ANDRÉA ARAUJO DE VASCONCELLOS

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FACULDADE DE ODONTOLOGIA DE PIRACICABA

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Orientador: Prof. Dr. Wander José da Silva

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutora em Clínica Odontológica, na área de Prótese Dental.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Dental Clinic, concentration area of Dental Prosthesis.

Este exemplar corresponde à versão final da tese defendida pela aluna Andréa Araujo de Vasconcellos e orientada pelo Prof. Dr. Wander José da Silva.

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A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 14 de Novembro de 2013, considerou a candidata ANDRÉA ARAUJO DE VASCONCELLOS aprovada.

Prof. Dr. WANDER JOSÉ DA SILVA

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ABSTRACT

The colonization of dental prosthesis by *Candida albicans* may contribute to the development of *Candida*-associated denture stomatitis. The pH changes that occur in the biofilm developed over the dental prosthesis may influence the amount of extracellular matrix and the structure of *C. albicans* biofilms. However, it is not clear how the pH could interfere in the extracellular matrix and specifically structural parameters of *C. albicans* biofilms. The object of this study was to evaluate the influence of environmental pH on the amount of extracellular matrix and structure in *C. albicans* biofilms. Poly (methylmethacrylate) resin discs with surface roughness standardized were used as substrate for biofilm formation. The discs were covered by the salivary pellicle, and *C. albicans* ATCC 90028 biofilms were developed in RPMI 1640 culture medium buffered at different pH values (4.0, 5.5 and 7.0) for 48 hours as experimental groups, and unbuffered culture medium (initial pH of 7.4) as control. The number of viable cells was quantified by serial dilution, and the amount of polysaccharide matrix was analyzed by phenol sulfuric method (n = 9). The morphological characteristics of biofilms were observed by scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) (n = 3). Data were analyzed by one-way ANOVA, followed by Tukey´s test, with significance set at 5%. It was observed a significant increase in the number of viable cells in biofilms developed at pH 4.0 and 5.5 ($p < 0.001$). The amount of polysaccharides was higher at pH 5.5, followed by biofilms developed at pH 4.0 ($p < 0.05$). The images obtained by SEM and CLSM showed higher hyphae formation by biofilms developed at pH 7.0 and in the control group. It can be concluded that acidic pH conditions influenced the formation of *C. albicans* biofilms, with higher viable cells and, consequently, higher amount of extracellular matrix. Furthermore, the pH significantly affected the morphology of *C. albicans* biofilms.

Key words: Hydrogen-Ion Concentration. *Candida*. Dental Prosthesis.

RESUMO

A colonização da prótese dental por *Candida albicans* pode contribuir para o desenvolvimento de candidose associada ao seu uso. As variações de pH que ocorrem no biofilme formado sobre a prótese dental podem influenciar a quantidade de matriz extracelular e a estrutura do biofilme de *C. albicans*. Entretanto, não está claro como o pH pode interferir na matriz extracelular e em determinados parâmetros estruturais nos biofilmes de *C. albicans*. O objetivo neste estudo foi avaliar a influência do pH na quantidade de matriz extracelular e na estrutura de biofilmes de *C. albicans*. Discos de resina à base de poli (metilmetacrilato) com a rugosidade de superfície padronizada foram utilizados como substrato para a formação dos biofilmes. Os discos foram cobertos por uma película de saliva e biofilmes de *C. albicans* ATCC 90028 foram desenvolvidos em meio de cultura RPMI 1640 tamponado em diferentes valores de pH (4,0, 5,5 e 7,0) por 48 horas como grupos experimentais, e meio de cultura sem o tamponamento (pH inicial de 7,4) como grupo controle. O número de células viáveis foi quantificado por diluição seriada, e a quantidade de matriz de polissacarídeos foi analisada pelo método fenol-sulfúrico (n = 9). As características morfológicas dos biofilmes foram observadas por meio de microscopia eletrônica de varredura e por microscopia confocal (n = 3). Os resultados foram submetidos à análise de variância com um fator, seguido pelo teste de Tukey, com nível de significância de 5%. Observou-se aumento significativo no número de células viáveis nos biofilmes desenvolvidos em pH 4,0 e 5,5 ($p < 0,001$). A quantidade de polissacarídeos foi maior em pH 5,5, seguida por biofilmes desenvolvidos em pH 4,0 ($p < 0,05$). As imagens obtidas por microscopia eletrônica de varredura e por microscopia confocal mostram maior formação de hifas pelos biofilmes quando desenvolvidos em pH 7,0 e no grupo controle. Pode ser concluído que condições de pH ácidos influenciaram a formação de biofilmes com o maior número de células viáveis e, consequentemente, maior quantidade de matriz extracelular. Além disso, o pH afetou consideravelmente a morfologia dos biofilmes de *C. albicans*.

Palavras-Chave: pH. *Candida.* Prótese Dentária.

SUMÁRIO

DEDICATÓRIA

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INTRODUÇÃO

Candida albicans são micro-organismos oportunistas que têm a capacidade de se desenvolver e colonizar sítios no hospedeiro humano com valores distintos de pH, como, por exemplo, a mucosa oro-faríngea, o trato gastrointestinal e o trato vaginal (Vylkova *et al*., 2011). Na cavidade bucal, o pH é aproximadamente 7,0 (Sosinska *et al*., 2011), embora alterações na dieta, o metabolismo dos micro-organismos e a redução do fluxo salivar podem levar a diminuição do pH (Bensen *et al*., 2004).

Nesse contexto, após a instalação de próteses dentais removíveis confeccionadas em poli (metilmetacrilato), em pacientes parcial ou totalmente edêntulos, a superfície das próteses dentais são expostas à saliva, o que favorece a adesão de micro-organismos (Gocke *et al*., 2002). Se uma higienização adequada da prótese dental não for realizada, a colonização por micro-organismos resultará no desenvolvimento de biofilmes tanto na superfície da mucosa bucal como na prótese dental, resultando em microambientes nos quais o pH pode variar e atingir valores ácidos (Samaranayake *et al*., 1983), que poderá levar ao desenvolvimento de candidose associada ao uso de prótese dental (Gendreal *et al*., 2011). Esta infecção está associada à presença de fungos de gênero *Candida spp*, tendo *Candida albicans* como a principal espécie envolvida na sua etiologia (Emami *et al*., 2012).

Os biofilmes são comunidades altamente estruturadas de micro-organismos heterogêneos envoltos por uma matriz extracelular (Harriott e Noverr, 2011). A matriz extracelular é constituída principalmente por polissacarídeos, embora proteínas, ácidos nucléicos e lipídeos também estejam presentes (Flemming e Wingender, 2010; Taff *et al*., 2012). Dentre suas diversas funções, destacam-se a capacidade de constituir o arcabouço para a arquitetura tridimensional dos biofilmes, importante para a adesão e coesão destes, o controle da desagregação das células e a proteção das mesmas contra o sistema imune do hospedeiro (Al-

Fattani e Douglas, 2006). Além disso, a matriz extracelular está relacionada ao processo de resistência dos biofilmes aos agentes antifúngicos, dificultando o acesso desses fármacos às camadas mais basais do biofilme (Al-Fattani e Douglas, 2006; Lal *et al*., 2010). Embora a presença de matriz extracelular seja uma característica comum aos biofilmes (Branda *et al*., 2005; Sardi *et al*., 2013), determinadas condições ambientais podem influenciar a sua formação, como o pH.

O pH pode influenciar ainda a célula fúngica de diferentes maneiras. As alterações no pH do ambiente podem afetar *C. albicans* em nível celular, particularmente na membrana plasmática e na remodelação da parede celular (Davis, 2003; Schmidt *et al*., 2008). Ainda, as proteínas fúngicas possuem um pH ótimo para sua atividade, podendo ser funcionais em um sítio no hospedeiro, mas não em outro (Davis, 2009). De acordo com Davis (2009), ambientes neutros e alcalinos geram estresses na célula fúngica relacionados principalmente à aquisição de nutrientes, tendo em vista que à medida que o pH aumenta, torna-se mais difícil estabelecer um gradiente de prótons na membrana plasmática para que haja a absorção de nutrientes. Por outro lado, ambientes ácidos também podem causar alteração na célula fúngica quando ácidos fracos estão presentes no ambiente, permeando a célula fúngica e dissociando-se no citoplasma, resultando em acidificação citosólica e morte celular. Além disso, o pH é um potente indutor na diferenciação morfológica de leveduras para hifas (Kucharikova *et al*., 2011), sendo importante para a virulência de *C. albicans* nos diversos nichos do hospedeiro (Bensen *et al*., 2004; Huang, 2012). Tem sido relatado que condições ácidas favorecem o crescimento de leveduras, enquanto as alcalinas favorecem o desenvolvimento de hifas (Kucharikova *et al*., 2011; Vylkova *et al*., 2011). Estudos mostram que enquanto as hifas estão relacionadas à maior produção de enzimas hidrolíticas, ao aumento da resistência antifúngica (Al-Fattani e Douglas, 2006; Vylkova *et al*., 2011) e à maior agressividade de infecção causada por *C. albicans* (Fonzi, 2002), as leveduras são de extrema importância

para a disseminação da infecção, especialmente nos estágios iniciais da doença (Saville *et al*., 2003).

Diante do exposto, observa-se que apesar do pH causar diversos efeitos na célula fúngica, ainda não está claro na literatura como o pH pode interferir na quantidade de matriz extracelular, considerada um importante mecanismo de resistência dos biofilmes aos agentes antifúngicos, e em determinados parâmetros estruturais dos biofilmes de *C. albicans*. Dessa forma, o objetivo neste trabalho foi avaliar a influência do pH no número de células viáveis, na quantidade de matriz extracelular e na estrutura de biofilmes maduros de *Candida albicans*.

CAPÍTULO 1*

Acidic pH conditions increased the amount of extracellular matrix in *Candida albicans* biofilms

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Abstract

Objective: The object of this *in vitro* study was to investigate the influence of environmental pH on the amount of extracellular matrix in *Candida albicans* biofilms. **Materials and methods:** Poly (methylmethacrylate) (PMMA) resin discs were fabricated and had their surface roughness standardized. Salivary pellicle was formed on the PMMA surface and, on the substrate, *C. albicans* ATCC 90028 biofilms were developed in RPMI 1640 medium buffered at different pH values (4.0, 5.5 and 7.0) for 48 h. Unbuffered culture medium was used as control, which has an initial pH of 7.4. The number of viable cells was quantified by serial dilution, and the amount of polysaccharide matrix was analyzed by phenol sulfuric method. The morphological characteristics of biofilms were evaluated by scanning electron microscopy (SEM) and by confocal laser scanning microscopy (CLSM). Data were analyzed by one-way ANOVA, followed by Tukey´s test, with significance set at 5%. **Results:** A significant increase in the number of viable cells was observed for biofilms developed at pH 4.0 and 5.5 in relation to other conditions ($p < 0.001$). The amount of polysaccharides matrix was higher at pH 5.5, followed by pH 4.0 ($p < 0.05$). The images obtained by SEM and CLSM showed higher hyphae formation by biofilms developed at pH 7.0 and in the control group. **Conclusion:** Thus, within the limitations of this study, it can be concluded that acidic pH conditions influenced the formation of *C. albicans* biofilms, with higher viable cells and, consequently, higher amount of extracellular matrix.

Key words: Hydrogen-Ion Concentration. *Candida*. Dental Prosthesis.

Introduction

The ability of *Candida albicans* to adapt to different environmental pH in the human host is essential to infect distinct mucosal surfaces, such as oral-pharyngeal, gastrointestinal and vaginal tracts (Vylkova *et al*., 2011). In the oral cavity, the pH is approximately 7.0 (Sosinska *et al*., 2011), however, changes in diet, metabolism of micro-organism and a reduction in the salivary flow markedly decrease the pH (Bensen *et al*., 2004; Ryu *et al*., 2010). In this context, *C. albicans* can colonize both oral mucosa and denture surfaces, resulting in acidic environment over maxillary dentures, which contribute to the development of *Candida*-associated denture stomatitis (Samaranayake *et al*., 1983).

In many pathogenic fungi, the environment pH is considered a potent virulence factor (Ullah *et al*., 2013). The phenotypic switching of *C. albicans* from budding yeast cell to hyphae growth is essential for its virulence, forming a morphologically complex biofilms, and this ability is mediated by environment pH (Davis, 2003; Ullah *et al*., 2013). In addition, the environmental pH interfere on biofilm formation (Kucharikova *et al*., 2011; Sardi *et al*., 2013), which is closely linked with the production of extracellular matrix (Flemming and Wingender, 2010). This matrix is composed by proteins, glycoproteins, glycolipids, extracellular DNA and, mainly, by polysaccharides (Al-Fattani and Douglas, 2006; Flemming and Wingender, 2010).

The extracellular matrix is relevant to preserve the architectural integrity of biofilms and to contribute to antifungal resistance (Taff *et al*., 2012), having been attributed the persistent and problematic nature of biofilms (Lal *et al*., 2010). Although the presence of extracellular matrix is a common feature of biofilms (Branda *et al*., 2005; Sardi *et al*., 2013), environmental conditions, such as pH, may influence their formation. However, it is still unclear how pH may interfere on the amount of extracellular matrix in *C. albicans* biofilms. Thus, this study aimed to

evaluate the influence of environmental pH on the amount of extracellular matrix in *Candida albicans* biofilms.

Materials and methods

Experimental design

This *in vitro* study had a randomized design. Poly (methylmethacrylate) (PMMA) resin discs were fabricated according to the manufacturer´s instructions. After this, the surface roughness was measured, and the discs were randomly divided into 4 groups for the biofilm assay. *C. albicans* ATCC 90028 biofilms were developed in RPMI 1640 medium buffered at pH 4.0, 5.5 and 7.0. Unbuffered culture medium was used as control, which has an initial pH of 7.4. The biofilms were developed for 48 h. The response variables were viable cells and polysaccharide matrix. The biofilm structure was analyzed by scanning electron microscopy (SEM) and by confocal laser scanning microscopy (CLSM). All of the experiments were performed in triplicate of three independent experiments on different days (n = 9). The data were analyzed by one-way ANOVA, considering the environmental pH as study factor, followed by Tukey´s test, with significance set at 5%.

Fabrication of PMMA discs

The discs (10 mm diameter, 2 mm thick) were fabricated using a hot water bath PMMA acrylic resin (QC-20 PMMA; Dentsply Ltd., Weybridge, England) according to manufacturers' recommendation using a metal mould. After, the discs were immersed in ultra-purified water for 48 h at 35 ^{o}C to release residual monomer (Pereira-Cenci *et al*., 2008). Then, 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 the discs was measured by a profilometer (Surfcoder SE 1700; Kosaka Laboratory Ltd., Kosaka, Japan) accurate to 0.01 µm with a total measurement length of 3.2 mm and 0.5 mm/s. The mean of three measurements for each disc was calculated, and the surface roughness was standardized at 0.31 ± 0.04 µm (Santana *et al*., 2013), in order to avoid any roughness interference with the *C. albicans* adherence and/or biofilm accumulation.

The discs were ultrasonically cleansed (Thornton T 740; Thornton-Inpec Eletronica Ltd., Vinhedo, São Paulo, Brazil) for 20 min to remove any contaminants, disinfected with sodium hypochlorite 0.5% for three minutes and washed three times with sterilized distilled water.

Preparation of Candida albicans suspension

C. albicans reference strain (ATCC 90028) was aerobically cultured on Sabouraud Dextrose Agar (SDA) (Difco, Michigan, USA) for 24 h at 35 ^{o}C. A loop of grown colonies were inoculated into Yeast Nitrogen Base culture medium (YNB; Difco) supplemented with 50 mM glucose under agitation for 18-20 h (exponential growth phase) at 35 ^{o}C. Then, *C. albicans* cells were washed twice with phosphate-buffered saline (PBS; pH 7.2). The cells were resuspended in RPMI 1640 culture medium (Invitrogen, Breda Netherlands) buffered with 0.165 M morpholinepropanesulfonic (MOPS; Sigma-Aldrich Co., St. Louis, MO, USA) at pH 4.0, 5.5, 7.0 or unbuferred medium, which has an initial pH of 7.4. The pH of the experimental groups was maintained for all experiment. On the other hand, after 24 of biofilm development, the pH of the control group was 4.8. A final suspension of ~10^{7} cells/mL was optically adjusted at 520 nm (OD=0.25) using a spectrophotometer (Spectronic 20; Bausch & Lomb, Rochester, NY, USA) (Gomes *et al*., 2011).

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, who had not used antibiotics, mouth rinses or any other medication that could affect salivary composition and flow in the past 3 months (Vieira *et al*., 2010). The volunteers provided written formal consent according to a protocol approved by the Ethics Committee in Research of Piracicaba Dental School (#113/2012). The saliva collected was centrifuged (10,000 x *g* 5 min at 4 °C), and the supernatant was filtered through a 0.22 µm membrane filter (Corning, NY, USA) and immediately used (Thein *et al*., 2007).

Under aseptic conditions, the discs were horizontally placed into a 24-well culture plate containing 2 mL of saliva and incubated under agitation for 1 h at 35 ^{o}C to form the salivary pellicle (Gomes *et al*., 2011). Saliva-coated discs were transferred to another 24-well culture plate containing 2 mL of *C. albicans* cell suspensions (10^7 cells/mL) for 1.5 h (adhesion phase).

The discs were washed twice with PBS and transferred to plates containing fresh RPMI 1640 medium. These sets were incubated aerobically under agitation at 35 ^{o}C. After 24 h of incubation, the biofilms were washed with PBS, and transferred to another 24-well culture plate with 2 mL of fresh medium. After 48 h, the biofilms were analyzed for cell counts, amount of matrix polysaccharide and morphological characteristics.

Viable cells

For cell counting, biofilm-containing discs 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 ^{o}C. Yeast cells were counted using a stereomicroscope (Coleman Comp. Imp., Santo André, São Paulo, Brazil), and the results were expressed in cells/mL.

Polysaccharide extraction

For polysaccharide extraction, an aliquot of the previously obtained sonicated biofilm suspension were centrifuged at 10,000 × *g* for 5 min at 4 ºC. The supernatant, containing the soluble extracellular polysaccharides (SEPs), was extracted. An aliquot of 1 M NaOH was added to the pellet, and the set was agitated for 15 min, centrifuges, and the supernatant was extract to get the insoluble extracellular polysaccharides (IEPs). Afterwards, an aliquot of 1 M NaOH was added to the residual pellet, and the tube was heated for 15 min at 100 °C in order to disrupt the fungal cell wall, centrifuged, and the supernatant was extract to obtain the intracellular polysaccharide (IPs) (Tenuta *et al*., 2006). Afterwards, three volumes of cold ethanol were added to the SEPs, IEPs and IPs. The tubes were maintained for 30 min at -20 °C. Next, the sets 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, using glucose as standard (Dubois *et al.,* 1951). The results were normalized by dry weight of biofilm (Tenuta *et al*., 2006).

Dry weight of biofilm

The eppendorfs were weighted on a precise balance to obtain the initial weight without the biofilm solution. Afterwards, an aliquot of the previously sonicated biofilm suspension was mixed with three volumes of cold 100% ethanol in each eppendorf. This set was maintained for 30 min at -20 °C. After, the eppendorfs were centrifuged (10,000 x *g* for 5 min at 4 °C), the supernatant was discarded, and the set was completely dried. The eppendorfs were weighted again, and the dry weight was determined by the difference between final and initial weight.

Scanning electron microscopy (SEM)

The discs were rinsed twice in PBS and transferred to the 24-well culture plate for fixation and dehydration. The discs were fixed overnight in Karnovsky solution (PBS, pH 7.2) and, therefore, dehydrated in a series of ethanol washes at room temperature (60%, 70%, 80%, 90% for 5 min, and 100% for 10 min). Then, they were dried under aseptic conditions at room temperature and gold-sputtered for analysis by SEM (Leo 435 VP, Carl Zeiss SMT, Oberkochen, Germany) at 15 kV. Images were obtained at 1,000× magnification (n = 3) (Cavalcanti *et al*., 2013).

Confocal laser scanning microscopy (CLSM)

The biofilm was evaluated by confocal laser-scanning microscopy (CLSM; Leica Microsystems CMS, Mannheim, Germany). 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).

The biofilms were incubated in the dark for 20 min at room temperature (Jin *et al*., 2005). A series of images were obtained at 1 µm intervals in the z-axis. At least five representative random optical fields were examined for each disc (n = 3) (Heydorn *et al*., 2000).

Statistical analysis

The statistical analysis was carried out using the SAS/LAB software package (SAS Software, version 9.0; Cary, NC, USA) with the significance level of 5%. 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 SEPs and IEPs data were transformed by Log 10, as suggested by the software. All data were analyzed by one-way ANOVA, followed by Tukey's HSD test. The significant level was fixed at 5%.

Results

A significant increase in the number of viable cells was observed for biofilms developed at pH 4.0 and 5.5 ($p < 0.001$).

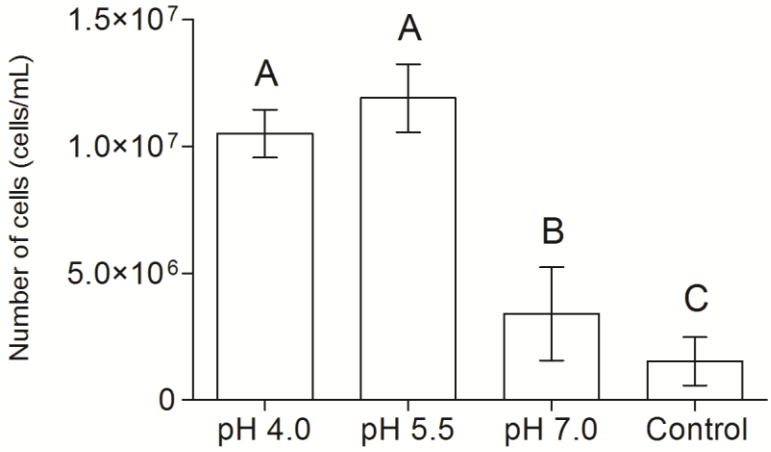

Figure 1. Number of cells (cells/mL) for C*. albicans* biofilms developed for 48 hours on PMMA surface. Distinct letters indicate significant differences between the groups ($p < 0.001$).

The results obtained by the phenol-sulfuric method indicated that biofilms developed at pH 5.5 resulted in the highest amount of polysaccharide matrix, followed by biofilms grown at pH 4.0 ($p < 0.05$, Figure 2).

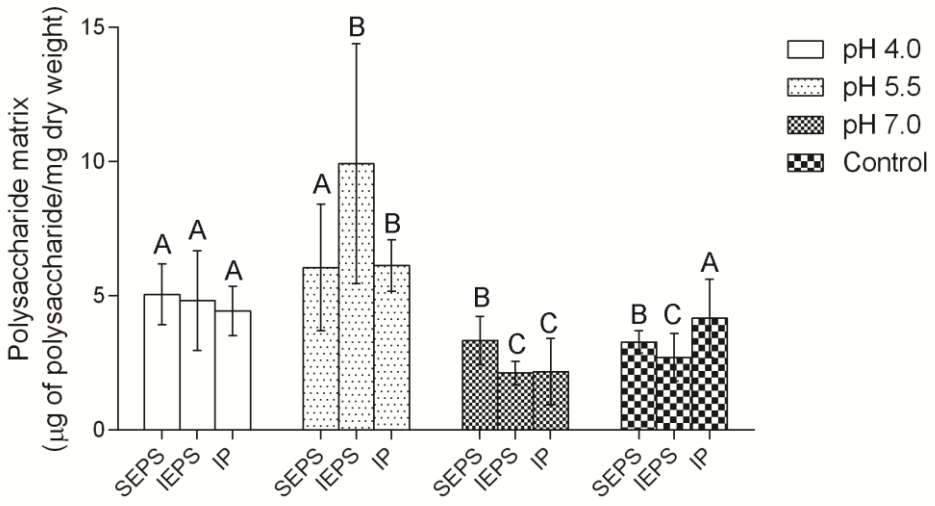

Figure 2. Polysaccharide matrix (µg of polysaccharide/mg biofilm dry weight) for C. *albicans* biofilms developed for 48 hours on PMMA surface. Distinct letters indicate significant differences between the environmental pH ($p < 0.05$).

SEM images indicate high amount of yeast growth in biofilms developed at pH 4.0 and 5.5. In contrast, a predominantly hyphae form could be found at pH 7.0 and in the control group.

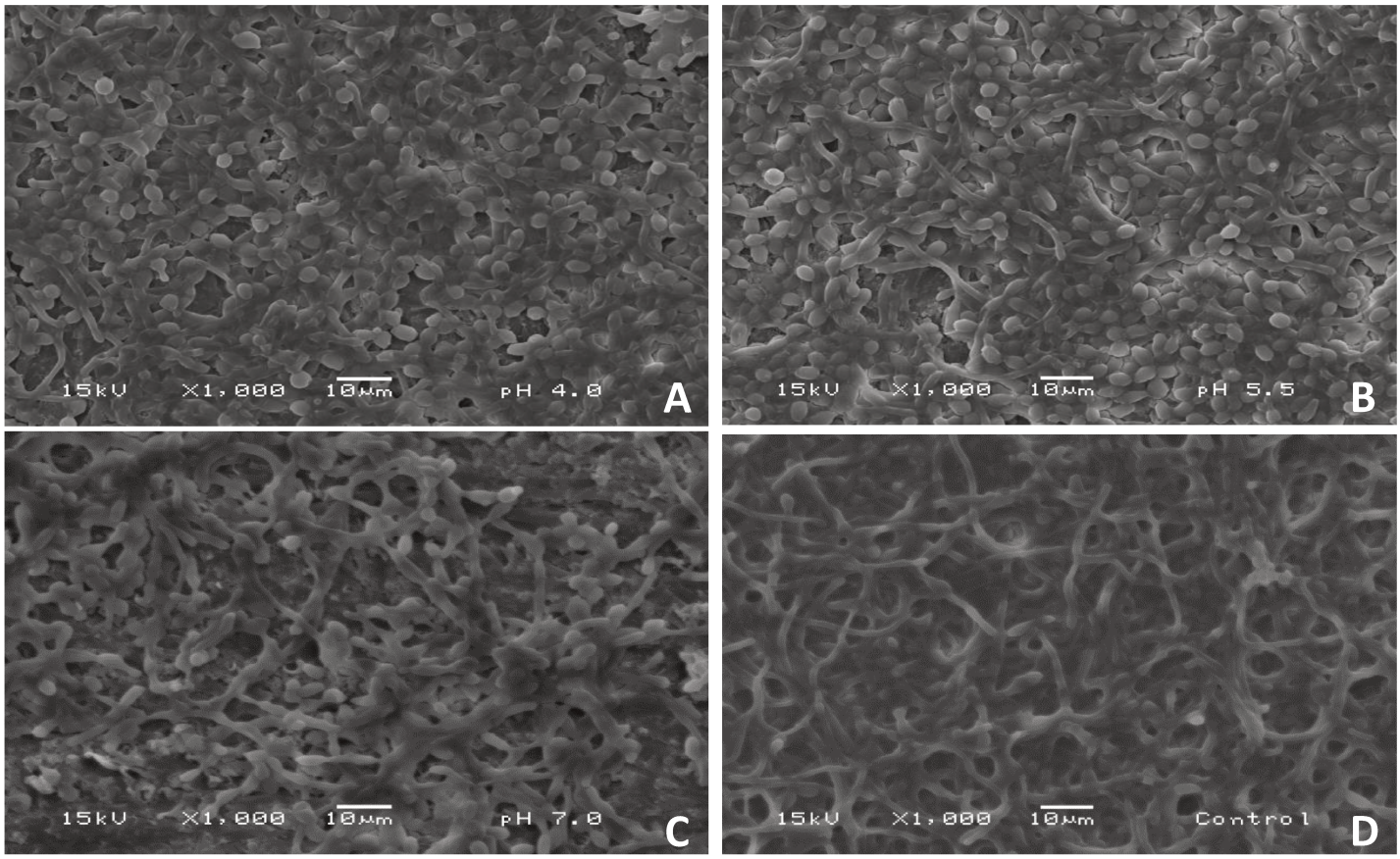

Figure 3. SEM visualization for C*. albicans* biofilms developed for 48 hours on PMMA surface. (A) pH 4.0; (B) pH 5.5; (C) pH 7.0; (D) Control group.

Similarly to observed at SEM visualization, CLSM images revealed a fully covered with yeast cells at pH 4.0 and 5.5, although at pH 7.0 and in the control group a higher yeast-to-hyphae transition was noted. In addition, in CLSM images can be observed a higher amount of dead cells at pH 7.0 and in the control group.

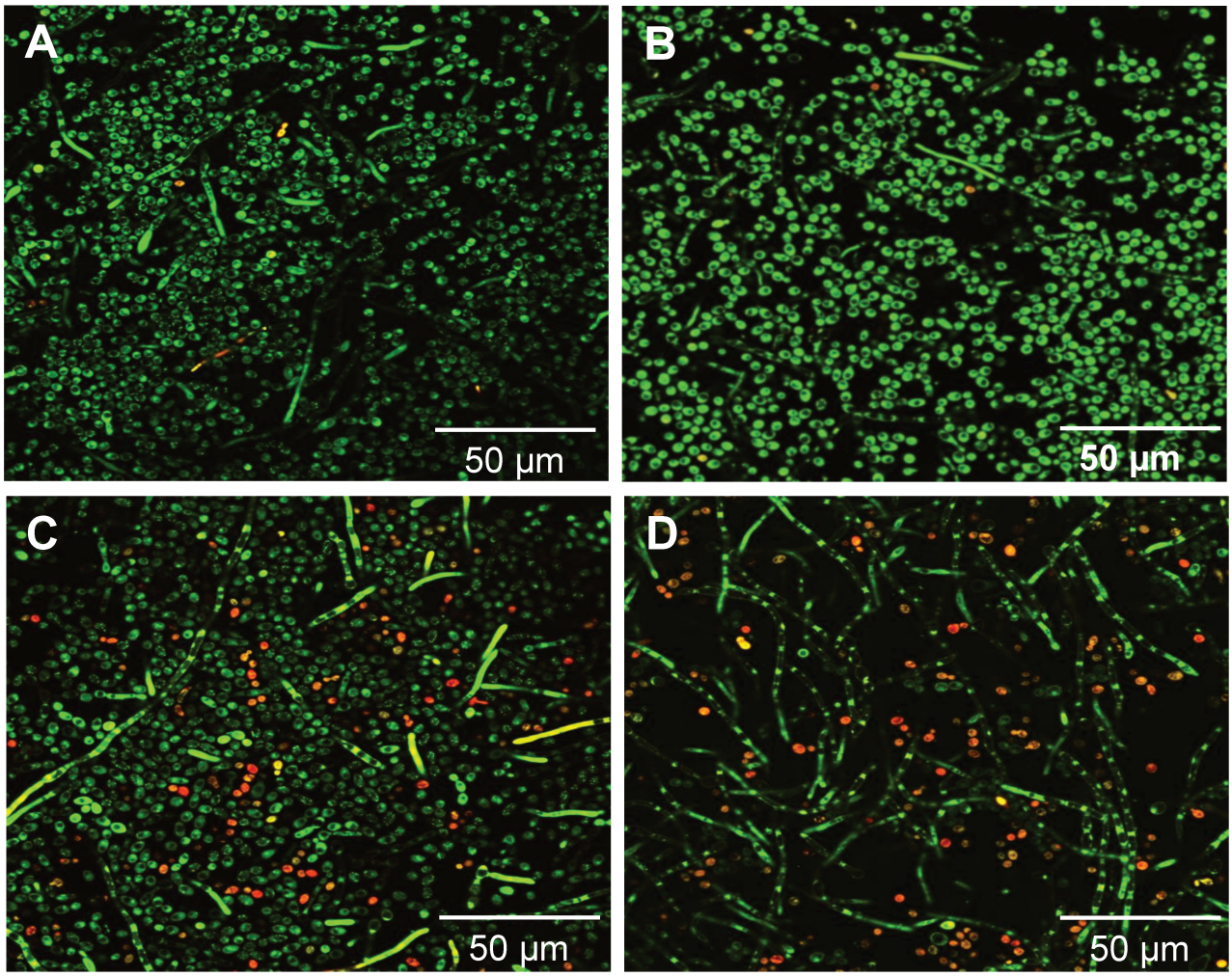

Figure 4. CLSM images for C. *albicans* biofilms developed for 48 hours on PMMA surface. (A) pH 4.0; (B) pH 5.5; (C) pH 7.0; (D) Control group. Live cells appear as green, while the dead cells appear as red.

Discussion

The knowledge about how pH can interfere on the amount of extracellular matrix is essential for the development of protective approaches to *Candida*-related diseases, considering that the main problem during fungal infections is the resistance of biofilms to antifungal agents, mainly associated with the extracellular matrix in which the biofilms are immersed (Lalla *et al*., 2013).

The present study evaluated different pH conditions that could be found at oral environment. The representative pH in the oral cavity is 7.0 (Sosinska *et al*., 2011). However, considering patients with *Candida*-associated denture stomatitis, an acidic environment could be detected in certain niches over maxillary denture surface (Samaranayake *et al*., 1983). In relation to culture medium, the RPMI 1640 medium was selected for this study, taking into account that it is a nutrient-rich for growth of biofilms, and mimics the composition of human fluids (Chandra *et al*., 2005). In addition, previous study showed that this culture medium stimulates the transition of yeast to hyphae and contributes to better adhesion of *C. albicans* biofilms (Kucharikova *et al*., 2011).

Our results showed that environmental pH may interfere on *C. albicans* biofilm at different manners. When the biofilms were developed at pH 4.0 and 5.5, higher amount of viable cells was found, although at pH 7.0 and in the control group considerably less viable cells was observed. Probably, acidic environment is more favorable to the development of *C. albicans* biofilms, being relevant to the progression of *Candida* infections, taking into account that the activity of hydrolytic enzymes that contribute to invasion of the micro-organisms in host tissues is maximized in acidic pH (Naglik *et al*., 2004; Tay *et al*., 2011). In addition, the lower values of viable cells in the control group could be related with the instability of pH during the biofilm development. In the control group, the initial pH was 7.4, although after 24 h of biofilm development, the pH was 4.8. Therefore, it may be

assumed that changes at plasma membrane may be occurred, considering that pH has dramatic effects on the *C. albicans* cell (Davis, 2003).

Another important aspect is the extracellular matrix of *C. albicans* biofilms, considering that the matrix provide a structural scaffold and protect the cells from hostile factors, such as host immunity and antifungal agents (Ramage *et al*., 2012; Taff *et al*. 2012). In our knowledge, the present study was the first to evaluate the influence of pH on the polysaccharide matrix of *C. albicans* biofilms. According our results, the acidic conditions act as a stress in C. *albicans* biofilms, resulting in higher amount of extracellular matrix in biofilms developed at pH 5.5, followed by biofilms grown at pH 4.0. Thereby, it is possibly that acidic conditions favor the production of extracellular glucans, those are one of the carbohydrates that has been linked to overall matrix production and drug resistance (Nett *et al*., 2010; Taff *et al*., 2012). In a clinical point of view, these pH conditions could be found in patients with *Candida*-associated denture stomatitis (Samaranayake *et al*., 1983), in which there are niches in the denture surface with low pH conditions, and, therefore, the resistance of the biofilms to antifungal agents may occur.

Regarding the images obtained by SEM and CLSI, it was noted that the environmental pH directly influence the morphology of *C. albicans* cells. While acidic conditions favored yeast cells, neutral pH was a potent inducer of the yeast-to-hyphae transition, in accordance with others studies (Kucharikova *et al*., 2011; Vylkova *et al*., 2011). Previous studies showed that more than 500 genes are pH regulated, which are important for different functions, including transporters for drug resistance, iron acquisition systems and oxidative stress response proteins (Moye-Rowley, 2002; Bensen *et al*., 2004). In addition, two cell wall proteins are differently expressed in response to environmental pH, the Phr1p and Phr2p (Bensen *et al*., 2004). While Phr1p is essential for systemic candidiasis (pH 7.4), the Phr2p is expressed in vaginal candidiasis (pH 4.0) (Ghannoum *et al*., 1995; De Bernardis *et al*., 1998). Thus, the regulations of gene expression according to

environmental signals, capacity for phenotypic switching and invasion to host tissues are essential for pathogenesis in the different host niches (Huang, 2012).

Overall, according to the results presented, it is clear that environmental pH may influence the extracellular matrix of *C. albicans* biofilms. However, it should be considered that in an *in vivo* condition the pH in the oral cavity is not static. Thus, although the clinical extrapolation of our results is limited, it is important to evaluate the cellular aspects such as plasma membrane and fungal cell wall of *C. albicans* developed in the same pH conditions studied.

Conclusions

Within the limitations of this study, it can be concluded that acidic pH conditions influenced the formation of *C. albicans* biofilms, with higher viable cells and, consequently, higher amount of extracellular matrix.

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Conflict of Interest

The authors have no conflict of interest to declare.

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CONCLUSÃO

Dentro das limitações desse estudo, pode ser concluído que condições de pH ácido influenciaram a formação de biofilmes de *C. albicans*, apresentando maior número de células viáveis e, consequentemente, maior quantidade de matriz extracelular. Além disso, o pH afetou consideravelmente a morfologia dos biofilmes de *C. albicans*.

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APÊNDICE – Análise estatística

a) Células viáveis

The GLM Procedure
Dependent Variable: cfu

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	3	6.302331E14	2.100777E14	120.15	<.0001
Error	28	4.8958496E13	1.7485177E12		
Corrected Total	31	6.791916E14			

R-Square	Coeff Var	Root MSE	cfu Mean
0.927917	19.40457	1322315	6814453

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ph	3	6.302331E14	2.100777E14	120.15	<.0001

Adjustment for Multiple Comparisons: Tukey

ph	LSMEAN cfu LSMEAN	Number
RPMI	1518750.0	1
ph4	10496875.0	2
ph5	11856250.0	3
ph7	3385937.5	4

Least Squares Means for effect ph
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: cfu

i/j	1	2	3	4
1		<.0001	<.0001	0.0406
2	<.0001		0.1923	<.0001
3	<.0001	0.1923		<.0001
4	0.0406	<.0001	<.0001	

b) Polissacarídeos extracelulares solúveis

The GLM Procedure
Dependent Variable: pecs_trans

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.37996200	0.12665400	9.30	0.0002
Error	28	0.38135338	0.01361976		
Corrected Total	31	0.76131537			

R-Square	Coeff Var	Root MSE	pecs_trans Mean
0.499086	18.89878	0.116704	0.617520

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ph	3	0.37996200	0.12665400	9.30	0.0002

ph	pecs_trans LSMEAN	LSMEAN Number
RPMI	0.51291379	1
ph4	0.69314382	2
ph5	0.75532738	3
ph7	0.50869467	4

Least Squares Means for effect ph
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: pecs_trans

i/j	1	2	3	4
1		0.0220	0.0015	0.9999
2	0.0220		0.7128	0.0186
3	0.0015	0.7128		0.0012
4	0.9999	0.0186	0.0012	

c) Polissacarídeos extracelulares insolúveis

The GLM Procedure
Dependent Variable: peci_trans

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	1.93631048	0.64543683	24.33	<.0001
Error	27	0.71639318	0.02653308		
Corrected Total	30	2.65270365			

R-Square	Coeff Var	Root MSE	peci_trans Mean
0.729938	27.95158	0.162890	0.582757

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ph	3	1.93631048	0.64543683	24.33	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

ph	peci_trans LSMEAN	LSMEAN Number
RPMI	0.41566051	1
ph4	0.64792279	2
ph5	0.95743817	3
ph7	0.31815194	4

Least Squares Means for effect ph
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: peci_trans

i/j	1	2	3	4
1		0.0480	<.0001	0.6338
2	0.0480		0.0054	0.0030
3	<.0001	0.0054		<.0001
4	0.6338	0.0030	<.0001	

d) Polissacarídeos intracelulares

The GLM Procedure
Dependent Variable: pic

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	62.9697000	20.9899000	15.44	<.0001
Error	28	38.0537201	1.3590614		
Corrected Total	31	101.0234201			

R-Square	Coeff Var	Root MSE	pic Mean
0.623318	27.57087	1.165788	4.228332

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ph	3	62.96969998	20.98989999	15.44	<.0001

ph	pic LSMEAN	LSMEAN Number
RPMI	4.16602163	1
ph4	4.43512863	2
ph5	6.13271800	3
ph7	2.17945938	4

Least Squares Means for effect ph
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: pic

i/j	1	2	3	4
1		0.9668	0.0111	0.0102
2	0.9668		0.0332	0.0032
3	0.0111	0.0332		<.0001
4	0.0102	0.0032	<.0001	

ANEXO 1 - Certificado do Comitê de Ética em Pesquisa

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 **"Influência do pH no desenvolvimento e na susceptibilidade ao fluconazol de biofilmes de Candida albicans"**, protocolo nº 113/2012, dos pesquisadores Andréa Araújo de Vasconcellos, 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 09/11/2012.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Influence of pH on development and susceptibility to fluconazole in Candida albicans biofilms"**, register number 113/2012, of Andréa Araújo de Vasconcellos, 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 11/09/2012.

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 2 - Comprovante de Submissão do Artigo

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Title: Acidic pH conditions increased the amount of extracellular matrix in *Candida albicans* biofilms

Authors: Vasconcellos, Andréa
Gonçalves, Leticia
Del Bel Cury, Altair
da Silva, Wander

Date Submitted: 10-Nov-2013

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