$), *C. albicans vs. C. krusei* ($p = 0.6625$), *C. tropicalis vs. C. glabrata* ($p = 0.0742$), *C. tropicalis vs. C. krusei* ($p = 0.9138$), *C. glabrata vs. C. krusei* ($p = 0.5859$), *C. albicans vs. C. albicans* and *C. tropicalis* ($p = 0.7269$), *C. albicans vs. C. albicans* and *C. glabrata* ($p = 0.2459$), *C. albicans vs. C. albicans* and *C. krusei* ($p < 0.0001$), *C. tropicalis vs. C. albicans* and *C. tropicalis* ($p = 0.8751$), *C. glabrata vs. C. albicans* and *C. glabrata* ($p = 0.3036$), *C. krusei vs. C. albicans* and *C. krusei* ($p < 0.0001$).

C. tropicalis, *C. glabrata,* and *C. krusei* was similar for the species studied. The results for the interactions between *C. albicans* and non-*albicans* species show that the highest metabolic activity was observed for the interaction between *C. albicans* and *C. glabrata*, and the lowest, for the interaction between *C. albicans* and *C. krusei.* When the single biofilm formed by *C. albicans* was compared with mixed biofilms formed by the *C. albicans* and non-*albicans* species, a statistically significant difference was found only for the interaction with *C. krusei* ($p < 0.0001$).

Discussion

Recent studies have shown that biofilms in nature are formed by multiple microbial species that are closely associated and interact with each other.[16] Some microorganisms have evolved mutualistic or even synergistic interactions to facilitate cohabitation on epithelial surfaces and to utilize metabolic by-products efficiently, whereas others have developed competitive antagonistic approaches during colonization.[23]

Species of the genus *Candida* are the most prevalent fungal pathogens in the oral cavity, and their ability to form biofilms is associated with their virulence.[24] *Candida albicans* is considered the most important species, and is found in approximately 70% of oral infections. Nonetheless, non-*albicans* species, such as *Candida glabrata*, *Candida tropicalis* and *Candida krusei,* are also frequently associated with the development of infections.[25] *Candida albicans* sometimes exists in a mixture with other microbial species in this environment.[26] The virulence of *C. albicans* may be influenced not only by its own properties but also by its interactions with cohabitating oral microorganisms.[20] Further research is required to understand the interactions that occur when different species of *Candida* coexist in a biofilm.

In the present study, the interactions between *C. albicans* and non-*albicans* species in mixed biofilms were evaluated by counting colony-forming units (CFU/mL) and by determining cell viability using the XTT colorimetric assay. The counting of CFU/mL was performed using a chromogenic medium (*Candida* HiChrome agar) that allows the colonies of *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. krusei* to be differentiated by color. Chromogenic media are widely used for presumptive identification of *Candida* species. Rajkumari et al.[27] compared the accuracy between the Automated VITEK 2 Compact system and a chromogenic medium (CHROMagar) for identification of 445 yeast isolates. VITEK 2 was able to correctly identify 354 (79.5%) isolates, whereas the chromogenic medium correctly identified 381 (85.6%) isolates.

The results obtained by counting CFU/mL show antagonistic interactions between *C. albicans* with *C. tropicalis*, *C. glabrata,* and *C. krusei*. El-Azizi et al.[14]

investigated the interactions between *C. albicans* and non-*albicans* species using *in vitro* models. *C. guilliermondii*, *C. krusei* or *C. lipolytica* were added to a preformed *C. albicans* biofilm. An increase in the biofilm growth of *Candida* spp. was observed in the *in vitro* model, except for *C. guilliermondii*. Similar results were observed when *C. albicans* was added simultaneously with non-*albicans* species to form a mixed biofilm. However, the number of *C. albicans* cells in the biofilm was significantly reduced when the cells were added to the preformed *C. guilliermondii*, *C. krusei* or *C. lipolytica* biofilms, indicating a competition for adhesion sites.

Among the non-*albicans Candida* species, *C. krusei* was the species with the highest inhibitory activity against *C. albicans*. Its greater number of CFU/mL suggests that *C. krusei* prevailed in this interaction. Using a biofilm model similar to that of this study and *Candida* reference strains, Rossoni et al.[21] verified that the inhibition of *C. albicans* was greater in the presence of *C. krusei* than in the presence of *C. glabrata*. Thein et al.[19] also studied the interaction between *C. albicans* and *C. krusei* using an *in vitro* biofilm model, and observed antagonism between the species; *C. krusei* suppressed approximately 85% of *C. albicans* growth. These results suggest that there may be competition for nutrients between these species or that *C. krusei* produces signaling molecules that inhibit the growth of *C. albicans*. Furthermore, it has been suggested that *C. krusei* has a higher initial rate of colonization, perhaps because of *C. krusei*'s very high cell surface hydrophobicity and resultant adherence to acrylic surfaces.[7] In addition, biofilm formation and coaggregation effects allow competing microorganisms to maximize the colonization surface area in an environment that is highly competitive for space and nutrients.[23]

To date, most previous studies have focused on analyzing biofilm formation by counting CFU/mL; however, Seneviratne et al.[10] suggest that biofilm development results should be analyzed by more than one method. In this study, in addition to colony counting, we used an XTT reduction assay that permits measurement of the metabolic activity of the microbial eukaryotic population present in biofilms. The intracellular reduction of XTT releases a formazan compound that can be quantified by colorimetric estimation, thereby allowing characterization of the metabolic activity of cells.[9]

The single biofilms formed by *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. krusei*, on flat-bottom 96-well microtiter plates for 48 h, showed similar metabolic activity, thus confirming the CFU counting method. Seneviratne et al.[10] evaluated the growth kinetics of *Candida* biofilms by counting CFU after 1.5, 24, 48 and 72 h of development. At an adherence time of 1.5 h, approximately 1-7 x 10^5 cells/mL had adhered to the well of the microtiter plate. After 48 h, the biofilm community reached a plateau of 0.3-2.2 x 10^8 cells/mL, and then declined by 72 hour. The spectrometric profile (520 nm) was also assessed and correlated with the number of CFU, showing maximum cell density at 48 h, declining thereafter.

In mixed biofilms, the results of the XTT colorimetric assay were compared with the single biofilm formed by *C. albicans*, because it is not possible to differentiate the cellular activity of the individual species, based only on the results of the XTT method. The results obtained in the single biofilms formed by *C. albicans* were similar to those of the biofilms mixed with *C. tropicalis* or *C. glabrata*. However, the presence of *C. krusei* in the mixed biofilm reduced cell viability, compared with the cell viability observed in the single biofilm formed by *C. albicans*. Pathak et al.[28] also studied the interactions among *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. krusei in vitro* through optical density values using the crystal violet assay. *C. tropicalis* decreased the biofilm production of non-*albicans* species. However, *C. krusei* negatively impacted the biofilm formation of multiple species, and the presence of *C. albicans* biofilms increased in the layer of heterotypic biofilms, indicating that *C. albicans* may provide a substrate for non-*albicans* species in or on acrylic materials.

In this study, we used clinical isolates from oropharyngeal candidiasis lesions of HIV-positive patients; however, only 1 clinical strain of each species was tested. Previous studies demonstrated that the clinical isolates of *Candida* exhibit intraspecies variability in relation to biofilm forming ability.[29,30] Muadcheingka and Tantivitayakul[31] evaluated the cell surface hydrophobicity (CSH) of 250 clinical isolates from oral candidiasis. CSH is an intrinsic property of

the external cell wall layer of *Candida*; it promotes strong binding and irreversible adherence to the mucosal membrane or other substrates, contributing to biofilm formation. These authors observed that the CSH values were strain dependent and not species-specific.

In brief, both the counting of CFU/mL and the XTT colorimetric assay results showed that *C. krusei* exerted strong inhibitory action on *C. albicans* during biofilm formation. This data confirms previous findings that the XTT assay yielded results similar to those of the CFU method, thereby making it a useful tool for studying biofilms formed *in vitro*. In addition, further studies are needed to elucidate the mechanisms of the antagonistic interaction between *C. krusei* and *C. albicans*, focusing especially on how *C. krusei* affects the quorum sensing-mediated molecules, the filamentation and the pathogenicity of *C. albicans*. This study should also be widened to include a larger number of clinical strains of *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. krusei*.

Conclusion

Considering the clinical *Candida* strains analyzed in this study, we concluded that *C. albicans* established antagonistic interactions with non-*albicans Candida* species in mixed biofilms. *C. krusei* significantly inhibited the growth and the metabolic activity of *C. albicans* in the mixed biofilm.

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