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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.

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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

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tissues (Pires *et al.* 2002; Pires-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

Table 1 – Distribution of patients studied.

Strains	Patients (number)																							
	Age range 20–49 y								Age range 50–69 y								Age range 70–99 y							
	Female				Male				Female				Male				Female				Male			
	Tp		Pp		T p		Pp		Tp		Pp		Tp		Pp		Tp		Pp		Tp		Pp	
	Col	NC	Col	NC	Col	NC	Col	NC	Col	NC	Col	NC	Col	NC	Col	NC	Col	NC	Col	NC	Col	NC	Col	NC
C. albicans	P3				P5				P2 P4 P6* P14* P15 P18* P28 P37 P44				P13 P21 P22 P24 P25* P34 P35		P9*		P11 P17* P36 P39** P40 P41* P42		P43		P10 P12 P16* P23* P26* P27 P32 P33			
C. glabrata									P18*				P8 P25*				P17* P39** P41*				P19			
C. krusei													P20**											
C. parapsilosis																					P16* P23*			
C. rugosa									P6*	P14*			P20**											
C. tropicalis			P29						P30 P38		P31		P20**		P9*		P39**				P26*			
Absent				P56				P60		P47 P48 P57 P66		P49 P55 P61 P64		P46 P51 P54 P01		P50 P63		P45 P62		P52 P58				P53 P59 P65

Tp: Total prosthesis; Pp: Partial prosthesis; Col: Colonized patient; NC: Non-colonized patient; P: patient; *: patient colonized with two *Candida* species; **: patient colonized with three *Candida* species.

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™ 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 chlamydospore 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

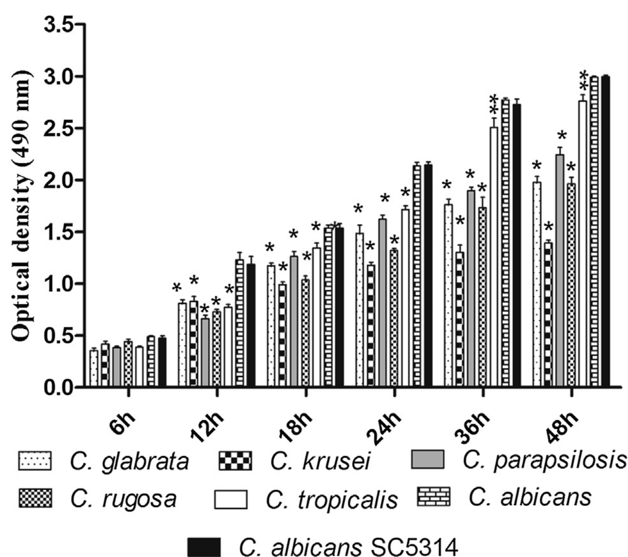

Fig 1 – Biofilm formation of *Candida* monospecies on microtitre plates. Biofilm viability was quantified by the XTT assay. At each time point of biofilm maturation, the optical density of *Candida* monospecies biofilms was compared with the optical density of *C. albicans* SC5314 (black columns, $^*p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

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

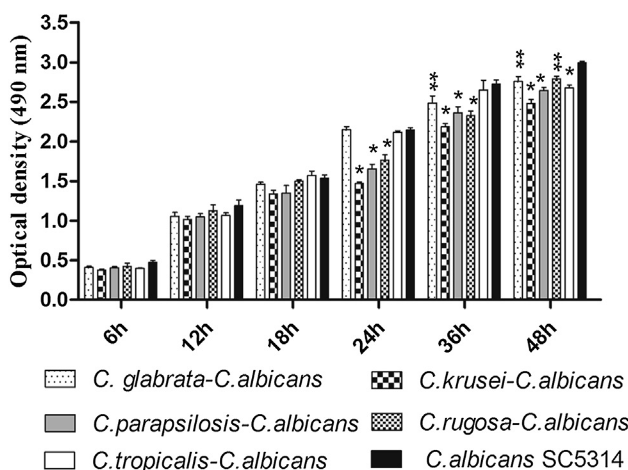

Fig 2 – Biofilm formation of *Candida* dual-species on microtitre plates. Biofilm viability was quantified by the XTT assay. At each time point of biofilm maturation, the optical density of mixed biofilms were compared with the optical density of *C. albicans* SC5314 (black columns, $^*p < 0.001$; $^{**}p < 0.05$) by ANOVA. Data are the means ± SD of experiments performed in quadruplicate and repeated three times.

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 OsO_4 at 1 % (w/v) for 1 h, dehydrated with the aid of an ethanol gradient, critical-point dried in CO_2 (Critical Point 264 Dryer, model CPD-030, Balzers, Oberkochen, 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 acrylic dentures; 11 (17 %) were 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; Filoche *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; Loster *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 1), 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

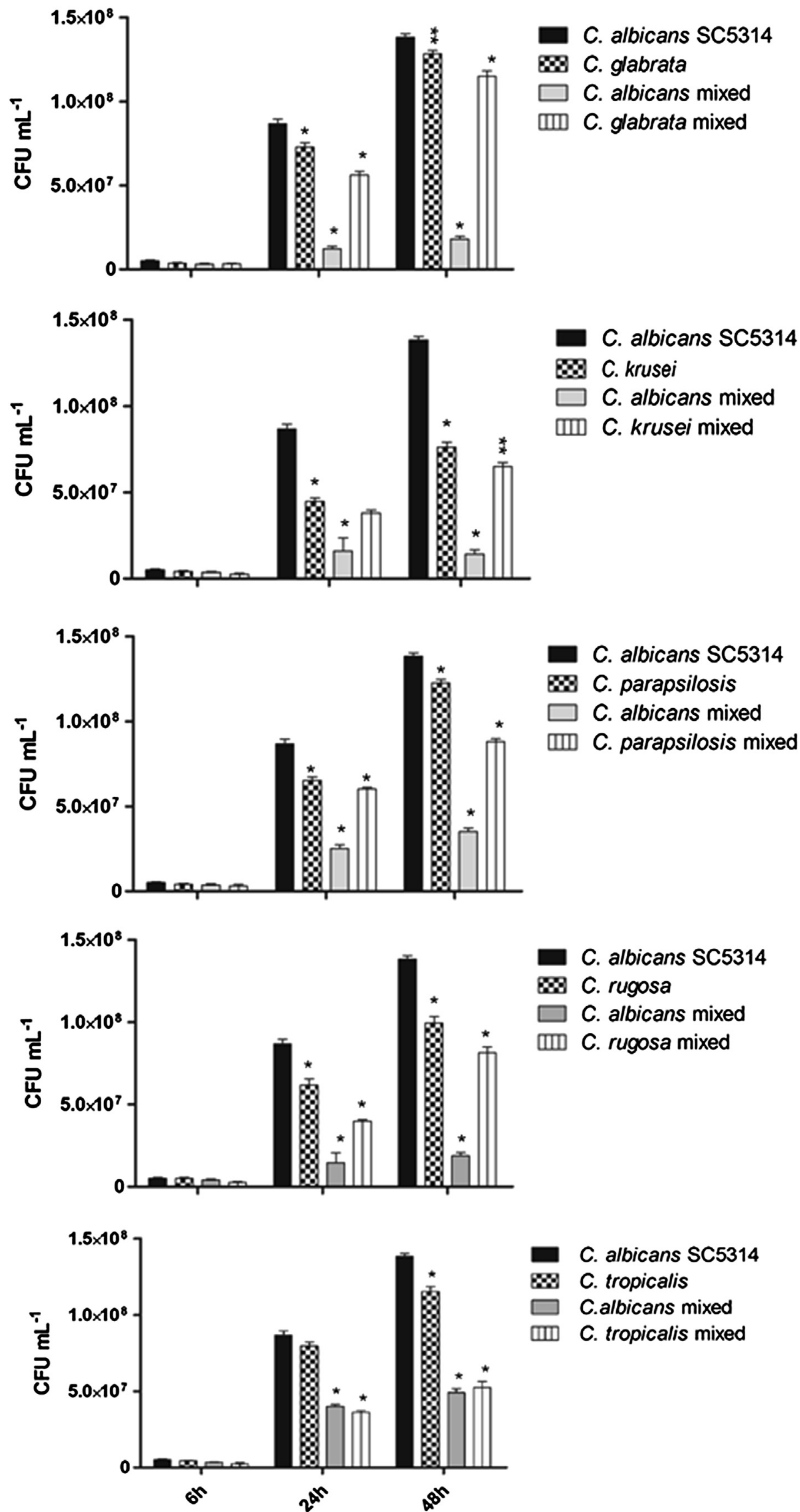

Fig 3 – Total cell numbers (CFU mL^{-1}) from monospecies and dual-species biofilms of *C. albicans* SC5314 and non-*albicans* *Candida* strains. The results were performed on 6, 24, and 48 h at 37 °C. When compared to *C. albicans* SC5314 at 24 h of

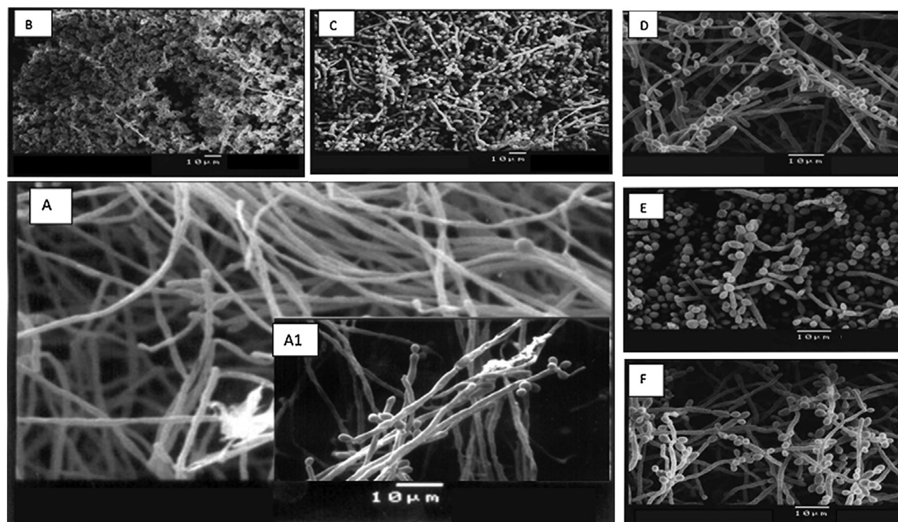

Fig 4 – Representative scanning electron microscopy images of a dual-species *Candida* biofilm as compared with the monospecies *C. albicans* SC5314 biofilm. Note that the *C. albicans* biofilms (A/A_1) consisted of a dense layer of hyphae whereas in dual-species *Candida* biofilms [(B) – *C. albicans* 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.

epithelium, competition between microorganisms for adherence, decreased pH, and increased potential for oxide reduction. As a result, fungal species demand rapid osmotic adaptation, oxidative stress response, and metabolic changes that promote biofilm formation (Sanchez-Vargas *et al.* 2013).

Fig 3 represents the mean values of CFU mL^{-1} obtained for monospecies and dual-species biofilms of *C. albicans* and *Candida* non-*albicans* produced at 6 h (early biofilms), 24 h (intermediate biofilms), and 48 h at 37 °C (mature biofilms). Compared to CFU mL^{-1} of *C. albicans* SC5314 no difference was observed to all non-*albicans Candida* biofilms at 6 h incubation time. At 24 h, CFU mL^{-1} from *C. glabrata*, *C. krusei* and *C. rugosa* differed significantly from the reference strains ($p < 0.001$), although for *C. parapsilosis* this difference was smaller ($p < 0.05$); no difference was observed with *C. tropicalis* (Fig 3). At 48 h incubation, all non-*albicans Candida* biofilms differed significantly from CFU mL^{-1} *C. albicans* SC5314 ($p < 0.001$; *C. glabrata*, $p < 0.05$) (Fig 3). 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 A_1). 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.*

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. Error bars represent the standard deviation of four replicates. *$p < 0.001$; **$p < 0.05$.

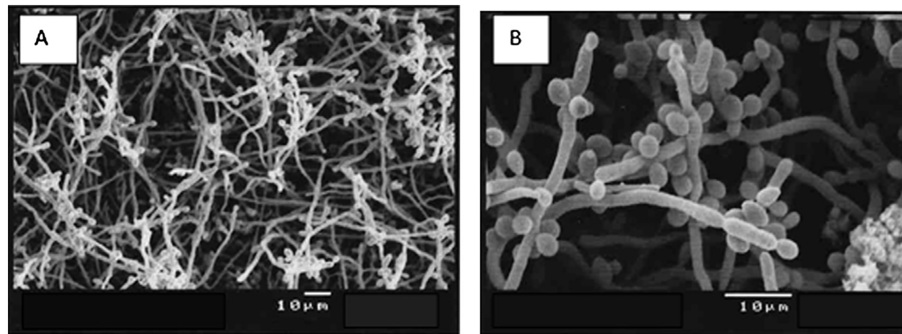

Fig 5 – Representative electron micrographs of dual-species *C. albicans/C. rugosa* biofilms. Note that the biofilms display blastospore, hyphae, and pseudohyphae elements. Magnification: A 750×; B 2000×.

rugosa strains (Fig 5). Infections caused by less common *Candida* species have increased in past years (Colombo *et al.* 2003; Pfaller *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; Ghosh *et al.* 2015). This species has been found in Latin America, particularly in Brazil (Colombo *et al.* 2003; Pfaller *et al.* 2010). Moreover, this fungus has lower susceptibility to fluconazole, amphotericin B, and echinocandins (Pfaller *et al.* 2010; Espinel-Ingroff *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-Ingroff *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*. All 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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