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Study of Microbial Interaction Formed by *Candida krusei* and *Candida glabrata: In Vitro* and *In Vivo* Studies

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Recently, the non-albicans Candida species have become recognized as an important source of infection and oral colonization by association of different species in a large number of immunosuppressed patients. The objective of this study was to evaluate the interactions between *C. krusei* and *C. glabrata* in biofilms formed *in vitro* and their ability to colonize the oral cavity of mouse model. Monospecies and mixed biofilms were developed of each strain, on 96-well microtiter plates for 48 h. These biofilms were analyzed by counting colony-forming units (CFU/mL) and by determining cell viability, using the XTT hydroxide colorimetric assay. For the *in vivo* study, twenty-four mice received topical applications of monospecie or mixed suspensions of each strain. After 48 h, yeasts were recovered from the mice and quantified by CFU/mL count. In the biofilm assays, the results for the CFU/mL count and the XTT assay showed that the two species studied were capable of forming high levels of *in vitro* monospecie biofilm. In mixed biofilm, the CFU of *C. krusei* increased (p=0.0001) and *C. glabrata* decreased (p=0.0001). The metabolic activity observed in XTT assay of mixed biofilm was significantly reduced compared with a single *C. glabrata* biofilm (p=0.0001). Agreeing with CFU *in vitro count, C. glabrata* CFU/mL values recovered from oral cavity of mice were statistically higher in the group with single infection (p=0.0001) than the group with mixed infection. We concluded that *C. krusei* inhibits *C. glabrata* and takes advantage to colonize the oral cavity and to form biofilms.

Key Words: *Candida krusei, Candida glabrata*, biofilms, experimental candidiasis, murine model.

Introduction

Some decades ago, *Candida albicans* represented 80% of the Candida species recovered from patients with oral and systemic candidiasis. Although *C. albicans* continues to be the most frequently isolated species, the number of infections caused by non-albicans species has increased significantly (1). Furthermore, *C. glabrata* has emerged as the second most common cause of candidemia, followed by *C. parapsilosis, C. tropicalis* and *C. krusei* (2).

One of the major factors contributing to the virulence of Candida is its ability in acclimatize to a variety of different habitats for growth and formation of surface-attached microbial communities known as "biofilms". Biofilms are defined as microbial communities encased in a matrix of extracellular polymeric substance (EPS), which display phenotypic features that differ from their planktonic or free-floating counterparts (3-5). It is known that *C. glabrata* exhibits intrinsically low susceptibility to azoles and develops resistance after exposure to these drugs (6) and *C. krusei* is naturally resistant to fluconazole, but in most cases, it is sensitive to voriconazole (cross-resistance is uncommon in this specie) (7,8), which complicates the treatment of candidiasis caused by this species. Because of that, it is important to know the behavior of these species when they grow together in a biofilm.

Recently, Rossoni et al. (9) and Barros et al. (10) evaluated the interaction of *C. albicans* associated with *C. krusei* and *C. glabrata* in biofilm formation. Both of the studies suggest that these interactions decrease the ability of *C. albicans* to form biofilm. Moreover, Santos et al. (11) studied the interaction between *C. albicans, C. krusei, C. glabrata* and *C. tropicalis.* Among the species tested, *C. krusei* exerted the highest inhibitory action against *C. albicans.* However, none of above studies evaluated the interaction between non-albicans Candida species.

There is just one study that investigated the *in vitro* interaction between *C. krusei and C. glabrata* (12) and our study is the first one that has additional *in vitro* data and also *in vivo*. In this context, the objective of this study was to evaluate the interactions between *C. krusei* and *C. glabrata* in *in vitro* biofilms and its ability to colonize the oral cavity of mice.

Material and Methods

Strains and Culture Conditions

For this study, we used *C. krusei* (ATCC 6258) and *C. glabrata* (ATCC 90030) strains provided from the Microbiology and Immunology Laboratory of the Institute of Science and Technology of São José dos Campos, UNESP-

Univ. Estadual Paulista, São Paulo, Brazil. The strains were stored as frozen stocks with 20% glycerol at -80 °C. The strains were routinely grown in Yeast Nitrogen Base (YNB, Himedia, Mumbai, India) liquid medium at 37 °C in a shaking incubator.

The strains were grown on Sabouraud Dextrose agar (Himedia) and incubated at 37 °C for 24 h. Next, a loopful of growth was inoculated in Yeast Nitrogen Base (YNB) broth supplemented with 100 mM glucose (Vetec, Rio de Janeiro, RJ, Brazil) and incubated at 37 °C for 18 h. The cells were collected by centrifugation (MPW Med. Instruments, Warsaw, Poland) and washed three times with phosphate buffered saline (PBS) (Laborclin, São José dos Pinhais, PR, Brazil). Yeast cells were counted using a Neubauer counting chamber (Laboroptik GmbH, Bad Homburg, Germany). The cell number was confirmed by determining CFU/mL on SDA plates (10^7 cells/mL).

Biofilm Formation In Vitro

The biofilm formation was performed as described by Seneviratne et al. (13), with some modifications. Standardized suspension containing 10^7 cells/mL was prepared for each strain of Candida. The monotypic biofilm were formed by adding 200 µL of standardized suspension of *C. krusei* or *C. glabrata* to each well of flat-bottom 96-well microtiter plates (Costar Corning, New York, NY, USA). For mixed biofilm were added 100 µL of standardized suspension of *C. krusei* and 100 µL of standardized suspension of *C. glabrata*. For the initial stage of adhesion the plates were incubated for 90 min at 37 °C at 75 rpm in an orbital shaker (Quimis, Diadema, SP, Brazil). After this step, the cell suspensions were carefully 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 of glucose were added, and the plates were incubated for 48 h at 37 °C in an orbital shaker. The liquid medium was replaced daily.

Analysis of Biofilm by Counting Colony Forming Units (CFU/mL)

After the biofilms formation, each well was washed four times with 200 µL of PBS. Next, 200 µL of PBS was added into each well plate and the biofilm cells were carefully scraped using a sterile pick. Subsequently, 100 µL aliquot was transferred to a tube (J Prolab, São José dos Pinhais, PR, Brazil) containing PBS. To disaggregate the cells on biofilms an ultrasonic homogenizer was used (Sonoplus HD 2200, Bandelin Electronic, Berlin, Germany) with output power of 50 W for 30 s. From the solution obtained in the microtubes, decimal dilutions of the biofilm suspension were performed and 100 mL aliquots of the dilutions were inoculated into Petri dishes containing chromogenic HiCromeCandida medium (Himedia), followed by incubation of the plates at 37 °C for 48 h. After incubation, Candida species from the mixed biofilms were differentiated by colony color on HiChrome Candida agar (cream for *C. glabrata* and purple for *C. krusei*) and the CFU/mL values were determined. For statistical analysis and comparison among the groups, the data of CFU/mL were converted to logarithmic form.

Analysis of Biofilm By Colorimetric Assay (XTT)

The biofilms formed were subjected to XTT assay (2-methoxy-4-nitro-5-sulfophenyl-5-phenylalanino-carbonyl-2H-tetrazolium hydroxide), which assessed the presence of metabolically active yeast (14). Each well containing the adhered biofilm was rinsed twice with 200 µL phosphate-buffered saline solution (PBS) to remove weakly adhered cells. Then, each of the wells was inoculated with 158 µL PBS, 40 µL XTT and 2 µL menadione. After incubation in the dark for 3 h at 37 °C, 100 µL of solution was transferred to a new plate and the colorimetric change of the solution was measured using a microplate reader (TP Reader; Thermoplate, Sdorf, Araras, SP, Brazil) at 490 nm (14).

Candida Interactions in Mice

The Animal Research Ethics Committee from the Institute of Science and Technology at UNESP, approved this study under protocol number 014/2011 -PA/CEP. Twenty-four adult male mice (Mus musculus, albinus, Swiss), weighing between 30 and 60 g were included in this study. Animals were divided into 3 groups: *C. krusei* (n=8), *C. glabrata* (n=8), and *C. krusei* + *C. glabrata* (n=8). The design of the study of interaction between Candida species was performed according Rossoni et al. (9) and Takakura et al. (15) and is shown in Table 1. In summary, animals were immunosuppressed with 3 subcutaneous injections of prednisolone (Depo-Medrol; Pfizer Laboratories Ltd., Guarulhos, SP, Brazil) at 100 mg/kg of body weight alternated with two inoculations of Candida. Tetracycline chloride (Terramicina; Pfizer) was administered in the

Table 1. Design of the study of the colonization between non-albicans Candida species in the immunosuppressed mice model

Day	Methodology
1	1st injection of prednisolone
2	Inoculation of Candida in the oral cavity of mice
3	2nd injection of prednisolone
4	Inoculation of Candida in the oral cavity of mice
5	3rd injection of prednisolone
6	Recovery of Candida from the tongue dorsum of mice euthanasia of the mice

drinking water of the animals at a concentration of 0.83 mg/mL, beginning one day before infection and was maintained throughout the experiment. A 50 µL intramuscular injection of chlorpromazine chloride, equivalent to 10 mg/kg of body weight (Amplictil; Sanofi Aventis, Suzano, SP, Brazil) in each thigh was used to sedate the animals.

Each strain of Candida was cultured for 24 h at 37 °C on Sabouraud Dextrose Agar (Himedia), and were re-suspended in 10 mL of PBS, subsequently being centrifuged at 358 xg for 10 min. The resulting pellet was re-suspended in 10 mL PBS and adjusted to $1x10^8$ cells/mL after counting in a Neubauer chamber (Laboroptik). A sterile swab (Absorve; Cral, São Paulo, SP, Brazil) soaked in the Candida suspension was used to inoculate the sedated mice by rubbing the swab for 3 min on the tongue dorsum. For the groups with mixed infections, the same procedure was performed, but the swab was soaked in a standard mixed suspension containing $1x10^8$ cells/mL for each Candida species.

In order to confirm the final concentration of each Candida species in the swab before the oral inoculation in mice, we performed another experiment for counting CFU/mL of Candida cells adhered to the swab. Therefore, after the sterile swab had been soaked in the Candida suspension, it was transferred to a falcon tube containing PBS and submitted to ultrasonic homogenizer for 30 s. A series of dilutions were made and plated in chromogenic medium HiCrome Candida (37 °C for 48 h) for quantification of CFU/mL for each Candida species.

Recovery of C. krusei and C. glabrata from the Tongue Dorsum of Mice

Samples from the oral cavity were collected with a swab and placed in a tube containing 0.9 mL of PBS and shaken for one min. Considering that the swab absorbed approximately 0.1 mL of saliva from the oral cavity of mice, this solution was estimated to be a 10^{-1} starting dilution of Candida from the soaked swab.

A series of dilutions were subsequently performed and 0.1 mL of each dilution was plated in duplicate onto the surface of plates containing chromogenic HiCrome Candida (Himedia) in order to differentiate the species recovered. Plates were incubated at 37 °C for 48 h and Candida colonies were counted to determine colony-forming units (CFU/mL).

Euthanasia of the Mice

The euthanasia of mice was performed within 2 days after the second inoculation with Candida, corresponding to 6 days of experiments. This procedure was performed by administration of an overdose of anesthetic.

Statistical Analysis

The CFU/mL data of *in vitro* biofilms of Candida and of experiments in mice were converted to logarithmic values and submitted Student t-test ($p \leq 0.05$).

Results

In biofilms formed *in vitro* at the bottom of 96-well plates it was observed that *C. krusei* and *C. glabrata* species were capable to form biofilms, reaching a median of 6.00 and 6.46 CFU/mL (log) in the monotypic biofilms, respectively. In the mixed biofilms, *C. krusei* showed significant higher number of CFU/mL ($p=0.0001$) and *C. glabrata* decreased ($p=0.0001$) compared to the single biofilm. These data indicate that the presence of *C. krusei* in the biofilm inhibited the growth of *C. glabrata*. The mean and standard deviation values of the CFU/mL obtained from the experiments described above are shown in Figure 1.

Regarding the colorimetric method based on XTT, which measured the metabolic activity of the biofilms formed, *C. glabrata* strain exhibited higher metabolic activity in relation to the *C. krusei* in single biofilms, with optical densities (OD490 nm) of 0.80 ± 0.103 and 0.21 ± 0.296, respectively (Fig. 2). When the above values were compared to the mixed biofilms (OD490 nm= 0.24 ± 0.168), a significant reduction was observed of the metabolic activity of *C. glabrata* ($p=0.0001$), while the values for *C. krusei* remained almost unchanged ($p=0.8116$).

Before the *in vivo* study, we did an assay to confirm

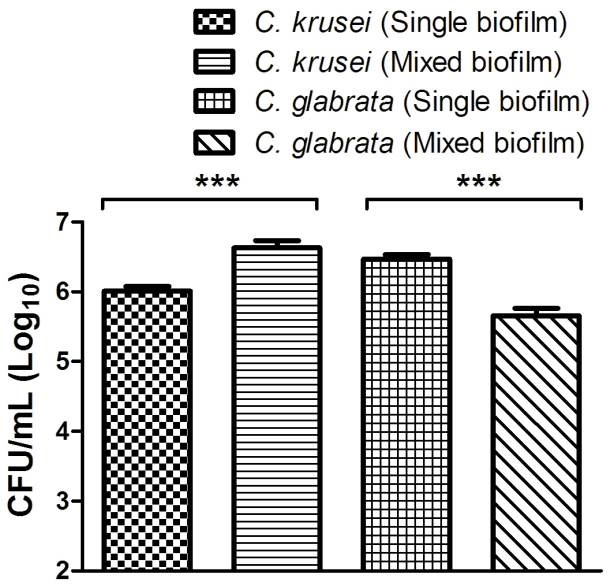

Figure 1. Quantification of colonies in biofilms formed at the bottom of 96-well plates (*in vitro* study). Mean values and standard deviation of CFU/mL (log10) of *C. krusei* and *C. glabrata*, organized in single and mixed biofilms. Student t-test. *C. krusei* counts: statistically significant difference between biofilm formed only by *C. krusei* and biofilm formed by *C. krusei*+*C. glabrata* (***$p<0.0001$). *C. glabrata* counts: statistically significant difference between biofilm formed only by *C. glabrata* and biofilm formed by *C. krusei*+*C. glabrata* (***$p<0.0001$).

the concentration of non-albicans Candida cells adhered to the swab. There was no difference on the capacity of C. krusei and C. glabrata to adhere the swab when they were soaked in both monotypic and heterotypic suspensions (p=0.5728) (Data not shown).

Samples from the oral cavity were collected and plated to count CFU/mL number. In the groups with single infections, we found values ranging from 6.05±0.27 CFU/mL (Log) for *C. krusei* and 4.49±0.22 CFU/mL (Log) for *C. glabrata* inoculations (Fig. 3). Both non-albicans species studied were capable to colonize the oral cavity of mice. Analyzing the *C. krusei* colonization profile in single and mixed infections, we observed that CFU/mL values were slightly higher in mixed infection (p=0.0913), with 6.30 CFU/mL values when compared to single infection (6.05 CFU/mL values). However, there was a significant reduction (p=0.0001) for *C. glabrata* in mixed infection (3.05 CFU/mL values) compared to single infection (4.49 CFU/mL values), agreeing with the *in vitro* results and suggesting an ecological interaction by competition between these two Candida species.

Discussion

C. glabrata is generally considered a species of low virulence but with a higher mortality rate than *C. albicans* and is the most common non-albicans Candida isolated species (16). The emergence of yeasts other than *C. albicans* and of mixed infections has suggested that the epidemiology of Candida infections is changing. Consequently, these infections may require higher doses of antifungal agents and may predispose patients to recurrent candidiasis, mainly because Candida species such as *C. glabrata* and *C. krusei* are becoming resistant to currently available antifungal treatments (17,18).

In this report, the interaction between *C. krusei* and *C. glabrata* was evaluated in *in vitro* biofilms and their ability to colonize the oral cavity of mice. In the *in vitro* study, the results obtained by counting CFU/mL show that *C. krusei* obtained higher growth when associated with *C. glabrata* compared to single species biofilm. However, *C. glabrata* was inhibited in the interaction compared with monotypic group. These data suggest that the growth of *C. krusei* was stimulated by the presence of *C. glabrata*. Most studies on polymicrobial biofilms have focused on the interaction between bacteria and Candida spp. or association between *Candida albicans* and non-albicans species. This is the first study that evaluate the interaction of *C. krusei* and *C. glabrata* in mixed biofilms.

In agreement with Kirkpatrick et al. (19) and Thein et al. (20), interaction of two Candida species seemed to suppress each other's growth, possibly because they competed for nutrients and/or one of the species generated toxic metabolites. Competitive inhibition may occur even in the initial step of adhesion onto a substrate during dual-species

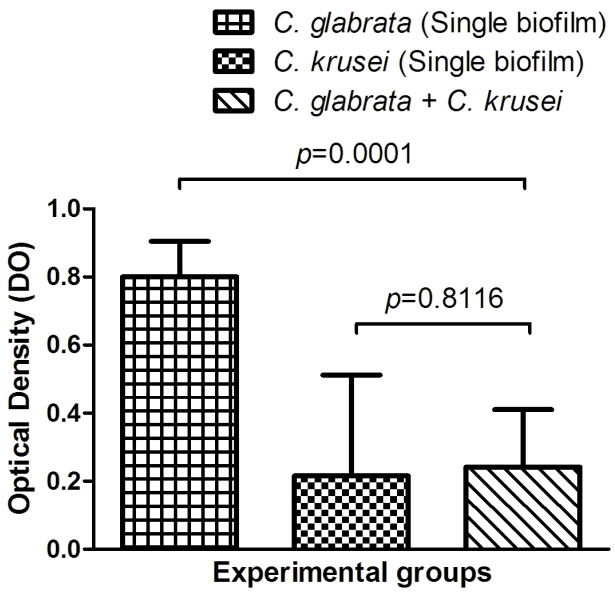

Figure 2. Mean values and standard deviation of the metabolic activity (XTT analysis) for single biofilms formed by *C. krusei* and *C. glabrata*, and for mixed biofilms formed by the interactions of *C. krusei*+*C. glabrata*. Student t-test: statistically significant differences between *C. glabrata* (single biofilm) vs. *C. glabrata*+*C. krusei* (p=0.0001) and no difference between *C. krusei* (single biofilm) vs. *C. glabrata*+*C. krusei* (p = 0.8116).

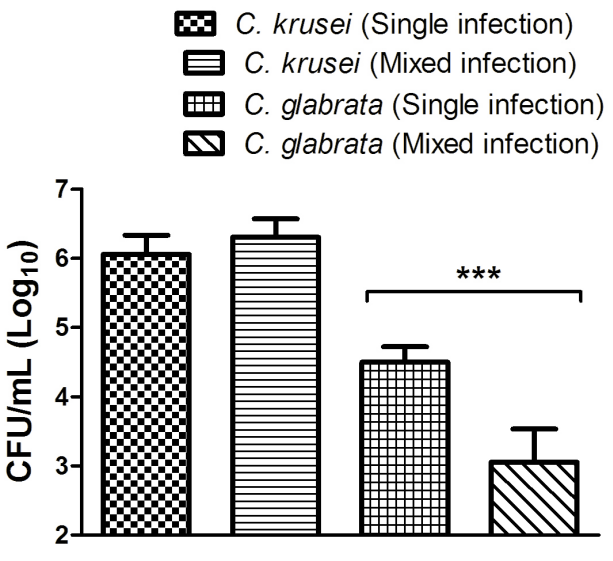

Figure 3. Quantification of fungal cells recovered from the buccal cavity of mice. Mean and standard deviation of the CFU/mL (Log) *of C. krusei* and *C. glabrata* recovered from the buccal cavity of immunosuppressed mice with single and mixed infections. Student-t test. CFU/mL of *C. krusei*: comparison between single infection by *C. krusei* and mixed infection *by C. krusei*+*C. glabrata* (p=0.0913); CFU/mL *of C. glabrata*: comparison between single infection by *C. glabrata* and mixed infection by *C. krusei*+*C. glabrata* (***p=0.0001).

Candida biofilm development (20).

C. glabrata is a non-dimorphic specie and its biofilm is composed of scattered chains of cells with multiple blastospores embedded in thin extracellular matrix. In contrast, *C. krusei* biofilm maintained its 'large American rice grain' form devoid of hyphal elements, which yielded a thick multi-layer ultrastructure enveloped by voluminous extracellular material (21).

Taff et al. (22) observed that XTT reduction activity is associated with the CFU in the biofilm. Furthermore, this method is the most accurate and reproducible assay for biofilm quantification. In this study, we selected the XTT activity method to determine biofilm forming activity of Candida spp. Single biofilms produced by *C. glabrata* showed the highest metabolic activity than that produced by *C. krusei*, although it produced less biomass. These results were similar with other reports (12,22-24). Consequently, *C. glabrata* cells in the biofilms seem to be metabolically more active than cells from other species that produce biofilms with more biomass. In mixed biofilms, the metabolic activity was reduced significantly in comparison with single biofilm of *C. glabrata*, and it was significantly similar to the single biofilm of *C. krusei*, suggesting a possible competition between the species. These findings corroborate with Pathak et al. (12) that evaluated the interaction of these species by XTT assay and found *C. krusei* decreased the biofilm production of *C. glabrata*.

Considering that *C. krusei* was stimulated in mixed biofilm and *C. glabrata* had reduction in CFU counts, the present study was expanded to encompass the oral colonization of these species in mice models. Based on the CFU/mL data analysis of yeast recovered from the buccal cavity of immunosuppressed mice with single infections, the recovery observed were equivalent to 6.05 log10 for groups infected with *C. krusei* and 4.49 log10 for groups infected with *C. glabrata*, showing that *C. krusei* had a higher colonization ability compared to *C. glabrata*. The low CFU count of *C. glabrata* is consistent with Tati et al. (25) who tried to establish a model of oropharyngeal candidiasis with *C. glabrata*. The authors used inocula size of ranging $1x10^{10}$ cells/mL and recovered $4\text{-}7x10^{2}$ cells/mL from the tongue. Moreover, it was not observed infection with *C. glabrata* resulted in no clinical appearance of disease or weight loss in animals.

Regarding the quantification of *C. glabrata* CFU/mL in mixed infections, we verified that the amount of yeast recovered was significantly lower (1.44 log10 of reduction) compared to single infection. The present study is pioneering in documenting of oral colonization by mixed infection of *C. krusei* and *C. glabrata* in mice models and indicates that further studies are required to investigate the interactions among Candida species using a larger number of strains in order to elucidating the mechanisms of how these species compete amongst themselves during the biofilm formation and oral colonization.

Within the parameters of this study, we concluded that *C. krusei* inhibits *C. glabrata* and takes advantage to colonize the oral cavity and to form biofilms.

Resumo

Recentemente, as espécies não albicans tem se tornado uma importante fonte de infecção e de colonização oral pela associação de espécies em um grande número de pacientes imunossuprimidos. O objetivo desse estudo foi avaliar a interação entre *C. krusei* e *C. glabrata* em biofilmes formados *in vitro* e sua capacidade em colonizar a cavidade oral em modelo de camundongo. Biofilmes monoespécies e mistos foram formados em placas de 96 poços por 48 h. Esses biofilmes foram analisados pela contagem de UFC/mL e pela determinação da viabilidade celular, usando ensaio de XTT. Para o estudo *in vivo*, vinte e quatro camundongos receberam aplicações tópicas de suspensões monoespécies e mistas de cada espécie. Após 48 h, as leveduras foram recuperadas dos camundongos e quantificadas por UFC/mL. Nos ensaios de biofilme, os resultados da contagem de UFC/mL e do ensaio de XTT mostraram que as duas espécies estudadas foram capazes de formar grande quantidade de biofilme monoespécie *in vitro*. Nos biofilmes mistos, a UFC/mL de *C. krusei* aumentou (p=0,0001) e de *C. glabrata* diminuiu (p=0,0001). A atividade metabólica observada no ensaio de XTT nos biofilmes mistos foi significantemente reduzida comparada com o biofilme formado apenas de *C. glabrata* (p=0,0001). Concordado com as contagens in vitro, os valores de UFC/mL de *C. glabrata* recuperados da cavidade oral dos camundongos foram estatisticamente maior no grupo com infecção simples (p=0,0001) do que do grupo com infecção mista. Nós concluímos que *C. krusei* inibe C. *glabrata* e possui vantagem em colonizar a cavidade oral e formar biofilmes.

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References

1. Silva S, Henriques M, Hayes A, Oliveira R, Azeredo J, Williams DW. *Candida glabrata* and *Candida albicans* co-infection of an in vitro oral epithelium. J Oral Pathol Med 2011;40:421-427.
2. MacPhail GL, Taylor GD, Buchanan-Chell M, Ross C, Wilson S, Kureishi A. Epidemiology, treatment and outcome of candidemia: a five-year review at three Canadian hospitals. Mycoses 2002;45:141-145.
3. Peeters E, Nelis HJ, Coenye T. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J Microbiol Methods 2008;72:157-165.
4. Pereira-Cenci T, Del Bel Cury AA, Crielaard W, Ten Cate JM. Development of Candida-associated denture stomatitis: new insights. J Appl Oral Sci 2008;16:86-94.
5. de Mello TP, de Souza Ramos L, Braga-Silva LA, Branquinha MH, Dos Santos AL. Fungal biofilm - A real obstacle against an efficient therapy: lessons from Candida. Curr Top in Med Chem 2017;17:1-18.
6. Tumbarello M, Sanguinetti M, Trecarichi EM, La Sorda M, Rossi M, de Carolis E, et al.. Fungaemia caused by *Candida glabrata* with reduced susceptibility to fluconazole due to altered gene expression: risk factors, antifungal treatment and outcome. J Antimicrob Chemother 2008;62:1379-1385.
7. Drago M, Scaltrito MM, Morace G; GISIA-2 Group. In vitro activity of voriconazole and other antifungal agents against clinical isolates of *Candida glabrata* and *Candida krusei*. Eur J Clin Microbiol Infect Dis 2004;23:619-624.
8. Freitas EM, Monteiro LC, Fernandes MB, Martelli Junior H, Bonan PR,

Nobre SA. Antifungal susceptibility in vitro determined by the Etest® for Candida obtained from the oral cavity of irradiated and elderly individuals. Braz Dent J 2015;26:99-104.

9. Rossoni RD, Barbosa JO, Vilela SFG, Santos JD, Barros PP, Prata MCA, et al.. Competitive interactions between *C. albicans*, *C. glabrata* and *C. krusei* during biofilm formation and development of experimental candidiasis. PLoS One 2015;10:e0131700.
10. Barros PP, Ribeiro FC, Rossoni RD, Junqueira JC, Jorge AOC. Influence of *Candida krusei* and *Candida glabrata* on *Candida albican*s gene expression in in vitro biofilms. Arch Oral Biol 2016;64:92-101.
11. Santos JD, Piva E, Vilela SFG, Jorge AOC, Junqueira JC. Mixed biofilms formed by *C. albicans* and non-albicans species: a study of microbial interactions. Braz Oral Res 2016;30. pii: S1806-83242016000100232.
12. Pathak AK, Sharma S, Shrivastva P. Multi-species biofilm of *Candida albicans* and non-*Candida albicans* Candida species on acrylic substrate. J Appl Oral Sci 2012;20:70-75.
13. Seneviratne CJ, Silva WJ, Jin LJ, Samaranayke LP. Architectural analysis, viability assessment and growth kinetics of *Candida albicans* and *Candida glabrata* biofilms. Arch Oral Biol 2009;54:2260-2269.
14. Jin Y, Samaranayake LP, Samaranayake Y, Yip HK. Biofilm formation of *Candida albicans* is variably affected by saliva and dietary sugars. Arch Oral Biol 2004;49:789-798
15. Takakura N, Sato Y, Ishibashi H, Oshima H, Uchida K, Yamaguchi H, et al.. A novel murine model of oral candidiasis with local symptoms characteristic of oral thrush. Microbiol Immunol 2003;47:321-326.
16. Savastano C, de Oliveira Silva E, Gonçalves LL, Nery JM, Silva NC, Dias ALT. *Candida glabrata* among Candida spp. from environmental health practitioners of a Brazilian Hospital. Braz J Microbiol 2016;47:367-372.
17. Thein ZM, Seneviratne CJ, Samaranayake YH, Samaranayake LP. Community lifestyle of Candida in mixed biofilms: a mini review. Mycoses 2009;52:467-475.
18. Guinea J. Global trends in the distribution of Candida species causing candidemia. Clin Microbiol Infec 2014;20:5-10.
19. Kirkpatrick WR, Lopez-Ribot JL, McAtee RK, Patterson TF. Growth competition between *Candida dubliniensis* and *Candida albicans* under broth and biofilm growing conditions. J Clin Microbiol 2000;38:902-904.
20. Thein ZM, Seneviratne CJ, Samaranayake YH, Samaranayake LP. Community lifestyle of Candida in mixed biofilms: a mini review. Mycoses 2009;52:467-475.
21. Parahitiyawa NB, Samaranayake YH, Samaranayake LP, Ye J, Tsang PW, Cheung BP, et al.. Interspecies variation in Candida biofilms formation studied using the Calgary biofilms device. APMIS 2006;114:298-306.
22. Taff HT, Nett JE, Andes DR. Comparative analysis of Candida biofilm quantitation assays. Med Mycol 2012;50:214-218.
23. Ferreira AV, Prado CG, Carvalho RR, Dias KS, Dias AL. *Candida albicans* and non-*C. albicans* Candida species: comparison of biofilm production and metabolic activity in biofilms, and putative virulence properties of isolates from hospital environments and infections. Mycopathologia 2013;175:265-272.
24. Muadcheingka T, Tantivitayakul P. Distribution of *Candida albicans* and non-albicans Candida species in oral candidiasis patients: correlation between cell surface hydrophobicity and biofilm forming activities. Arch Oral Biol 2015;60:894-901.
25. Tati S, Davidow P, McCall A, Hwang-Wong E, Rojas IG, Cormack B, et al.. *Candida glabrata* binding to *Candida albican*s hyphae enables its development in oropharyngeal candidiasis. PLoS Pathog 2016;12:e1005522.

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