LETÍCIA MACHADO GONÇALVES

"DEVELOPMENT OF *Candida albicans* BIOFILMS IN THE PRESENCE OF FLUCONAZOLE: EFFECTS ON EXTRACELLULAR MATRIX"

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Orientador: Prof. Dr. Wander José da Silva

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Este exemplar corresponde à versão final da tese defendida pela aluna Letícia Machado Gonçalves e orientada pelo Prof. Dr. Wander José da Silva.

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RESUMO

O fluconazol (FLZ) é um antifúngico amplamente utilizado no tratamento da candidose associada ao uso de prótese dental. No entanto, o sucesso desta terapia pode ser dificultado pela presença da matriz extracelular dos biofilmes de *Candida albicans*. Estudos já foram conduzidos avaliando o efeito do FLZ em biofilmes de *C. albicans*, no entanto, estudos que simulem uma condição na qual os biofilmes são desenvolvidos na presença do FLZ são escassos. Ainda, na tentativa de avaliar a organização tridimensional destes biofilmes, várias técnicas de microscopia já foram descritas, apesar de pouca atenção ser dada na análise da matriz extracelular. Frente ao exposto, o objetivo deste trabalho foi avaliar a matriz extracelular de biofilmes de *C. albicans* na presença de concentração salivar de FLZ. Dois estudos foram conduzidos e, em ambos, discos (10 mm x 2 mm) de resina acrílica à base de poli(metil metacrilato) foram confeccionados e cobertos com uma película de saliva sobre a qual biofilmes de *C. albicans* foram desenvolvidos. O primeiro estudo foi conduzido com o objetivo de definir uma metodologia para análise da matriz extracelular. Biofilmes de *C. albicans* ATCC 90028 foram desenvolvidos em meio de cultura sem suplementação (controle) ou suplementado com glicose ou sacarose, por 72 horas. Durante o desenvolvimento dos biofilmes foi adicionado o corante Concanavalina A (ConA) para a marcação da matriz extracelular. Após o desenvolvimento, os biofilmes foram corados com SYTO 9 para a marcação das células. As imagens obtidas por microscopia confocal foram analisadas pelo software COMSTAT. Para confirmação dos resultados, os biofilmes foram coletados e analisados bioquimicamente para determinação da composição da matriz de polissacarídeos pelo método fenol-sulfúrico. Os dados foram analisados por ANOVA seguido do teste de Tukey com nível de significância de 5%. Para o segundo estudo, biofilmes de *C. albicans* (ATCC 90028) e de dois isolados clínicos (P01 e P34) foram desenvolvidos em meio de cultura contendo FLZ na concentração biodisponível na saliva (2,56 µg/mL), durante 48h. O grupo controle foi desenvolvido sem a presença de

fluconazol. O número de células viáveis foi quantificado por diluição seriada, e a matriz extracelular analisada bioquimicamente, além da avaliação por microscopia confocal. Os dados foram analisados pelo teste t de Student com nível de significância de 5%. No primeiro estudo, observou-se que o uso de ConA permitiu uma marcação eficaz da matriz extracelular, confirmada pelo análise bioquímica ($p < 0,05$). No segundo estudo, houve uma redução de 80% no número de células viáveis dos biofilmes desenvolvidos na presença de FLZ ($p < 0,001$). Considerando-se a proporção de polissacarídeos produzidos pelo número de células viáveis, observou-se que nos grupos experimentais, *C. albicans* foi capaz de produzir mais matriz extracelular do que os grupos controle ($p < 0,05$). Este resultado foi confirmado pelas imagens obtidas por microscopia confocal. Conclui-se que a microscopia confocal é uma ferramenta efetiva na análise da matriz extracelular de biofilmes de *C. albicans*, e que os biofilmes de *C. albicans* desenvolvidos na presença de FLZ apresentaram maior produção de matriz extracelular.

Palavras-Chave: *Candida albicans*. Biofilmes. Prótese Dentária. Microscopia Confocal. Fluconazol.

ABSTRACT

Fluconazole (FLZ) is a commonly used antifungal agent to treat patients with *Candida*-associated denture stomatitis. However, the success of this therapy may be compromised by the presence of extracellular matrix of *C. albicans* biofilms. Although studies have been conducting evaluating the effect of FLZ on *C. albicans* biofilms, studies that simulate the condition in which biofilms were allowed to grow in the presence of FLZ are limited. Additionally, in order to evaluate the three-dimensional organization of these biofilms, several microscopic techniques have been described, although little attempt has been paid to assess the extracellular matrix. The objective of this study was to investigate the extracellular matrix of *C. albicans* biofilms in the presence of salivary concentration of FLZ. For this, two studies were conducted. For both studies, discs (10 mm x 2 mm) were fabricated using poly(methyl methacrylate). Salivary pellicle was formed on disc surface and *C. albicans* biofilms were developed. In the first study a methodology for analyzing the extracellular matrix was defined. *C. albicans* ATCC 90028 biofilms were developed in culture media without (control) or with supplementation by glucose or sucrose for 72 hours. During development, biofilms were stained with Concanavalin A (ConA) in order to label the extracellular matrix. After, cells were also labeled with SYTO-9. Images obtained by confocal microscopy were analyzed by COMSTAT software. In order to confirm the results, biofilms were subjected by the biochemical phenol-sulfuric method. Data were analyzed by ANOVA followed by Tukey's test at a significance level set at 5%. For the second study, biofilms of a ATCC 90028 and two clinical isolates (, P01 and P34) were developed for 48 hours. FLZ at 2.56 µg/mL, concentration bioavailable in saliva, was added to culture media of experimental groups. Biofilms were investigated for the number of viable cells by serial dilution and extracellular matrix production was analyzed by phenol-sulfuric method and confocal microscopy. Data were analyzed by Student's t-test, with significance level set at 5%. In the first study, the use of ConA provided an effective labeling of extracellular matrix, which was confirmed by phenol-sulfuric

method ($p < 0.05$). In the second study, a reduction of 80% was observed in the number of viable cells for *C. albicans* biofilms developed in the presence of FLZ ($p < 0.001$). Considering the proportion of polysaccharides produced by the number of viable cells, it was observed that in the experimental groups, *C. albicans* was able to produce more extracellular matrix than the control groups ($p < 0.05$). This result was confirmed by confocal images. It was concluded that confocal microscopy is an effective tool to investigate the extracellular matrix of *C. albicans* biofilms; and *C. albicans* biofilms developed in the presence of FLZ present increased extracellular matrix production.

Keywords: *Candida albicans*. Biofilms. Dental Prosthesis. Microscopy, Confocal. Fluconazole.

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INTRODUÇÃO

A reabilitação com próteses dentais removíveis confeccionadas em poli (metil metacrilato) constitui um tratamento bastante utilizado, seja em pacientes parcial (Zitzmann *et al.,* 2007) ou totalmente edêntulos (Carlsson & Omar, 2010). Após a instalação na cavidade bucal, as próteses dentais são expostas à saliva, sendo passíveis de formação de uma película de proteínas salivares, a qual favorece a adesão de microrganismos (Gocke *et al.,* 2002). A adesão destes microrganismos na superfície das próteses, caso não seja interrompido através de higienização adequada ou por tratamento com antifúngicos ou antimicrobianos (Chandra *et al.,* 2001a), resultará no desenvolvimento de comunidades microbianas conhecidas como biofilmes (Glass *et al.,* 2010).

O acúmulo dos biofilmes na superfície da prótese, além de ser antiestético e contribuir para halitose (de Oliveira *et al.,* 2011), funciona como reservatório de microrganismos com potencial de causar infecções locais e/ou sistêmicas (Glass *et al.,* 2010; Emami *et al.,* 2012). Dentre as infecções locais, a candidose associada ao uso de próteses é a mais comumente encontrada em usuários de próteses dentais removíveis, podendo afetar até 65% desta população (Gendreau & Loewy, 2011). Esta infecção está associada à presença de fungos do gênero *Candida* spp., tendo como principal espécie envolvida em sua etiologia a *Candida albicans* (Emami *et al.,* 2012). Do ponto de vista sistêmico, os microrganismos encontrados no biofilme das próteses podem ser deglutidos ou aspirados, e levar ao desenvolvimento de infecções sistêmicas, tais como a pneumonia aspirativa (Coulthwaite & Verran, 2007; Glass *et al.,* 2010).

Os biofilmes de *C. albicans* constituem comunidades complexas de leveduras e/ou hifas e pseudo-hifas (Theilade *et al.,* 1983; Kulak *et al.,* 1997), as quais interagem entre si durante o processo de colonização da superfície das próteses (Budtz-Jørgensen, 2000). Além disso, os exopolissacarídeos produzidos por estas células são componentes importantes dos biofilmes (Lal *et al.,* 2010; Silva *et al.,* 2012), os quais podem auxiliar no processo de fixação das células

fúngicas ao substrato (Al-Fattani & Douglas 2006). Os exopolissacarídeos envolvem os microrganismos formando uma matriz extracelular, a qual é capaz de conferir proteção diante de possíveis variações ambientais (Flemming & Wingender, 2010).

Na tentativa de restabelecer a saúde bucal de pacientes com candidose associada ao uso de próteses, tem-se preconizado a utilização de fármacos, dentre os quais se destaca o uso do fluconazol (Spellberg *et al.,* 2006). O amplo uso deste antifúngico é atribuído à biodisponibilidade na saliva e/ou plasma, baixa hepatotoxicidade e possibilidade de administração oral ou intravenosa (Garcia-Hermoso *et al.,* 1995; Force & Nahata, 1995; Koks *et al.,* 1996). O mecanismo de ação do fluconazol está relacionado com a inibição da síntese de ergosterol, componente essencial para a manutenção da membrana celular fúngica, afetando sua permeabilidade, alterando sua atividade metabólica e, consequentemente, inibindo o desenvolvimento dos biofilmes (Niimi *et al.,* 2010; Ramage *et al.,* 2012).

Considerando o mecanismo de ação do fluconazol, espera-se que após sua administração, as concentrações biodisponíveis na cavidade bucal sejam suficientes para o êxito no tratamento da candidose associada ao uso de prótese (Force & Nahata, 1995; Mann *et al.,* 2009). Entretanto, algumas dificuldades são mencionadas, como a resistência adquirida pelas cepas e a complexa estrutura dos biofilmes formados na superfície das próteses (Baillie & Douglas, 1999; Bonhomme & d'Enfert 2013). Neste caso, a presença da matriz extracelular parece dificultar a difusão do agente antifúngico para as camadas basais dos biofilmes, limitando sua ação (Niimi *et al.,* 2010; da Silva *et al.,* 2012).

Além de funcionar como barreira física à difusão antifúngica, tem-se sugerido que a matriz extracelular também funciona como barreira química (Nett *et al.,* 2007; Nett *et al.,* 2010; Vediyappan *et al.,* 2010). Neste sentido, componentes da matriz, como os β-glucanos, são capazes de ligar-se quimicamente ao fluconazol (Vediyappan *et al.,* 2010), resultando em acúmulo das drogas na matriz e, consequentemente, permitindo que os microrganismos possam se desenvolver ainda que em elevadas concentrações antifúngicas (Nett *et al.,* 2010). No entanto,

as possíveis alterações na quantidade e qualidade da matriz produzida, bem como organização tridimensional desta quando exposta ao fluconazol, ainda não foram completamente elucidadas.

Na tentativa de avaliar a organização tridimensional dos biofilmes de *C. albicans*, inúmeras técnicas de microscopia já foram utilizadas na literatura (Hawser *et al.,* 1998; Chandra *et al.,* 2001b; Kuhn *et al.,* 2002; Jin *et al.,* 2005). A microscopia confocal a laser, por exemplo, tem sido amplamente empregada na análise de biofilmes fúngicos (Chandra *et al.,* 2001b; Jin *et al.,* 2005; Goncalves *et al.,* 2012; da Silva *et al.,* 2012) por ser uma técnica não-destrutiva e permitir a visualização tridimensional dos biofilmes em condição de hidratação (Mukherjee *et al.,* 2005). No entanto, estudos prévios envolvendo a microscopia confocal têm focado apenas na viabilidade celular (Chandra *et al.,* 2001b; Jin *et al.,* 2005; Goncalves *et al.,* 2012; da Silva *et al.,* 2012), e pouca atenção é dada na investigação da matriz extracelular. Além disso, o uso combinado de corantes que permitam uma marcação eficiente das células de *C. albicans* e polissacarídeos da matriz ainda permanece como limitação.

É importante destacar que vários estudos já foram conduzidos avaliando o efeito do fluconazol em biofilmes de *C. albicans* (Chandra *et al.,* 2001b; Konopka *et al.,* 2010; da Silva *et al.,* 2012). Porém, estes estudos primeiramente formaram os biofilmes e, somente quando estes estavam desenvolvidos, eram submetidos à aplicação única do fluconazol. Estudos que simulem uma condição na qual os biofilmes são desenvolvidos sobre as superfícies das próteses dentais durante uma terapia antifúngica, ou seja, na presença do fluconazol, são escassos (Gomes *et al.,* 2011).

Dessa maneira, utilizando-se biofilmes de *C. albicans* formados em superfície de poli (metil metacrilato), foi simulada uma condição de terapia antifúngica utilizando-se o fluconazol, droga comumente administrada na clínica odontológica. Assim, pode ser hipotetizado que a presença do antifúngico possa alterar o microambiente dos biofilmes, modificando sua organização tridimensional. Neste sentido, visualizar a organização tridimensional dos

microrganismos e da matriz extracelular através de microscopia confocal pode ajudar a elucidar o papel desta como uma barreira física (Niimi *et al.,* 2010). Além disso, sabendo do papel da matriz extracelular como barreira química à ação antifúngica (Nett *et al.,* 2010), é possível que a presença do fluconazol possa interferir na produção da matriz. Estas definições nas alterações da matriz podem gerar importantes subsídios para o desenvolvimento de medidas terapêuticas. Dessa forma, a projeção de agentes que possa romper esta interação antifúngico-matriz pode ajudar a prevenir a recorrência das infecções fúngicas (Nett *et al.,* 2010).

Diante disto, o presente estudo teve por objetivo investigar a matriz extracelular de biofilmes de *C. albicans* desenvolvidos em resina a base de poli (metil metacrilato) na presença de concentração salivar de fluconazol. No capítulo 1 foi apresentada a descrição de uma metodologia para análise da matriz extracelular de biofilmes de *C. albicans* utilizando-se microscopia confocal. Já o capítulo 2, utilizando a metodologia descrita no capítulo anterior, objetivou investigar a matriz extracelular de biofilmes de *C. albicans* desenvolvidos na presença de fluconazol.

CAPÍTULO 1*

Confocal analysis of the exopolysaccharide matrix of *Candida albicans* biofilms

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Abstract

The confocal laser-scanning microscopy (CLSM) was carried out to investigate the exopolysaccharide matrix of *Candida albicans* biofilms developed on denture material under dietary carbohydrate exposure. Biofilms were developed on poly (methyl methacrylate) discs in culture media without (control) or with supplementation by glucose or sucrose for 72 h. For CLSM analysis, biofilms were labeled with concavalin A (ConA) during its development. After, biofilms were also labeled with SYTO-9. To confirm the results, the matrix was investigated by phenol-sulfuric method. Data were analyzed by ANOVA followed by Tukey's test with the level of significance set at 5%. The use of ConA during biofilm development provided an effective labeling of exopolysaccharide matrix. The exposure to sucrose resulted in biofilms with the highest exopolysaccharide matrix biovolume ($p < 0.05$). The characterization obtained by CLSM was confirmed by phenol-sulfuric method. CLSM was an effective tool to investigate the exopolysaccharide matrix of *C. albicans* biofilms, and the exposure to sucrose resulted in increased matrix production.

Keywords: Confocal laser scanning microscopy, Fungal infections, *Candida albicans*, Biofilms, Extracellular Matrix and Denture bases.

Introduction

Candida albicans is the main pathogen responsible for the development of *Candida*-associated denture stomatitis (CADS), a common infection observed in denture wearers (Emami *et al.*, 2012). When colonizing the acrylic denture surface and also the mucosa underlying denture, the yeast cells are predominantly organized as biofilms, communities encased within an extracellular matrix (Mukherjee *et al.*, 2005; Seneviratne *et al.*, 2008; Ramage *et al.*, 2009). This matrix is composed mainly of polysaccharides and small amounts of proteins (Baillie &

Douglas, 2000; Al-Fattani & Douglas, 2006; Lal *et al.*, 2010), and it creates a three-dimensional environment that is important for both biofilm integrity and its resistance to antifungal agents (Ramage *et al.*, 2009; Tobudic *et al.*, 2012). Although it is known that *C. albicans* is capable to produce exopolysaccharide matrix (Hawser *et al.*, 1998; Al-Fattani & Douglas, 2006), it is important to highlight that the amount produced is dependent of environmental factors, being particularly relevant the availability of nutrients (Seneviratne *et al.*, 2008).

In the oral cavity, the carbohydrates consumed in the diet are the primary and preferred nutrient sources for *C. albicans* (Samaranayake & MacFarlane, 1982; Jin *et al.,* 2004; Seneviratne *et al.,* 2009; Santana *et al.,* 2013). Additionally, denture wearers tend to choose a carbohydrate-rich diet because of difficulty in chewing (Lyon *et al.,* 2006). It is known that the constant supply of sugars may create an environment conductive to *C. albicans* colonization (Jin *et al.,* 2004; Seneviratne *et al.,* 2009). Also, it is expected that dietary carbohydrates could provoke structural changes on *C. albicans* biofilms (Heydorn *et al.,* 2000; Santana *et al.,* 2013). Among these sugars, it has been showed that fermentable carbohydrates such as sucrose and glucose serve as substrates for exopolysaccharide matrix synthesis in bacterial biofilms models (Cury *et al.,* 2000; Aires *et al.,* 2008; Xiao & Koo, 2010). So far, however, there has been little discussion about the effects of carbohydrates in the structural basis and also production of exopolysaccharide matrix using a *C. albicans* biofilm model.

In order to examine the structure of biofilms, a number of microscopic techniques have been used (Hawser *et al.,*1998; Chandra *et al.,* 2001; Kuhn *et al.,* 2002; Jin *et al.,* 2005). In this context, the confocal-laser scanning microscopy (CLSM) surged as a valuable tool for studying both bacterial (Klein *et al.,* 2009; Xiao & Koo, 2010) and fungal biofilms (Chandra *et al.,* 2001; Jin *et al.,*2005; da Silva *et al.,* 2010; Goncalves *et al.,* 2012), allowing a three-dimensional visualization of hydrated and undisturbed biofilms (Mukherjee *et al.,* 2005). However, previous studies using CLSM technology in candidal biofilms had focused mainly on cell viability (Chandra *et al.,* 2001; da Silva *et al.,* 2010;

Goncalves *et al.,* 2012), and little attempt has been paid to assess the exopolysaccharide matrix (Chandra *et al.,* 2001). Furthermore, the combined use of fluorescent stains which permit an effective labeling of both yeast cells and matrix still remains as a limitation. Considering this, the objective of this study was to investigate the exopolysaccharide matrix of *C. albicans* biofilms developed on the denture surface under dietary carbohydrate exposure by CLSM.

Materials and methods

Study design

This *in vitro* study had a randomized and blinded design. Poly (methyl methacrylate) (PMMA) acrylic resin discs with standardized surface roughness were used as substrata for *C. albicans* biofilm development. Biofilms were developed in culture media without (control) or with supplementation by glucose or sucrose for 72 h. During development, biofilms were exposed to Concanavalin A (ConA) in order to label the exopolysaccharide matrix. After development, yeast cells were also labeled with SYTO-9. For CLSM analysis, the biofilms were scanned in a multi-stack mode. The z-slices were subjected to the COMSTAT software, which allowed the quantification of structural parameters such as biovolume and distribution on disc surface. In order to confirm the CLSM results, it was performed a biochemical analysis of the exopolysaccharide matrix using the phenol-sulfuric method. Data were analyzed by ANOVA followed by Tukey's test at a significance level set at 5%. All of the experiments were performed in five replicates of three independent experiments on different days (n = 15).

Fabrication of discs

Discs were fabricated using a water bath PMMA acrylic resin (QC-20; Dentsply Ltd., Weybridge, England) according to manufacturers' directions. Prior to polymerization cycle (100^{o}C for 20 min), the resin mass in the plastic phase was packed in a metal mould to standardize the disc dimensions (10 mm in diameter, 2

mm in thickness). Processed discs were immersed in distilled water for 48 h at ± 35 ^{o}C to release residual monomer (Moura *et al.,* 2006). To simulate the inner side of a denture and to standardize the surface roughness in 0.30 ± 0.04 µm (Quirynen et al. 1990), the discs surfaces were grounded in a horizontal polisher (model APL-4; Arotec, São Paulo, Brazil) by using progressively smoother aluminum oxide papers (320, 400, and 600 grit). The surface roughness was analyzed by a profilometer (Surfcoder SE 1700; Kosaka Laboratory Ltd., Kosaka, Japan) accurate to the nearest 0.01-µm, calibrated at a disc length of 0.8 mm, 2.4-mm percussion of measure, and 0.5 mm/s. The mean of three measurements of each disc was calculated. After this measurement, the discs were ultrasonically cleansed (Thornton T 740; Thornton-Inpec Eletronica Ltd., São Paulo, Brazil) in distilled water for 20 min to remove any contaminants and artifacts. The discs were disinfected with sodium hypochlorite 0.5% for three minutes and washed three times with sterilized water.

Preparation of C. albicans suspensions

C. albicans (ATCC 90028, reference strain) was aerobically cultured from their original broth by its incubation in Sabouraud Dextrose Agar (SDA; Difco, Michigan, USA) for 24 h at ± 35 °C. A loop of grown colonies was inoculated into Yeast Nitrogen Base culture medium (YNB; Difco) supplemented with 50 mM glucose and incubated aerobically in an orbital shaker (NT 151; Nova Tecnica Laboratory, São Paulo, Brazil) at ± 35 °C. When *C. albicans* cells were in the exponential growth phase, which occurred after 18-20 h of incubation, they were washed twice with phosphate-buffered saline (PBS; pH 7.2). Then, suspensions were prepared in YNB culture medium without (control group) or supplemented with 100 mM glucose or 100 mM sucrose. These suspensions were standardized spectrophotometrically (Spectronic 20; Bausch & Lomb, New York, USA) at approximately 10^{7} cells/mL (0.25 at 520 nm).

Biofilm development

In order to mimic the oral cavity, the discs were coated with human saliva prior to biofilm development. For this, human saliva was collected from a single volunteer, who had not used antibiotics, mouth rinses or any other medication that may affect salivary composition and flow in the past 3 months (Vieira *et al.,* 2010). The volunteer provided written formal consent according to a protocol approved by the Local Ethical Committee in Research (#082/2010). For every experiment, the saliva sample was collected at the same time of the day and the volume was limited to 50 mL per collection period. Saliva was collected during masticatory stimulation with flexible film in an ice-chilled polypropylene tube and clarified by centrifugation (10.000 x g for 5 min at 4 ^{o}C). The supernatant was filter-sterilized and immediately used. Under aseptic conditions, each disc was placed inside a 24-well culture plate and an aliquot of saliva was added to each well. The plate was incubated aerobically in an orbital shaker for 1 h at ± 35 °C to form the salivary pellicle.

Next, saliva-coated discs were transferred to another 24-well culture plate, and the *C. albicans* suspension previously prepared was added to each well. These sets were incubated aerobically in an orbital shaker for 90 min at ± 35 °C (adhesion phase). Thereafter, the discs were carefully washed with PBS and transferred to new 24-well culture plates containing YNB culture medium without carbohydrate supplementation or supplemented with glucose or sucrose, as stated before. At the end of each 24 h, the discs were washed with PBS followed by the addition of fresh media. At each media change, 50 μM Concanavalin A conjugate (ConA-tetramethylrhodamine; Invitrogen Molecular Probes Inc., Oregon, USA) was added to the culture media to label the exopolysaccharide matrix during its synthesis. The concentration of ConA was determined in pilot studies (data not show). The plates were incubated aerobically in an orbital shaker, protected from light, for 72 h at ± 35 °C.

Confocal laser scanning microscopy analysis (CLSM)

For CLSM analysis, the biofilm-containing discs were carefully washed with purified water and, afterwards, the yeast cells were labeled using 2.5 µM SYTO 9 fluorescent stain (Invitrogen Molecular Probes Inc.). Then, the biofilms were incubated protected from light for 20 min at ± 35°C. As described before, the exopolysaccharide matrix was labeled previously with 50 µM ConA.

The biofilm structural organization was examined using a Leica microscope TCS SP5 (Leica Microsystems CMS, Mannheim, Germany) with a 63x, 0.8 numerical aperture oil-immersion objective lens. This CLSM was equipped with an argon laser tuned at 488 nm and helium-neon laser tuned at 543 nm for simultaneous measurement of SYTO-9 (green; 480/500) and ConA (red; 555/580) in a multi-stack mode. The images were acquired by the software LAS AF (Leica Microsystems CMS, Mannheim, GE) at a resolution of 1024 x 1024 pixels and line average of 16. To assess the structure of the biofilms, a series of optical sections at 1-µm intervals in the z-axis were taken throughout the full depth of the biofilm. At least 5 representative randomly optical fields were scanned for each disc.

The biofilm three-dimensional reconstruction was processed using the ImageJ software (Research Services Branch, Bethesda, Maryland, USA). The series of images were imported to the software and three-dimensional reconstructions were created using the interactive 3D surface plot plugin. Additionally, the confocal image stacks were subjected to the image-processing COMSTAT software (Heydorn *et al.,* 2000). In this study, the structural parameters investigated were the biovolume ($\mu m^3/\mu m^2$) and biofilm coverage (µm) on disc surface.

Exopolysaccharide matrix analysis by phenol-sulfuric method

In order to confirm CLSM results, it was used a well-documented biochemical methodology to analyze the exopolysaccharide matrix (Dubois *et al.,* 1951). For this, biofilms were developed for 72 h, as described before without ConA labeling. Thereafter, the biofilms were washed twice and transferred to

plastic tubes containing PBS. This set was sonicated at 7 W for 30 s. An aliquot of this solution was centrifuged (10.000 x *g* for 5 min at 4 °C) and the supernatant containing the soluble extracellular polysaccharides (SEPs) was transferred to a plastic tube named SEPs. To the residual pellet, an aliquot of 1 M NaOH was added for the insoluble extracellular polysaccharides (IEPs) extraction. The tube was agitated for 15 min, centrifuged, and the supernatant was transferred to a plastic tube named IEPs. To the SEPs and IEPs plastic tube, 3 volumes of cold ethanol were added and maintained for 30 min at – 20 °C. Next, the tubes were centrifuged and the pellet was washed twice with cold 75% ethanol. Polysaccharides precipitated were resuspended in 1 M NaOH, and the total carbohydrate was estimated by the phenol-sulfuric method (Dubois et al. 1951), using glucose as standard. The results were normalized by dry weight of biofilm. The sum of SEPs and IEPs values was used to estimate the total amount of exopolysaccharide matrix.

Statistical analysis

All analyses were performed using the SAS software (SAS Institute Inc., version 9.0; Cary, NC, USA) with the level of significance set at 5%. The assumptions of equality of variances and normal distribution of errors were checked and the data were transformed as suggested by the software: biovolume (exponentiation, y2) and extracellular polysaccharide (Log10). All data were analyzed using one-way ANOVA followed by Tukey's test.

Results

Representative confocal images and three-dimensional reconstructions of *C. albicans* biofilms were showed in Figure 1. The combined used of SYTO-9 and ConA provided an effective labeling of both yeast cells and exopolysaccharide matrix under the different experimental conditions. CLSM images and reconstructions showed that biofilms developed in the presence of sucrose tend to be more densely cellularized compared to the other groups. In addition, the exposure to sucrose also resulted in large amounts of exopolysaccharide matrix.

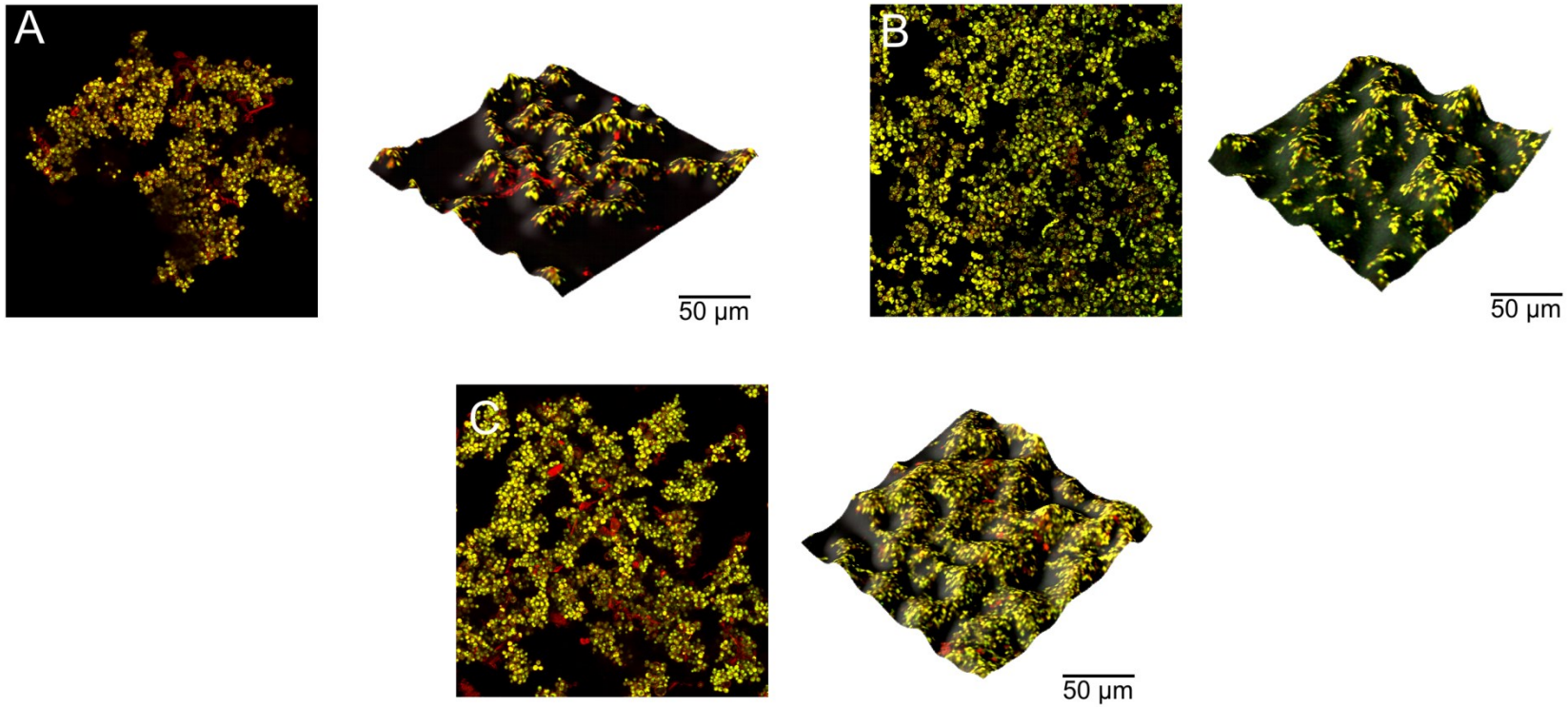

Figure 1. Confocal images and three-dimensional reconstructions of *C. albicans* biofilms developed on denture material: **(A)** Control; **(B)** Glucose; **(C)** Sucrose. SYTO-9 stained the yeast cells in green, while ConA highlighted the cell walls and stained the exopolysaccharide matrix in red.

The biovolume of *C. albicans* biofilms was significantly affected by the type of carbohydrate exposure ($p < 0.05$, Table 1). COMSTAT analysis confirmed that there was an increase in the biovolume of both yeast cells and exopolysaccharide matrix in biofilms developed in the presence of sucrose ($p < 0.05$). The control group presented the lowest bio-volume of yeast cells ($p < 0.05$). The presence of glucose showed similar production of exopolysaccharide matrix when compared to the control group ($p < 0.05$). The results obtained by the phenol-sulfuric method indicated that the biofilms exposed to sucrose resulted in the highest production of exopolysaccharide matrix ($p < 0.001$, Table 2), confirming the CLSM results.

Table 1. Biovolume of *C. albicans* biofilms developed on denture material obtained by three-dimensional computerized analysis (mean ± s.d., n=15).

Carbohydrate Group	**Biovolume ($\mu m^3.\mu m^{-2}$)**		
	Yeast cells	**Exopolysaccharide matrix**	**Total biofilm**
Control	0.68 ± 0.25 A	0.79 ± 0.22 A	1.47 ± 0.47 A
Glucose	1.55 ± 0.53 B	0.58 ± 0.06 A	2.13 ± 0.56 B
Sucrose	2.39 ± 0.29 C	1.31 ± 0.13 B	3.70 ± 0.39 C

Different letters indicate that are significant differences between the groups tested (ANOVA, Tukey HDS, $p < 0.05$).

Table 2. Exopolysaccharide matrix (µg of polysaccharides/mg dry weight) of *C. albicans* biofilms developed on denture material obtained by phenol-sulfuric method (Means ± s.d., n=15).

Carbohydrate Group	**Exopolysaccharide matrix**
Control	3.14 ± 1.24 A
Glucose	4.67 ± 1.12 A
Sucrose	12.22 ± 4.27 B

Different letters indicate that are significant differences between the groups tested (ANOVA, Tukey HDS, $p < 0.05$).

Regarding the biofilm distribution, the control group revealed a poor coverage of PMMA surface discs; while the presence of sucrose resulted in the highest biofilm coverage. Furthermore, the control was the only group that showed the exopolysaccharide matrix involving the yeast cells. In contrast, in glucose and sucrose groups the exopolysaccharide matrix was found interspersed between the yeast cells (Figure 2).

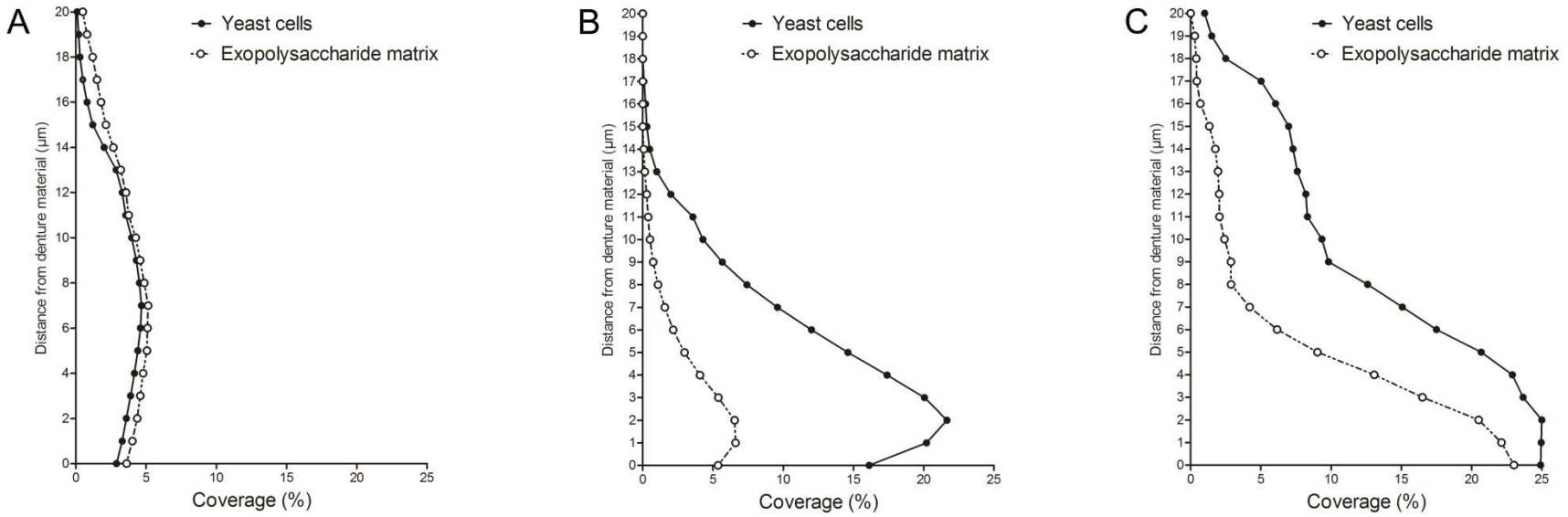

Figure 2. Yeast cells and exopolysaccharide matrix distribution of *C. albicans* biofilms developed on denture material obtained by three-dimensional computerized analysis: **(A)** Control; **(B)** Glucose; **(C)** Sucrose. In image A, the exopolysaccharide matrix is found involving the yeast cells. In images B and C, the exopolysaccharide matrix is found interspersed between the yeast cells.

Discussion

Several microscopic techniques have been used for examination of candidal biofilm structure, such as scanning electron microscopy in combination with an ice-freezing technique (Hawser *et al.*, 1998) and fluorescence microscopy (Chandra *et al.,* 2001; Kuhn *et al.,* 2002). However, these techniques are time-consuming, and neither can demonstrate the three-dimensional distribution of exopolysaccharide matrix or differentiate matrix components from the yeast cells (Jin *et al.,* 2005). In the present study it was employed the CLSM technique, a widely used tool in *C. albicans* biofilms (Chandra *et al.,* 2001; Jin *et al.,* 2005; da Silva *et al.,* 2010; Goncalves *et al.,* 2012), which is particularly advantageous in analyzing biofilms without disturbing its structure (Mukherjee *et al.,* 2005). In the *C. albicans* biofilm model it was simulated a clinical condition of CADS, in which the biofilms were constantly exposed to carbohydrates consumed in diet. Thus, aiming to analyze the effects of such carbon sources on exopolysaccharide matrix production, it was combined the use of SYTO-9 and ConA as stains, which allowed an effective labeling of both yeast cells and exopolysaccharide matrix, respectively.

SYTO-9 is a small molecule with the capacity to penetrate the fungal cell wall with relative ease and stain the cells in green (Jin *et al.,* 2005). Previous studies have used FUN-1, a yeast-specific fluorescent stain (Kuhn *et al.,* 2002; Suci & Tyler, 2003), however this stain leads the yeast cells to produce cylindrical intra-vacuolar structures (CIVS) to emit fluorescence. Also, it was noted that in the presence of hyphae forms, FUN-1 can yield multiple CIVS in a same cell, leading an overestimated population (Suci & Tyler, 2003). Additionally, during FUN-1 staining, the presence of nutrients is a prerequisite for CIVS production (Kuhn *et al.,* 2002; Jin *et al.,* 2005), which is a particularly important limitation, considering that different carbohydrates sources provided during biofilm development could affect the labeling process (Seneviratne *et al.,* 2009). When labeled with SYTO-9 the whole cell is uniformly stained regardless its morphology. Additionally, the use of SYTO-9 is based on the membrane integrity instead of the metabolic activity of

cells (Jin *et al.,* 2005) and therefore, it was a suitable choice for cells visualization in the present study.

In the present investigation, it was also used ConA for exopolysaccharide matrix examinations. It has been showed that the extracellular matrix isolated from *C. albicans* biofilms is mainly consisted by glucose and mannose residues (Baillie & Douglas, 2000; Al-Fattani & Douglas, 2006; Lal *et al.,* 2010). Thus, considering that ConA specifically binds to carbohydrates residues of the cell wall membrane, it was an ideal candidate for the exopolysaccharide matrix visualization in the experimental conditions tested, as confirmed by the CLSM images obtained. In fact, ConA has been commonly used for this purpose (Chandra *et al.,* 2001; Suci & Tyler, 2003; Andes *et al.,* 2004; Jin et al. 2005; Lal *et al.,* 2010). However, it has not been used labeling exopolysaccharide matrix during the biofilm development, neither in combination with SYTO-9. The fluorescent stain served as a primer being simultaneously incorporated during exopolysaccharide matrix synthesis over the course of biofilm development (Klein *et al.,* 2009). Although ConA staining was also performed only after biofilm development in the pilot studies (data not shown), the better results were found when this stain was incorporated during matrix synthesis.

Regarding the images obtained by the combined use of SYTO-9 and ConA, it is notorious that CLSM was an effective tool in analyzing the structural changes of *C. albicans* biofilms under the conditions tested. As the images clearly shows, the exopolysaccharide matrix produced was represented as a cell wall-like projection of carbohydrates residues. There was an uneven exopolysaccharide matrix distribution in the glucose and sucrose groups, which demonstrates a heterogeneous synthesis within the biofilm compartments. In contrast, a more homogeneous synthesis was observed in the control group, where the matrix is mainly found involving the yeast cells. Furthermore, considering the biofilm global structure, the images also showed a typical micro-colony and water channel architecture, as previously described (da Silva *et al.,* 2010).

To gain refined knowledge about the biofilm characterization, the z-slices images were also subjected to the COMSTAT software (Heydorn *et al.,* 2000). According to this mathematical model, the biovolume provides an estimation of biofilm biomass, which can be defined as the biomass volume divided by substratum area. On the other hand, the biofilm distribution could also be assessed measuring the percentage of coverage of the substrate by the biofilm (Heydorn *et al.,* 2000). Thus, separating the channels provided by SYTO-9, ConA and also overlaying them, we estimated the biovolume and distribution of the yeast cells, exopolysaccharide matrix and total biofilm.

It was observed that sucrose allowed the development of robust biofilms, presenting not only the highest biovolume, but also the highest coverage of disc surface. This result was expected, since the hydrolysis of this disaccharide into glucose requires little energy expenditure, while provides great amount of easily metabolizable carbon sources (Samaranayake & MacFarlane, 1982). Furthermore, similar with previous reports using bacterial biofilm models (Cury *et al.,* 2000; Aires *et al.,* 2008; Xiao & Koo, 2010), it seems that sucrose served as the main important substrate for the synthesis of the exopolysaccharide matrix in candidal biofilms. These results were also confirmed by phenol-sulfuric analysis (Dubois *et al.,* 1951). This result seems to be very important, given that the exopolysaccharide matrix creates a 3D environment that is important for both the biofilm integrity and its resistance to antifungal agents (Ramage *et al.,* 2009; Tobudic *et al.,* 2012). It may be that the sucrose can trigger *C. albicans* responses at the transcriptional level (Klein *et al.,* 2009; Santana *et al.,* 2013), modulating the expression of genes associated with exopolysaccharide matrix formation. Nevertheless, the exact mechanism by which dietary carbohydrates enhance exopolysaccharide matrix production by *C. albicans* biofilms requires future investigation.

It was also noted that biofilms developed in the control group presented similar biovolume of exopolysaccharide matrix compared to glucose. Particularly in the control group, it was possible that the lack of nutrients could lead a stress situation, and consequently, candidal cells secreted great amounts of

exopolysaccharide matrix in order to protect itself from other environmental challenges (Ene *et al.,* 2012). Overall, it is supposed that these structural changes are beneficial for survival under nutrient-limited conditions (Santana *et al.,* 2013).

Taken together, the results suggested that the presence of carbohydrates markedly affected the exopolysaccharide matrix production of *C. albicans* biofilms. The results inferred that the use of ConA during biofilm development combined with SYTO-9 can be successfully used for visualization and quantification of both yeast cells and exopolysaccharide matrix.

Conclusions

Within the limits of this study, it was concluded that CLSM was an effective tool to investigate the exopolysaccharide matrix of *C. albicans* biofilms developed on the denture material, and the exposure to sucrose resulted in increased exopolysaccharide matrix production.

Conflict of interest

The author(s) declare no conflicts of interest with respect to the authorship and/or publication of this article.

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CAPÍTULO 2*

Candida albicans biofilms developed in the presence of fluconazole increase exopolysaccharide matrix production

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Abstract

Objective: The purpose of this study was to investigate whether the presence of fluconazole (FLZ) could affect the exopolysaccharide matrix produced by *Candida albicans* biofilms. Materials and methods: Poly (methyl methacrylate) acrylic resin discs were fabricated and had their surface roughness standardized. Salivary pellicle was formed on the PMMA surface, and biofilms of a reference strain (ATCC 90028) and two clinical isolates of *C. albicans* (P01 and P34) were developed. FLZ at 2.56 µg/mL concentration was added to the culture media of the experimental groups. The culture media was changed each 24 h until 48 h of biofilm development. Biofilms were investigated for number of viable cells by serial dilution and production of exopolysaccharide matrix by phenol sulfuric method and confocal microscopy. Data were analyzed by the independent Student's t-test, with the significance level set at 5%. Results: A significant reduction in the number of viable cells was found for *C. albicans* biofilms developed in the presence of FLZ ($p < 0.001$). Confocal images also demonstrated the increased number of dead cells in the experimental group. Considering the proportion between the number of viable cells and the production of exopolysaccharide, it was observed that in the experimental groups, *C. albicans* was able to produce more exopolysaccharide than the control groups ($p < 0.05$), as seen in confocal analysis. Conclusion: *C. albicans* biofilms developed in the presence of FLZ increased the exopolysaccharide matrix production.

Keywords: Biofilms, *Candida albicans*, Extracellular Matrix and Fluconazole.

Introduction

A large percentage of denture wearers are affected by *Candida*-associated denture stomatitis (CADS), with *C. albicans* being identified as the main pathogen of this condition (Gendreau & Loewy, 2011; Emami *et al.,* 2012). After colonizing the acrylic denture surface, the yeast cells are predominantly organized as biofilms, communities involved within an extracellular matrix (Mukherjee *et al.,* 2005; Ramage *et al.,* 2009). The extracellular matrix is composed mainly of polysaccharides and small amounts of proteins (Al-Fattani & Douglas, 2006; Lal *et al.,* 2010), creating a three-dimensional environment that preserve the architectural integrity of fungal biofilms and contributes to their antifungal resistance (Flemming & Wingender, 2010; Tobudic *et al.,* 2012; Bonhomme & d'Enfert, 2013).

Fluconazole (FLZ) is a commonly used antifungal agent to treat patients with CADS (Konopka *et al.,* 2010) due to its high bioavailability, low hepatotoxicity, reduced cost and the possibility of being administrated orally or intravenously (Spellberg *et al.,* 2006). Its mechanism of action is direct related to alterations in biofilm structural levels (Chandra *et al.,* 2001; Ramage *et al.,* 2012) acting as a fungistatic agent that interferes in the ergosterol biosynthesis and, consequently, the fungal cell permeability (Ramage *et al.,* 2012).

Yet, studies have emphasized an important role of the exopolysaccharide matrix in the tolerance of *C. albicans* biofilms to FLZ (Nett *et al.,* 2007; Vediyappan *et al.,* 2010; Nett *et al.,* 2010; da Silva *et al.,* 2012). The exopolysaccharide matrix could act as a physical barrier and/or sequestering azole antifungals, therefore preventing their access to biofilm cells and the elicitation of cellular responses (Nett *et al.,* 2010). It is important to highlight that these studies evaluating the effect of FLZ on *C. albicans* biofilms have been conducted using FLZ after biofilm development, condition where the exopolysaccharide matrix is already structured.

So far however, studies that have simulated the condition in which *C. albicans* biofilms were allowed to grow on denture surfaces whilst the patients were undergoing FLZ therapy are poorly found in the current literature (Gomes *et al.,*

2011). Considering the mechanism of action of FLZ, it is expected that the presence of FLZ could provoke changes in biofilm development, particularly in the structural organization and exopolysaccharide matrix production. Thus, the purpose of this study was to investigate whether the presence of FLZ could affect the exopolysaccharide matrix produced by *C. albicans*.

Materials and methods

Experimental design

This *in vitro* study had a randomized and blinded design. Discs were fabricated using a water bath poly (methyl methacrylate) (PMMA) acrylic resin. For standard purposes, the surface roughness of all discs was measured. Salivary pellicle was formed on the PMMA surface, and biofilms of a reference strain (ATCC 90028) and two clinical isolates of *C. albicans* (P01 and P34) were developed. FLZ at the bioavailable concentration in saliva (2.56 µg/mL) was added to the culture media of experimental groups. The culture media of control and experimental groups were changed after 24 h until 48 h of development. Biofilms were investigated for the number of viable cells and production of extracellular matrix. All of the experiments were performed in four replicates of three independent experiments on different days (n = 12). Data were analyzed by the independent Student's t-test, with the significance level set at 5%.

Fabrication of discs

Discs (10 mm diameter, 2 mm thickness) were fabricated using a water bath PMMA acrylic resin (QC-20; Dentsply Ltd., Weybridge, England) according to manufacturers' directions using a metal mould. Processed discs were immersed in distilled water for 48 h at 35 °C to release residual monomer (Moura *et al.,* 2006). To simulate the inner side of a denture, the disc surfaces were ground in an horizontal polisher (model APL-4; Arotec, São Paulo, Brazil) by using progressively smoother aluminum oxide papers (320, 400, and 600 grit). The surface roughness

of all discs was analyzed by a profilometer (Surfcoder SE 1700; Kosaka Laboratory Ltd., Kosaka, Japan) accurate to the nearest 0.01 μm and calibrated at a disc length of 0.8 mm, 2.4 mm percussion of measure, and 0.5 mm/s. The mean of three measurements for each disc was calculated, and the surface roughness was standardized at 0.30 ± 0.04 μm (Santana *et al.,* 2013).

After this measurement had been made, the discs were ultrasonically cleansed (Thornton T 740; Thornton-Inpec Eletronica Ltd., Vinhedo, São Paulo, Brazil) in purified water for 20 min to remove any contaminants and artifacts. The discs were also disinfected with sodium hypochlorite 0.5% for three minutes and washed three times with sterilized water.

Preparation of C. albicans suspension

C. albicans reference strain (ATCC 90028) and two clinical isolates (P01 and P34) were selected for this study. The clinical isolates were obtained from the surface of dentures of patients without symptoms of CADS. Before the experimental procedures, the identity of all isolates was reconfirmed by the CHROMagar® *Candida* test (Difco Laboratories, Detroit, MI, USA) and the carbohydrate assimilation test using Vitek-2 identification system (bioMe´rieux, Marcy l'Etoile, France).

C. albicans strains were grown aerobically on Sabouraud Dextrose Agar (SDA; Difco) for 24 h at 35 °C. A loop of yeast cells was inoculated into Yeast Nitrogen Base culture medium (YNB; Difco) supplemented with 50 mM glucose and incubated aerobically under agitation at 35 °C. During the exponential growth phase (*i.e.*, after 18–20 h of incubation), *C. albicans* cells were washed twice with phosphate-buffered saline (PBS; pH 7.2). Next, the yeast cells were resuspended in YNB medium supplemented with 100 mM glucose, and the suspensions were standardized spectrophotometrically (Spectronic 20; Bausch & Lomb, Rochester, NY, US) to ~10^7 cells/mL (OD = 0.25 at 520 nm).

Biofilm development

To mimic the oral cavity, the discs were coated with human salivary pellicle prior to biofilm development. For this, stimulated human saliva was collected from two healthy volunteers, centrifuged (10,000 × *g* for 5 min at 4 ºC), and the supernatant was filtered-sterilized and immediately used. The volunteers provided written formal consent according to a protocol approved by the Ethics Committee in Research of Piracicaba Dental School (#042/2008). Under aseptic conditions, each disc was placed in a 24-well culture plate and two milliliters of saliva were added to each well. The plate was incubated aerobically under agitation for 1 h at 35 °C to form the salivary pellicle.

Saliva-coated discs were transferred to another 24-well culture plate, and 2 mL of standard suspensions (~10^7 cells/mL) were added to each well. The sets were incubated aerobically under agitation at 35 °C for 1.5 h (adhesion phase). After the adhesion phase, the discs were carefully washed twice with PBS. Next, 2 mL of YNB medium with 100 mM glucose was added to the control groups and a mixture of YNB with 100 mM glucose and FLZ (Sigma–Aldrich Corp, St. Louis, MO, USA) at 2.56 mg/mL (concentration bioavailable in saliva) was added to the experimental groups (Force & Nahata 1995). The plates were incubated aerobically under agitation at 35 °C. After the first 24 h of incubation, the biofilms were washed twice with PBS, and transferred to another 24-well culture plate with 2 mL of culture medium (control group) or culture medium with FLZ (experimental group). Then, the biofilms were returned to an orbital shaker for an additional 24 h prior to analysis.

Number of viable cells

Biofilm-containing discs were washed twice with PBS, and, afterwards, were immersed in PBS, and sonicated (7 W, for 30 s) to disrupt the biofilm structure. The sonicated suspensions were serially diluted in PBS, and samples were plated in triplicate onto SDA. The plates were incubated aerobically for 24 h at 35 °C. Yeast

cells were counted on a stereomicroscope (Coleman Comp. Imp., Santo André, São Paulo, Brazil) and expressed by cells/mL.

Polysaccharide extraction

For polysaccharide extraction, 400 µL of the previously obtained sonicated biofilm suspension were centrifuged (10,000 × *g* for 5 min at 4 ºC). The supernatant, containing the soluble extracellular polysaccharides (SEPs), was transferred to a tube named SEPs. An aliquot of 1 M NaOH was added to the pellet to extract the insoluble extracellular polysaccharides (IEPs). The tube was agitated for 15 min, centrifuged, and the supernatant was transferred to another tube named IEPs. Finally, an aliquot of 1 M NaOH was added to the residual pellet to extract the intracellular polysaccharide (IPs) (Tenuta *et al.,* 2006). The tube was heated for 15 min at 100 °C, centrifuged, and the supernatant was transferred to another tube named IPs.

Three volumes of cold ethanol were added to the tubes containing SEPs, IEPs and IPs. The tubes were maintained for 30 min at -20 °C. Next, the tubes were centrifuged, and the pellets were washed twice with cold 75% ethanol. The precipitated polysaccharides were resuspended in 1 M NaOH. The total carbohydrate was estimated by the phenol-sulfuric method (Dubois *et al.,* 1951). The results were normalized by dry weight of biofilm.

Confocal laser scanning microscopy (CLSM)

In order to visualize the live and dead cells, the biofilm-containing discs were stained by SYTO-9 and propidium iodide with the Live/Dead *BacLight* viability kit (Invitrogen Molecular Probes, Eugene, OR, USA). In order to visualize the extracellular matrix, additional biofilm-containing discs were stained with SYTO 9 fluorescent stain (Invitrogen Molecular Probes Inc, Eugene, OR, USA) and 50 µM Concanavalin A conjugate (ConA-tetramethylrhodamine; Invitrogen Molecular Probes). ConA was added to the culture media during biofilm development in order to label the extracellular matrix during its synthesis.

The biofilms were incubated with SYTO 9 and propidium iodide for 20 min at 35 °C, with care taken to protect the samples from light. The biofilms structural organization was examined using a Leica microscope TCS SP5 (Leica Microsystems CMS, Mannheim, GE) with a 63x, 0.8 numerical aperture oil-immersion objective lens. The images were acquired by the software LAS AF (Leica Microsystems CMS, Mannheim, GE) at a resolution of 1024 x 1024 pixels and line average of 16. To assess the structure of the biofilms, a series of optical sections at 1-µm intervals in the z-axis were taken throughout the full depth of the biofilm. At least 5 representative randomly optical fields were scanned for each disc. Additionally, the confocal image stacks were subjected by the image-processing software COMSTAT (Heydorn *et al.,* 2000). In this study, the structural parameter of biofilm coverage (µm) on disc surface was investigated.

Statistical analysis

The results were statistically analyzed by the SAS/LAB software package (SAS Software, version 9.0; SAS Institute Inc., Cary, NC, USA). The assumptions of equality of variances and normal distribution of errors were checked, and when violated, the data were transformed as suggested by the software. The cell viability, SEPs, IEPs and IP data were transformed by Log 10. The comparison between control and experimental groups, for each strain, was performed using the Student's *t*-test. The significant level was fixed at 5%.

Results

A significant reduction in the number of viable cells was found for reference strain and clinical isolates *C. albicans* biofilms developed in the constant presence of FLZ ($p < 0.001$, Figure 1A). Figures 1B and 1C show representative *C. albicans* biofilms stained with the Live/Dead *BacLight* viability kit. These confocal images clearly illustrate the increased number of dead cells in the experimental group. Additionally, in the z-slices of *C. albicans* biofilms, increases in the cell volume and the amount of black spaces were observed in the presence of FLZ (Figures 1B and 1C).

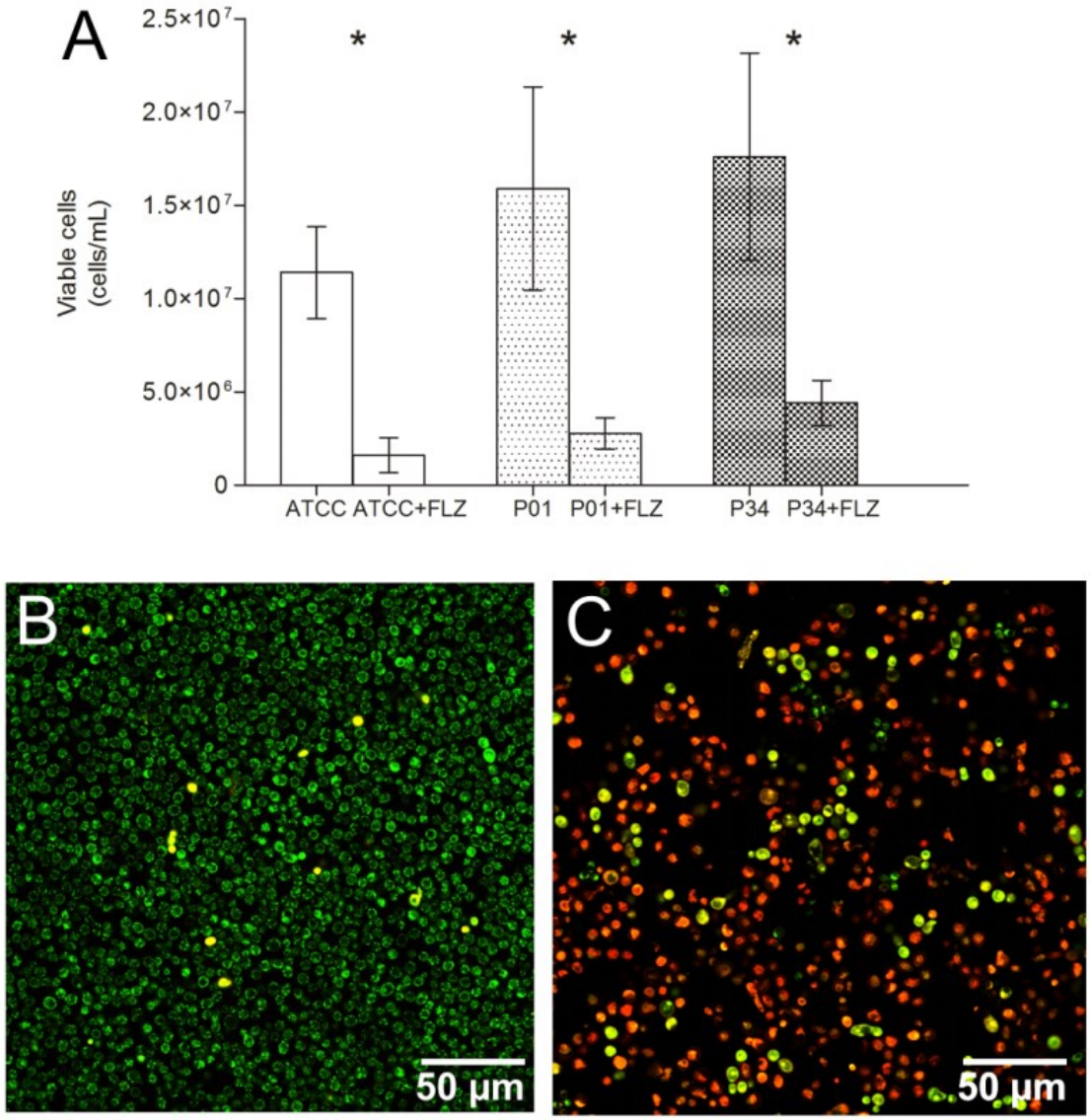

Figure 1. **(A)** Cell viability of *C. albicans* reference strain and clinical isolates biofilms developed in the constant presence of FLZ. Representative confocal images of *C. albicans* ATCC 90028 in the **(B)** control and **(C)** experimental groups. Live cells appear green and dead cells appear red. Symbol (*) indicates statistically significant differences between control and experimental groups within each *C. albicans* strain (*t*-test, $p < 0.05$).

The amount (µg/mg biofilm dry weight) of SEPs, IEPs and IP were also significantly lower when biofilms were grown in the constant presence of FLZ (p < 0.001; Figure 2A, 2B and 2C). However, considering the proportion of SEPs, IEPs and IP produced by the number of viable cells, it was observed that in the experimental groups, *C. albicans* was able to produce more extracellular and intracellular polysaccharides than the control groups (p < 0.05; Figure 3A, 3B and 3C).

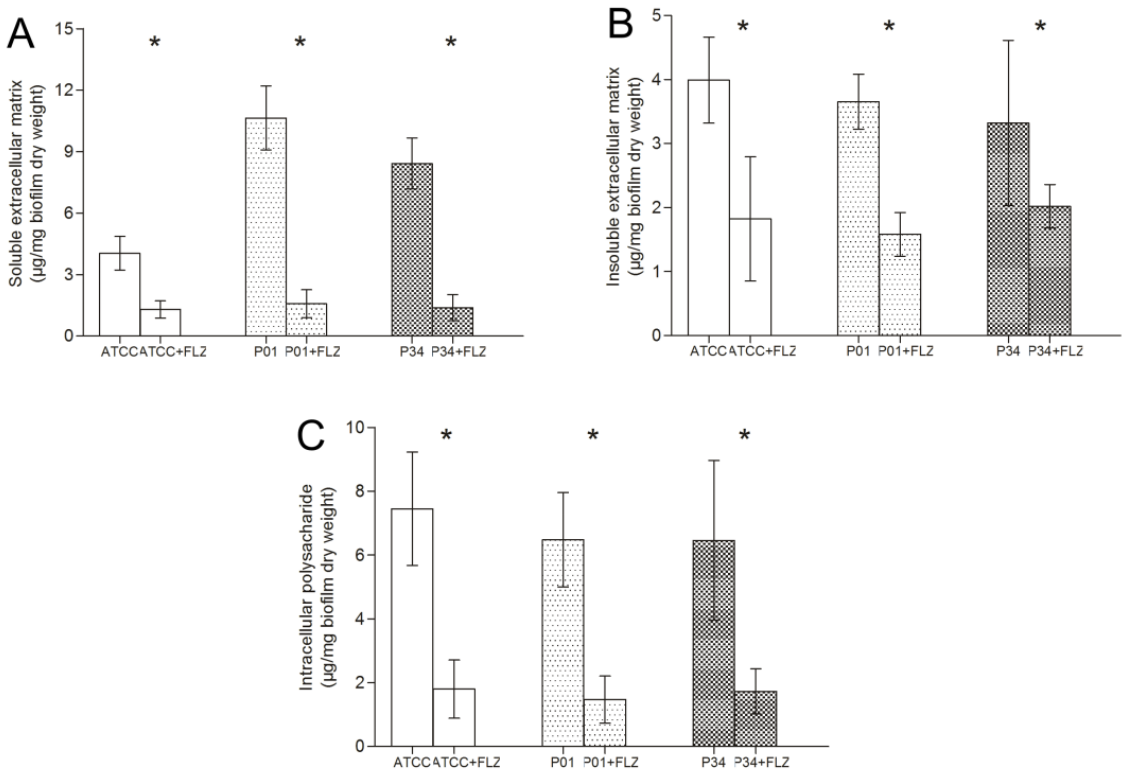

Figure 2. **(A)** SEPs, **(B)** IEPs and **(C)** IPs of *C. albicans* reference strain and clinical isolates biofilms developed in the constant presence of FLZ. Symbol (*) indicates statistically significant differences between control and experimental groups within each *C. albicans* strain (t-test, p < 0.05).

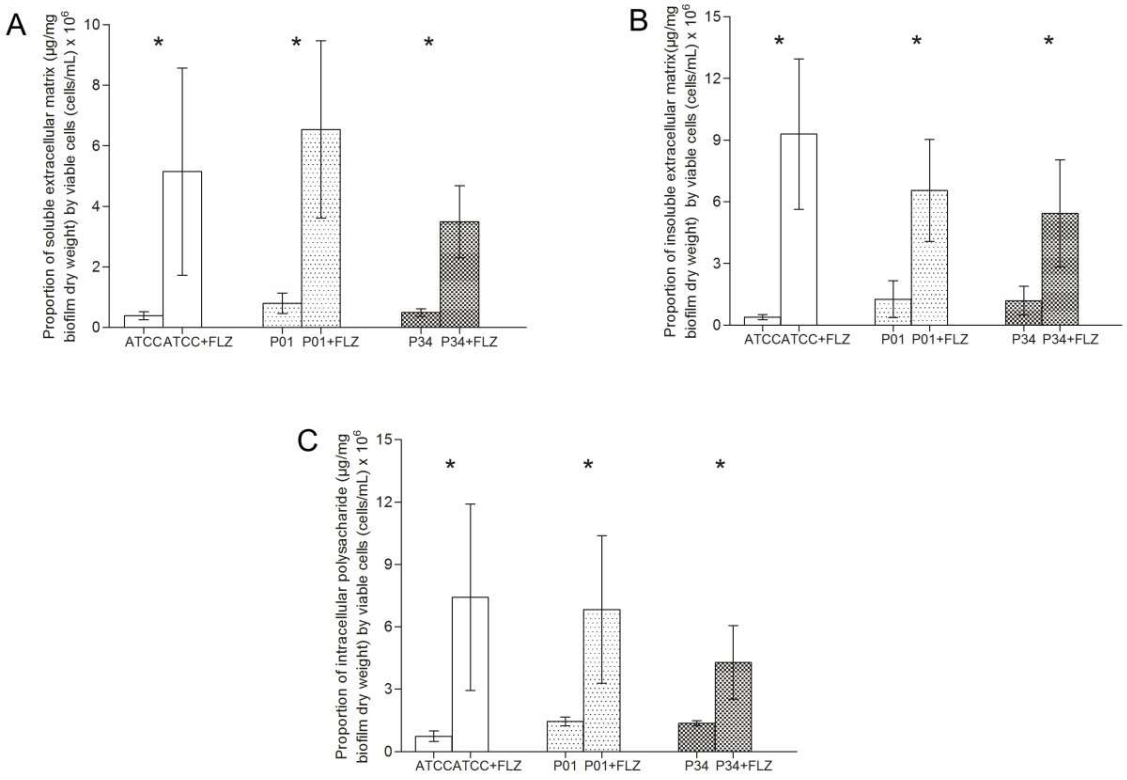

Figure 3. Proportion of **(A)** SEPs, **(B)** IEPs and **(C)** IPs produced by viable *C. albicans* cells developed in the presence of FLZ. Symbol (*) indicates statistically significant differences between control and experimental groups within each *C. albicans* strain (*t*-test, $p < 0.05$).

Figures 4A and 4B show representative *C. albicans* biofilms stained with ConA in order to visualize the extracellular matrix. Although the number of cells was much lower in the experimental group, both control and experimental groups stained great amounts of extracellular matrix, visualized as red. Representation of cells and extracellular matrix coverage on disc surface also confirms that despite the reduced biofilm coverage in the experimental group, the production of extracellular matrix was higher considering the quantity of yeast cells. Additionally, it was observed that for both control and experimental group, the extracellular matrix was found interspersed between yeast cells (Figure 4C and 4D).

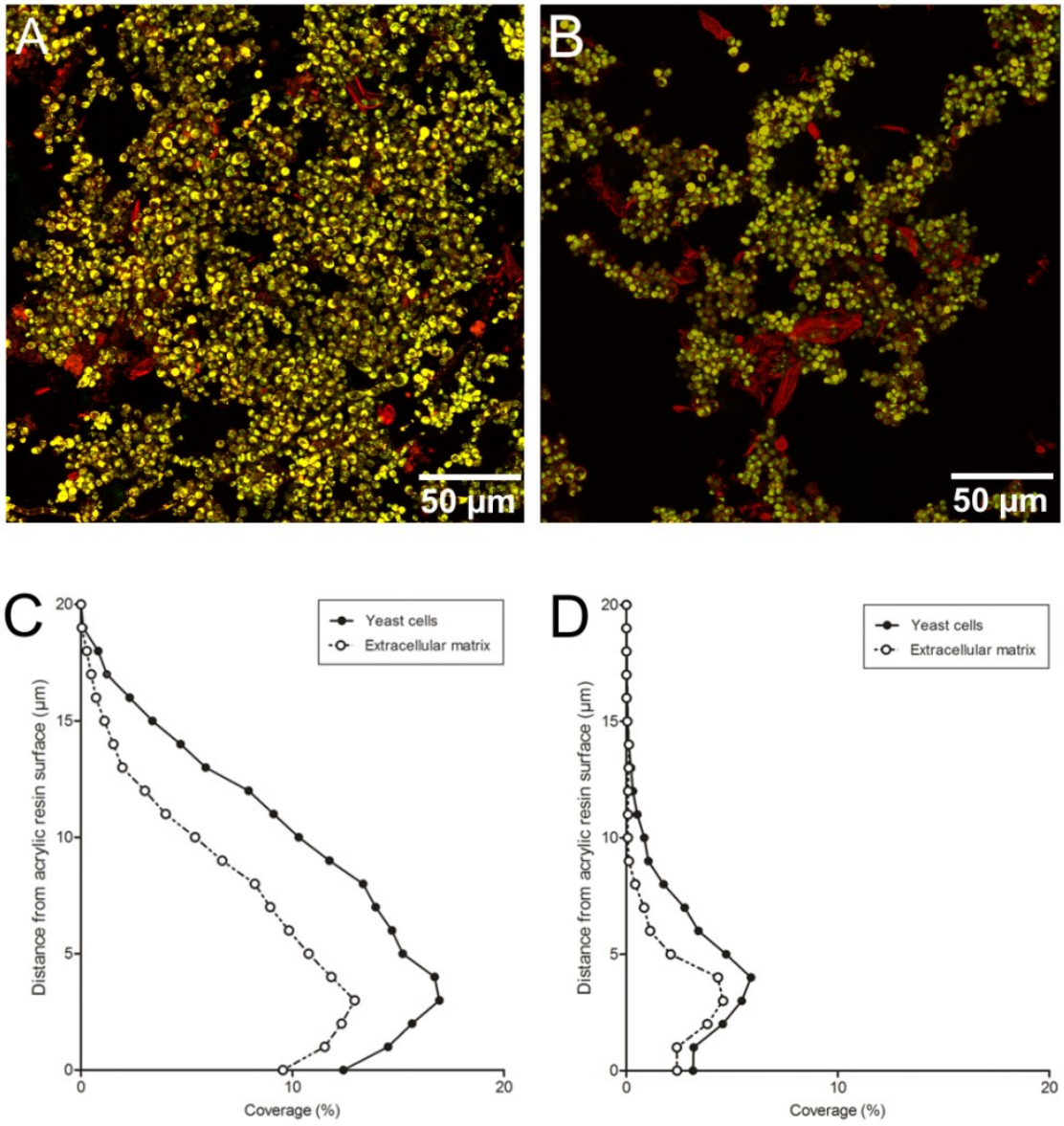

Figure 4. Representative confocal images of *C. albicans* ATCC 90028 in the **(A)** control and **(B)** experimental groups. Cells appear green and extracellular matrix appears red. *C. albicans* ATCC 90028 biofilm coverage on disc surface in the **(C)** control and **(D)** experimental groups.

Discussion

Although the effect of FLZ on *C. albicans* biofilms has been extensively investigated (Nett *et al.,* 2007; Vediyappan *et al.,* 2010; Nett *et al.,* 2010; da Silva *et al.,* 2012), there is little information regarding biofilms developed in the constant presence of FLZ (Gomes *et al.,* 2011).The present biofilm growth model simulated a condition in which patients wearing dentures are under a FLZ therapy regimen. Despite FLZ treatment, in some cases biofilms continued to develop over the dentures. Understanding the behavior of *C. albicans* biofilm growth under FLZ therapy, as well as their mechanism of resistance related to the exopolysaccharide matrix production, may be important for the development of protective approaches to *Candida*-related diseases.

The present study showed that *C. albicans* biofilms developed in the presence of FLZ, at the bioavailable concentration in saliva (2.56 µg/mL), reduced the number of viable cells approximately 80% for both reference strain and clinical isolates. This result was also confirmed after analyzing the confocal images which showed that the number of dead cells was higher in the experimental groups. Previous studies showed that when using concentrations less that 64 µg/mL, *C. albicans* biofilms did not reach a 50% reduction in viability (Chandra *et al.,* 2001; Konopka *et al.,* 2010). However, the fact that biofilms were grown in the constant presence of FLZ may have influenced the lower cell quantification in the experimental group, whilst the previous authors grew the biofilms first, and afterwards exposed these biofilms to FLZ.

Actually, the mechanism of action of FLZ in *C. albicans* biofilms is related to a fungistatic action (Ramage *et al.,* 2012). However, it is possible that the constant presence of FLZ at a concentration higher than the minimal inhibitory concentration (*i.e*. 0.5 µg/mL) (da Silva *et al.,* 2012) could lead the drug to a toxic effect. Furthermore, it is already known that *C. albicans* cells grown in the presence of FLZ have profound alterations in cellular structure (Gomes *et al.,* 2011). As previously described the cells cultured in the presence of FLZ seemed larger,

which was confirmed by the confocal images, with an altered structure with deformed nucleus and a significant increase in the number of vacuoles (Gomes *et al.,* 2011). This increased number of vacuoles could be correlated to the action of FLZ which inhibits ergosterol biosynthesis, a component of the fungal cell membranes. With this inhibition, toxic substances that are ergosterol precursors could accumulate in the cells, probably in these vacuoles (Niimi *et al.,* 2010), resulting in the fungal death observed in our study. Also, it is important to highlight that in the experimental group we changed the FLZ every 24 h, considering that the half-life of FLZ ranges from 27 to 37 h (Egusa *et al.,* 2008).

Considering that the exopolysaccharidematrix represents an important component related to biofilm architecture and antifungal tolerance (Flemming & Wingender 2010; Tobudic *et al.,* 2012; Bonhomme & d'Enfert 2013), the present study was the first to evaluate the production exopolysaccharide matrix in biofilms grown in the presence of FLZ, using phenol-sulfuric and confocal methodologies. Both methods used in the present study considered only the carbohydrates present, considering that the carbohydrate part, commonly referred as “exopolysaccharide”, is regard as the major structural component of the matrix and provides a framework to the biofilm complex (Lal *et al.,* 2010). The phenol-sulfuric is a colorimetric method that quantifies in µg the polysaccharide present in the matrix. On the other hand, the ConA specifically binds to carbohydrates residues of the cell wall membrane, allowing the matrix visualization.

The phenol-sulfuric method demonstrated that the presence of FLZ resulted in the lowest amounts of soluble and insoluble extracellular polysaccharides. However, these results should be interpreted carefully. Considering the amount of exopolysaccharidematrix produced by the number of viable cells, it is concluded that the experimental groups produced proportionally more extracellular matrix than the control groups. The results of biofilm coverage and confocal images also corroborates with this inference, considering the large amounts of matrix stained in the experimental group despite the reduced number of cells. These results seem to be very important given the role of the exopolysaccharidematrix in the antifungal

tolerance. It is possible to speculate that the presence of FLZ serve as a stress situation capable to stimulate cells to produce extracellular glucans, which was deposited in the matrix in an attempt to protect themselves (da Silva *et al.,* 2012). These extracellular glucans are important for biofilm tolerance and acts by sequestering antifungals such as FLZ, rendering cells resistant to their action and preventing its intracellular penetration (Nett *et al.,* 2010). Also, matrix components could act as a physical barrier to the diffusion of FLZ, thereby limiting its access to cells located at basal layers. Additionally, the experimental groups produced proportionally more intracellular polysaccharide than the control groups, which represents the energetic cells reserve. These results seem to be closely related to the increase in cell volume identified in the confocal images, as previously described (Gomes *et al.,* 2011).

Overall, according to the results presented it is clear that although the constant presence of FLZ resulted in decreased biofilm formation but great exopolysaccharide matrix production, this drug was not able to inhibit the development of these biofilms. However, it is important to point out that, in an *in vivo* condition, this residual biofilm may not necessarily represents the endurance of *Candida*-related infection. Thus, although the clinical extrapolation of our results is limited, it is extremely important in the foundation of future *in vivo* investigations that should be conducted to confirm these findings.

Conclusion

Within the limits of this study it can be concluded that *C. albicans* biofilms developed in the presence of FLZ, at the bioavailable concentration present in saliva, increased the extracellular matrix production.

Conflict of interest

The author(s) declare no conflicts of interest with respect to the authorship and/or publication of this article.

Ethical approval

The study was approved by the Ethics Committee of Piracicaba Dental School (042/2008), and all subjects volunteered to participate and signed and written informed consent form.

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CONCLUSÃO

A partir dos resultados foi possível concluir que a microscopia confocal é uma ferramenta efetiva na análise da matriz extracelular de biofilmes de *C. albicans*. Além disso, conclui-se que, proporcionalmente ao número de células viáveis, os biofilmes de *C. albicans* desenvolvidos na presença de fluconazol apresentaram elevada produção de matriz extracelular.

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ANEXO 1 - Certificado do Comitê de Ética em Pesquisa 1

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Formação e desenvolvimento de biofilme de Candida spp. sobre a superfície de resina a base de poli(metilmetacrilato) na presença de saliva, plasma e agente antifúngico: efeitos sobre fatores de virulência, morfologia e quantificação do biofilme"**, protocolo nº 042/2008, dos pesquisadores Altair Antoninha Del Bel Cury, Priscila Nogueira Gomes, Wander José da Silva e William Custodio, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 04/02/2009.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Formation and development of biofilm of Candida spp. on the surface of poly(methylmetacilate) containing resin in the presence of saliva, plasma and antifungic agent: effects on virulence factors, morphology and quantification of biofilm"**, register number 042/2008, of Altair Antoninha Del Bel Cury, Priscila Nogueira Gomes, Wander José da Silva and William Custodio, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 02/04/2009.

Prof. Dr. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 2 - Certificado do Comitê de Ética em Pesquisa 2

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Efeito de diferentes fontes de energia no desenvolvimento do biofilme de Candida albicans sobre a superfície de poli (metilmetacrilato)"**, protocolo nº 105/2011, dos pesquisadores Ivone Lima Santana, Altair Antoninha Del Bel Cury, Letícia Machado Gonçalves e Wander José da Silva, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 21/10/2011.

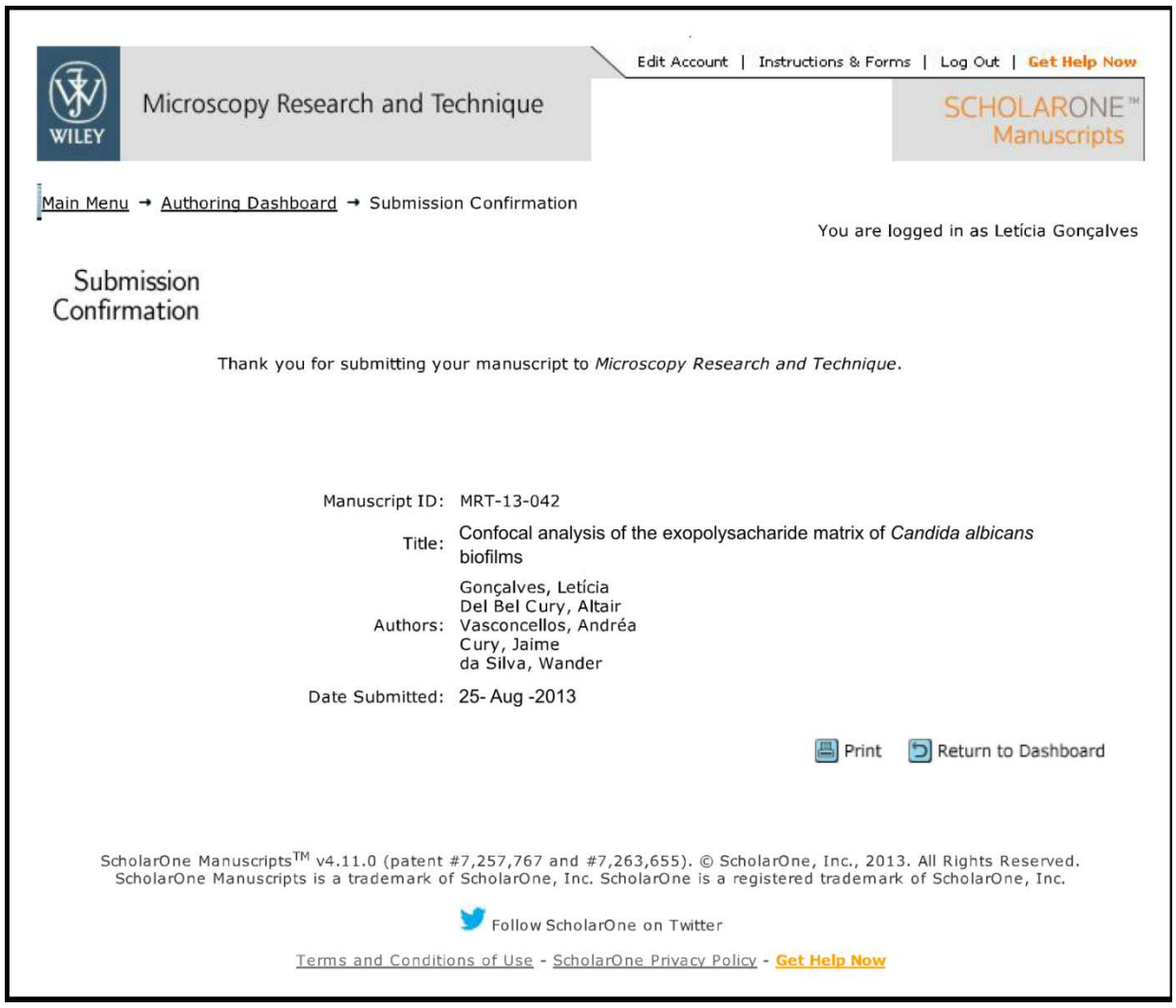
The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Effect of diferent energy sources in the development of Candida albicans biofilm on the surface of poly (methylmethacrylate)"**, register number 105/2011, of Ivone Lima Santana, Altair Antoninha Del Bel Cury, Letícia Machado Gonçalves and Wander José da Silva, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 10/21/2011.

Profa. Dra. Lívia Maria Andaló Tenuta
Secretária
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 3 – Confirmação de submissão de manuscrito



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