Candida/Candida biofilms. First description of dual-species Candida albicans/C. rugosa biofilm

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ABSTRACT

Denture liners have physical properties that favour plaque accumulation and colonization by Candida species, irritating oral tissues and causing denture stomatitis. To isolate and determine the incidence of oral Candida species in dental prostheses, oral swabs were collected from the dental prostheses of 66 patients. All the strains were screened for their ability to form biofilms; both monospecies and dual-species combinations were tested. Candida albicans (63 %) was the most frequently isolated microorganism; Candida tropicalis (14 %), Candida glabrata (13 %), Candida rugosa (5 %), Candida parapsilosis (3 %), and Candida krusei (2 %) were also detected. The XTT assay showed that C. albicans SC5314 possessed a biofilm-forming ability significantly higher (p < 0.001) than non-albicans Candida strains, after 6 h 37°C. The total C. albicans CFU from a dual-species biofilm was less than the total CFU of a monospecies C. albicans biofilm. In contrast to the profuse hyphae verified in monospecies C. albicans biofilms, micrographies showed that the C. albicans/non-albicans Candida biofilms consisted of sparse yeast forms and profuse budding yeast cells that generated a network. These results suggested that C. albicans and the tested Candida species could co-exist in biofilms displaying apparent antagonism. The study provide the first description of C. albicans/C. rugosa mixed biofilm.

Introduction

Candida species inhabit the human oral cavity. Candida albicans is the species that is predominantly associated with mucosal and systemic fungal infections elicited by yeasts (Miceli et al. 2011). Nevertheless non-albicans Candida species have arisen a major opportunistic pathogens. Candida glabrata, Candida parapsilosis, Candida tropicalis, and Candida krusei have been frequently isolated from the oral cavity and are often related to biofilm formation on the surface of medical devices and
tissues (Fires et al. 2002; Fires-Gonçalves et al. 2007; Zahir & Himratul-Aznita 2013).

Biofilms constitute a structured community of microbial cells enveloped in a self-produced polymeric matrix consisting of extracellular DNA, carbohydrates, proteins, hexosamine, phosphorus, and uronic acid adhered to an inert or living surface (Flemming & Wingender 2010). Biofilm cells inherently have limited exposure to immunological defense and antimicrobial drugs, which could account for the emergence of resistant microorganisms (Lewis 2012). Slow growth, altered cell metabolism regulation due to nutrient limitation and stress conditions, and cell density are other suggested mechanisms of biofilm resistance (Seneviratne et al. 2010). In addition, biofilms represent a significant predictor of mortality (Tumbarello et al. 2007, 2012; Silva et al. 2011) and species that produce biofilms have been correlated with poor outcome of infected patients (Tumbarello et al. 2012). For example, Candida cells surviving on dentures after chemical or mechanical treatment constitute a highly tolerant cell subpopulation (persistent) that restores the biofilm in the presence of nutrients and causes a relapse of chronic infection (Lewis 2012).

Furthermore, the oral cavity harbours a vast range of microbial species. The residents themselves might interact extensively while forming the biofilm structures, to carry out physiological functions and induce microbial pathogenesis (Kuramitsu et al. 2007; Thein et al. 2009; Kolenbrander et al. 2010). The residents may (i) compete for nutrients; (ii) interact synergistically to stimulate the growth or survival of one or more residents; (iii) produce antagonist compounds that inhibit the growth of another resident; (iv) neutralize a virulence factor produced by another resident; and (v) interfere in the growth-dependent signaling mechanisms of another resident (Kuramitsu et al. 2007). It is likely that resident microorganisms interact closely in this scenario, allowing a mixed-species biofilm to grow.

Most studies on polymicrobial biofilms have focused on the relationship between bacteria and Candida spp. However, Candida–Candida co-existence within biofilms has been poorly demonstrated (Kirkpatrick et al. 2000; Coco et al. 2008; Cuellar-Cruz et al. 2012). Thus, this study aimed to examine the frequency of Candida spp. on prostheses from denture wearers and to assess the formation of both monospecies and dual-species biofilms consisting of C. albicans and C. non-albicans strains. To our knowledge, this is the first report of mixed biofilm formation by C. albicans and Candida rugosa.

### Materials and methods

#### Sample

Dental prostheses from 66 patients (33 males and 33 females, aged from 29 to 92 y) attending the outpatient clinics of the Dentistry School of the University of Franca, State of São Paulo, Brazil, were used in this study. All the subjects provided informed consent according to the protocols approved by the Human Subjects Committee of the
University of Franca. The patients generally self-reported good general health without severe medical or psychological conditions.

Yeast isolates

Sample collection was conducted mid morning, at least 2 h after eating, drinking, or postdorm hygiene procedures. A sterile swab was rubbed in the fitting surface of the dental prostheses and deposited in a tube containing Stuart medium for transport. The swabs were then inoculated on CHROMagar Candida™/C228 medium (Becton Dickinson, Sparks, MD) and incubated at 37 °C for 48 h. Following the manufacturer’s instructions, the API ID 32 C™ (bio-Merieux, Marcy-l’Etoile, France) and Vitek Yeast Biochemical Card™ (bio-Merieux, Marcy-l’Etoile, France) identified the Candida species. Candida albicans was confirmed by germ tube and chlamydo-sphere formation on cornmeal agar (Difco, Detroit, Mich, USA) 0.33 % Tween 80. Reference strains of C. albicans (ATCC 90028), Candida glabrata (ATCC 2001), Candida parapsilosis (ATCC 22019), Candida krusei (ATCC 34135), Candida rugosa (ATCC 10571), Candida tropicalis (ATCC 13803) from the American Type Culture Collection (ATCC), and C. albicans SC5314 were also included.

Biofilm formation

Each Candida strain or each isolate of non-albicans Candida was co-incubated with Candida albicans SC5314 on a polystyrene surface following the protocol described by Ramage et al. (2001). Briefly, the Candida overnight culture, grown in yeast–peptone–glucose (YPG) broth [1 % (w/v) yeast extract, 2 % (w/v) peptone, and 2 % (w/v) glucose], was diluted to 10^6 cells mL^{-1} in RPMI 1640 medium supplemented with L-glutamine, buffered with morpholinepropanesulfonic acid (MOPS – Sigma, St. Louis, MO) at 0.165 M, pH 7.0, and added to glucose at 0.9 %. A total of 100 μL (individually) or 50 μL (for each strain, in case of dual-species biofilm) of the Candida suspension was added to the wells of a 96-well microtitre plate (Corning Inc., Corning, NY, USA). Biofilm formation was quantified at 6, 12, 18, 24, and 48 h at 37 °C. After incubation, the medium in the wells was removed and washed three times with sterile phosphate buffer solution (PBS). Candida albicans SC5314 was used as the control strain in all the experiments. All non-albicans Candida isolates found co-existing with C. albicans on dental prostheses were used for dual-species biofilm analysis. All isolates were assessed in quadruplicate and repeated at least three times.

Biofilm quantification

For the assessment of metabolic activity, 100 μL of the XTT solution [XTT (0.5 mg mL^{-1} in PBS), menadione (0.1 mM in acetone)] was added to each well of the microtitre plates. After incubation at 37 °C for 5 h, 75 μL of the solution was transferred to a new well of a 96-well plate, and XTT-formazan was determined at 490 nm. In addition, aliquots (100 μL) of scrapped biofilm were sonicated, serially diluted in PBS (1 mL), plated onto CHROMagar Candida™ medium, and incubated at 37 °C for 48 h. Colony-forming units (CFUs) of each Candida species were determined. All the isolates were assessed in quadruplicate, and the assays were repeated at least three times. The readings for the isolates of each Candida species or each Candida dual-species were averaged together, which provided the reading for the species or the pair. The

![Fig 1 – Biofilm formation of Candida monospecies on microtitre plates. Biofilm viability was quantified by the XTT assay. At each time point of biofilm maturation, the optical density of Candida monospecies biofilms was compared with the optical density of C. albicans SC5314 (black columns, *p < 0.001; **p < 0.01) by ANOVA. Data are the means ± SD of experiments performed in quadruplicate and repeated three times.](image1)

![Fig 2 – Biofilm formation of Candida dual-species on microtitre plates. Biofilm viability was quantified by the XTT assay. At each time point of biofilm maturation, the optical density of mixed biofilms were compared with the optical density of C. albicans SC5314 (black columns, *p < 0.001; **p < 0.05) by ANOVA. Data are the means ± SD of experiments performed in quadruplicate and repeated three times.](image2)
optical densities (ODs) of the Candida species that formed a biofilm were statistically compared with the ODs of a Candida albicans SC5314 biofilm produced in each time point. Strains with maximal OD at 6 h lower than or equal to 0.120 (three standard deviations (0.023) above the mean OD (0.050) of a clean tissue culture plate) were not considered to form a biofilm.

Scanning electron microscopy (SEM)

SEM imaging employed the best biofilm-forming strains of each Candida species shown in XTT assay after 48 h. Monospecies or dual-species biofilms were formed on sterile polyvinyl chloride (PVC) disks within 24-well microtitre plates (Corning). The disks were then removed, washed with potassium phosphate buffer 0.1 M, pH 7.2–7.4, and placed in a fixative [4 % formaldehyde (v/v), 1 % glutaraldehyde (v/v) in potassium phosphate buffer 0.1 M] solution overnight. The samples were rinsed twice for 3 min in potassium phosphate buffer 0.1 M, post-fixed with OsO₄ at 1 % (w/v) for 1 h, dehydrated with the aid of an ethanol gradient, critical-point dried in CO₂ (Critical Point 264 Dryer, model CPD-030, Balzers, Oberko- chen, Germany), and gold-coated by sputtering (Denton Vacuum, model Desk II, Freehold, NJ, USA). After this process, samples were observed under a scanning electron microscope (JEOL, model JSM 5410, Japan) operating in the high vacuum mode, at 15 kV. Experiments were performed in quadruplicate and repeated at least three times.

Statistic analysis

Data were analyzed using parametric analysis of variance (ANOVA) followed by a post-hoc Bonferroni test. Graph Pad Prisma 5 (La Jolla CA, USA) was employed. All the data are reported as the means ± the standard deviation. Significance was set at p < 0.05.

Results and discussion

Forty-four (66 %) patients had their dental prostheses colonized with Candida species. Candida isolates were present in dental prostheses from both females (n = 23; 35 %) and males (n = 21; 32 %). As depicted in Table 1, the age range 70–99 y (50 %) showed the highest prevalence of colonization, followed by the range 50–69 y (42 %). Of the 66 studied individuals, 55 (83 %) used complete removable partial prostheses. Most of the elderly individuals had been using the prostheses up to 10–20 y (39 %), followed by 6–10 y (32 %). Increased age of dental prostheses leads to wear of the material increasing porosity, roughness, grooves, and cracks, which create a niche for microbial colonization/adhesion (Fanello et al. 2006). In addition, predisposing factors associated with patients included advanced age associated with decline in the immune defense or systemic diseases and socioeconomic status (Shulman et al. 2005; Filöche et al. 2010).

The identified species corresponded to a total of 56 yeast isolates that included Candida albicans (35; 63 %), Candida tropicalis (8; 14 %), Candida glabrata (7; 13 %), Candida rugosa (3; 5 %), Candida parapsilosis (2; 3 %), and Candida krusei (1; 2 %) (Table 1). In agreement with the findings of other authors (Zaremba et al. 2006; Loser et al. 2012; Pereira et al. 2013), the majority of the yeast isolates verified in the swabs corresponded to C. albicans (63 %), often recovered in association with other yeasts (Table 1).

Twelve (27 %) of the 44 positive cultures contained mixed Candida species. The most usual Candida dual-species consisted of C. albicans and C. glabrata (Table 3), detected in 5 % of the samples, in agreement with Cavaleiro et al. (2013). Interestingly, in a recently published study (Mario et al. 2012), C. glabrata emerged as the fifth most commonly isolated Candida species in Brazil. Other researchers have also reported on cultures containing mixed Candida species (Torres et al. 2003; Alt-Epping et al. 2012). 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 (Thein et al. 2009; Guinea 2014).

Candida strains grown on polystyrene formed biofilms. A total of 48 strains generated biofilms. The XTT assay showed that the clinical and ATCC strains of C. albicans (Fig 1) did not differ significantly in terms of biofilm production. After 6 h at 37 °C, C. albicans SC5314 presented significantly higher (p < 0.001) biofilm-forming ability than non-albicans Candida strains (Fig 1). In the case of C. tropicalis, at 36 h and 48 h, the difference was smaller (p < 0.01).

Compared with the monospecies C. albicans SC5314 biofilms, the dual-species C. albicans/non-albicans Candida biofilms had significantly different metabolic activity from 18 h (Fig 2). Candida albicans/C. glabrata biofilms presented different metabolic activity after 36–48 h (p < 0.05). For C. albicans/C. krusei and C. albicans/C. parapsilosis biofilms, different metabolic activities were observed from 24 h to 48 h (p < 0.001). Candida albicans/C. rugosa co-cultures have different metabolic activity from 24 h to 36 h (p < 0.001) and from 48 h (p < 0.05). The dual-species C. albicans/C. tropicalis biofilms only presented significantly different metabolic activity after 48 h of incubation at 37 °C (p < 0.001). Overall, analysis of these biofilms showed that C. tropicalis in monoculture or co-cultured with C. albicans formed denser biofilms (Figs 1 and 2). In agreement with previous studies, C. tropicalis was more successful at forming biofilms in comparison with other Candida species (Silva et al. 2011; Marcos-Zambrano et al. 2014). In addition, the biofilm-forming ability and structure are microbial strain- and species-dependent (Silva et al. 2011).

Biofilm formation is an important virulence factor in numerous Candida species (Tumbarello et al. 2007). Katragkou et al. (2010) demonstrated that reduced cytokine production occurs in a biofilm-phagocyte co-culture as compared with a mixture of planktonic cells and phagocytes (phagocytes become entrapped within the structured network of cells and matrix, so they cannot internalize cells within the biofilms). Furthermore, in edentulous patients, prostheses can cause multiple alterations in the oral environment including changes in saliva and in phosphate, calcium, and protein deposits on the acrylic surfaces that are in contact with the
Fig 3 – Total cell numbers (CFU mL⁻¹) from monospecies and dual-species biofilms of C. albicans SC5314 and non-albicans Candida strains. The results were performed on 6, 24, and 48 h at 37 °C. When compared to C. albicans SC5314 at 24 h of
maturation, the results showed a significant decrease of the number of non-albicans Candida in monospecies biofilms, except for C. tropicalis strains. At 48 h, all non-albicans Candida monospecies biofilms present significant decrease in the CFU mL\(^{-1}\). In all the co-cultured biofilms, the total CFU mL\(^{-1}\) of each organism was significantly different from the total CFU mL\(^{-1}\) of the corresponding monospecies Candida biofilms over 24 h–48 h of biofilm generation (\(p < 0.05; p < 0.001\)). In addition, the non-albicans Candida species dominated in all the co-culture biofilms, although the initial concentrations of the two species in the inocula were the same. These results were consistent with the results published by El-Azizi et al. (2004), who reported that addition of C. albicans to preformed C. krusei, Candida lipolytica, or Candida guilliermondii biofilms and the co-incubation of C. albicans with other Candida species like C. krusei, C. glabrata, C. lipolytica, C. guilliermondii, or C. parapsilosis diminishes the number of C. albicans cells within the resulting mixed biofilms. Competitive inhibition may occur even in the initial step of adhesion onto a substrate during dual-species Candida biofilm development (Thein et al. 2009).

Cell numbers from biofilms confirmed visual differences in the biofilm organism constitution. SEM analysis of mixed-species Candida biofilms (Fig 4B–F) displayed a low abundance of hyphae and profuse distribution of blastospores as compared with the reference monospecies C. albicans biofilm (Fig 4A and A1). In agreement with Kirkpatrick et al. (2000) and Thein et al. (2009), interaction of the 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. Recently, Wright et al. (2013), demonstrated that the transition from the planktonic growth mode to a biofilm community prompts major transcriptional and proteomic changes. These modifications occur in response to sensing of diffusible signals, such as autoinducer molecules, and to contact with host tissues or other microbial cells. In addition, the presence of C. glabrata seemed to reduce hyphal development by C. albicans in previous studies (Agwu et al. 2012; Tournu & Van Dijck 2012).

The present study has been the first to report on the in vitro formation of a mixed biofilm containing C. albicans and C. albicans SC5314 biofilm. Note that the C. albicans biofilms (A/A1) consisted of a dense layer of hyphae whereas in dual-species Candida biofilms [(B) – C. albicans plus C. glabrata (P17); (C) – C. albicans plus C. tropicalis (P38); (D) – C. albicans plus C. krusei (P20); (E) – C. albicans plus C. parapsilosis (P16); (F) – C. albicans plus C. rugosa (P14)] single cells (blastospores) predominated.

Fig 4 – Representative scanning electron microscopy images of a dual-species Candida biofilm as compared with the monospecies C. albicans SC5314 biofilm. Note that the C. albicans biofilms (A/A1) consisted of a dense layer of hyphae whereas in dual-species Candida biofilms [(B) – C. albicans plus C. glabrata (P17); (C) – C. albicans plus C. tropicalis (P38); (D) – C. albicans plus C. krusei (P20); (E) – C. albicans plus C. parapsilosis (P16); (F) – C. albicans plus C. rugosa (P14)] single cells (blastospores) predominated.
rugosa strains (Fig 5). Infections caused by less common Candida species have increased in past years (Colombo et al. 2003; Pfaffer et al. 2010; Mohammadi et al. 2015), and C. rugosa has been recognized as an emerging fungal pathogen (Pfaller et al. 2010; Mohammadi et al. 2015; Gosh et al. 2015). This species has been found in Latin America, particularly in Brazil (Colombo et al. 2003; Pfaffer et al. 2010). Moreover, this fungus has lower susceptibility to fluconazole, amphotericin B, and echinocandins (Pfaller et al. 2010; Espinel-Ingoff et al. 2014).

In agreement with Colombo et al. (2003) and Tay et al. (2011), this work obtained isolates from elderly patients, which confirmed that older individuals are at higher risk of infection with C. rugosa. Tay et al. (2011) had previously reported on the ability of C. rugosa isolates to form biofilms on plastic surfaces in vitro. Some authors have associated C. rugosa with catheter-related infections and resistance against therapy (Colombo et al. 2003; Espinel-Ingoff et al. 2014).

This study attested that C. albicans was the Candida species most often associated with colonization of dental prostheses. Non-albicans species like C. tropicalis, C. parapsilosis, C. glabrata, C. krusei, and C. rugosa did not emerge as single colonizers but rather in co-colonization with C. albicans. All the recovered non-albicans Candida species displayed monospecies biofilm-forming ability and formed mixed Candida biofilms with reference strains C. albicans SC5314 in vitro. The mixed communities apparently engaged in an antagonistic relationship because the presence of hyphae (a more invasive form of C. albicans) decreased as compared with monospecies C. albicans biofilms. In addition, the CFU mL⁻¹ of C. albicans SC5314 in all the dual-species biofilms decreased significantly when compared with C. albicans monospecies biofilms. Such information could cast light on Candida species interactions in biofilm communities from dental prostheses isolates and lead to novel approaches for the control of such biofilms.

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References


Fig 5 — Representative electron micrographs of dual-species C. albicans/C. rugosa biofilms. Note that the biofilms display blastospore, hyphae, and pseudohyphae elements. Magnification: A 750×; B 2000×.
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