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Influência da superfície de resinas de poli (metil metacrilato) na estrutura de biofilmes de *Candida albicans*

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"Que importa a nuvem no horizonte,

chuva de amanhã?

Hoje o sol inunda o meu dia."

Helena Kolody

RESUMO

Os biofilmes de *Candida albicans* formados sobre a superfície de resina de poli (metil metacrilato) (PMMA) apresentam alta virulência em função da liberação de enzimas hidrolíticas e são responsáveis pela candidose oral, infecção fúngica mais comum em usuários de próteses dentais removíveis. A organização do biofilme em várias camadas celulares envoltos por matriz de polissacarídeos extracelulares leva estas camadas celulares a estado metabólicos diferenciados e, portanto o uso da técnica de redução XTT (2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide), cuja reação é dependente da atividade celular, pode ser questionada. Assim, objetivou-se no primeiro estudo que compõem este trabalho, padronizar e aperfeiçoar esta técnica através da suplementação de glicose. Para isso, biofilmes de *Candida albicans* ATCC 90028 com tempos de crescimento de 24, 48 e 72 h foram avaliados. Para estabelecer o melhor tempo de incubação do XTT, este foi mantido a temperatura de 37 °C, em tempos de 90, 180 e 270 minutos. A fórmula padrão do teste XTT (controle) foi modificada com a adição de 50, 100 e 200 mM de glicose para os grupos experimentais. Os melhores resultados para a incubação foram observados com tempo de 180 minutos e para a suplementação de glicose à concentração de 200 mM ($p<0,001$). Uma vez determinada a melhor condição de leitura da bioatividade dos biofilmes, foi idealizado o segundo estudo, que objetivou a mensuração da bioatividade e a análise morfológica de biofilmes de *Candida albicans* desenvolvidos sobre a superfície de PMMA. Duas resinas a base de PMMA (banho de água e microondas) foram utilizadas como substrato para o desenvolvimento de biofilmes de duas cepas de *Candida albicans* (ATCC 90028 e SC5314). A atividade metabólica destes biofilmes foi mensurada com a técnica modificada de redução mitocondrial do XTT. A análise da morfologia da fase de adesão e dos biofilmes de 24, 48 e 72 horas foi avaliada com o auxílio do microscópio de varredura confocal a laser e o microscópio eletrônico de transmissão. A combinação das análises da morfologia com a mensuração da bioatividade, permitiu verificar o desenvolvimento do biofilme, desde a fase de adesão, com alta atividade metabólica, evoluindo para a constituição de uma comunidade microbiana de alta complexidade estrutural com a presença de matriz de polissacarídeos extracelulares, através da qual os nutrientes se difundem, porém dotados de pouca atividade metabólica. O método

de ativação das resinas não interferiu na bioatividade do biofilme e com a morfologia do biofilme formado para a cepa de *Candida albicans* SC 5314. Com base nestes estudos concluiu-se que a incubação de 180 minutos utilizando a suplementação de 200 mM de glicose apresenta resultados de atividade metabólica celular com a menor variação para o estudo de biofilmes de *Candida albicans* e que a morfologia do biofilme de ambas as espécies foi fator fundamental para as diferenças na estrutura do biofilme.

Palavras-chave: poli (metil metacrilato); biofilme, *Candida albicans*.

ABSTRACT

Candida albicans biofilms, developed on poly (methyl methacrylate) (PMMA) resin surfaces show high virulence index due to hydrolytic enzyme secretion and are responsible for oral candidosis in removable denture wearer. Since biofilms are surrounded by an extracellular polysaccharides matrix, the nutrients diffusion across the cell layers is difficulted, occasioning cell with different metabolic states. Thus, considering that the XTT reduction assay is dependent of cellular activity, its use for evaluating mature biofilms may lead to inaccuracies since biofilm bottom cells layers tends to be relatively quiescent at later stages of biofilm formation. The aim of the first study was the improvement of the XTT reduction assay by using glucose supplements in the standard XTT formulation. *Candida albicans* ATCC 90028 was used to form 24, 48 and 72 hours biofilm. The oxidative activity at 90, 180 and 270 minutes of incubation was evaluated. The standard XTT composition was modified by the addition of 50, 100 and 200 mM of glucose. The control comprised XTT formulation without glucose supplements. Biofilm growth yield after 180 min incubation, when evaluated with the 200 mM glucose supplemented XTT produced the most consistent readings on repetitive testing ($p<0.001$). After the results of the determination of the oxidative activity assay using XTT supplemented by 200 mM glucose second study was conducted in which it was evaluated the characteristics of biofilm development of *Candida albicans* on two different poly (methyl methacrilate) resins (PMMA). Thus, two different *Candida albicans* strain (ATCC 90028 and SC5314) were allowed to develop its biofilm in two PMMA surfaces with different polymerization method: waterbath (WB) and microwave (MW). Different time points (adhesion, 24, 48 and 72 hours) were compared. Surface roughness (SR) and surface free energy (SFE) of PMMA specimens were previously measured. Oxidative activity was measured by XTT. Confocal images were used to measure bio-volume, thickness, biofilm roughness, diffusion and the of live/dead cells proportion in the different biofilm development stages. Cell counts were used to estimate the number of cells, and a combination of these techniques allowed to describe the *Candida albicans* biofilms development, starting from a small amount of cells with high oxidative activity, reaching to microbial community of complexity structural prosperities, with the presence of a matrix of polysaccharide,

however with low oxidative activity. WB and MW showed no difference regards SR and SFE means ($p>0.05$) and oxidative activity showed different results for each specie ($p<0.001$). Based on both studies, it can be conclude that 180 min incubation with the 200 mM glucose supplemented XTT generates more accuracy in oxidative activity results and also that the different polymerization method of PMMA are not able to interfere with biofilm development and main differences detected were related to the intrinsic characteristic of each *Candida* strain and biofilm development time.

Key words: poly (methyl metacrylate); Candida albicans; biofilm.

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1. INTRODUÇÃO

Nos últimos 20 anos constatou-se uma alteração na epidemiologia das infecções fúngicas. Uma vasta lista de condições médicas como transplantes, cânceres, terapias imunosupressivas, AIDS, idade avançada e grandes cirurgias (1, 2) levam ao uso crônico e/ou profilático de agentes antifúngicos acarretando a seleção dos microrganismos mais resistentes. Além dessas alterações epidemiológicas, o fenômeno da inversão da pirâmide populacional, devido o aumento da expectativa de vida da população mundial e brasileira, acarreta maior número de pacientes reabilitados com próteses dentais removíveis e/ou implanto suportadas (3, 4), sendo estes os mais propensos a desenvolvimento de doenças fúngicas.

Entre as doenças fúngicas que acometem os usuários de próteses removíveis, destaca-se a candidose, ou estomatite induzida por prótese, na qual a *Candida albicans* atua como o patógeno mais importante e mais virulento (5). A prevalência de candidose situa-se entre 11 e 67% dos usuários de próteses dentárias removíveis (6) e a associação da *Candida albicans* como principal patógeno está diretamente relacionada à sua eficiência de aderir e colonizar as superfícies de próteses dentárias. O sucesso da colonização sobre a superfície da resina poli (metil metacrilato) (PMMA) se relaciona com as propriedades físicas apresentadas por este material, tais como energia livre de superfície e rugosidade de superfície, que podem interferir na adesão destes microorganismos.

Na matriz polimérica das resinas de poli (metil metacrilato), as cadeias internas apresentam-se em equilíbrio dinâmico, uma vez que suas cadeias circundantes exercem uma atração de mesma intensidade em sentido contrário, o que não ocorre nas cadeias externas onde esse equilíbrio é inexistente. Esta diferença de atração molecular nas cadeias externas apresenta uma força resultante denominada energia livre de superfície, que se relaciona com a energia eletrostática da parede celular do microrganismo (5, 7, 8), que através de interações químicas, promove a adesão inicial com a posterior formação de biofilme (5, 9).

Quando a resina acrílica apresenta rugosidade de superfície acima de 0,02 μm, esta se torna passível de colonização (5, 10-12), pois o crescimento em forma micelial

de *Candida* spp faz com que esse fungo se desenvolva e se aloje no interior das ranhuras presentes na superfície de PMMA, onde estará livre de forças externas de remoção, como exemplo o efeito limpante da saliva, proporcionando um reservatório de fungos para uma posterior reinfecção (13).

O desenvolvimento de biofilme de *Candida spp* sobre a superfície de resinas de poli (metil metacrilato) transcorre em diferentes fases, iniciando-se pela adesão, onde ocorre interação entre a superfície do material e a *Candida* spp. que utiliza seus filamentos extracelulares para a fixação e adesão. Após esse primeiro contato com a superfície, inicia-se a fase de colonização, onde ocorre a formação de micro-colônias. O desenvolvimento celular e maturação destas colônias levam a formação de uma comunidade microbiológica altamente especializada, denominada de biofilme (5). Esta comunidade encontra-se envolta por uma matriz de polissacarídeos extracelulares, conferindo ao biofilme sustentação e proteção (14-16).

A formação de uma estrutura espacial complexa, em função da presença da matriz de polissacarídeos extracelular resulta em uma distribuição desigual dos nutrientes ao longo das camadas que compõem o biofilme, ocasionando diversidade na atividade metabólica. Enquanto as camadas superiores do biofilme são ativas em função da grande riqueza de nutrientes, as camadas basais encontram-se em estado de quiescência (17-19). Assim, como a matriz de polissacarídeos extracelular dificulta a difusão de nutrientes ao longo do biofilme, o mesmo ocorre para os agentes antimicrobianos, conferindo uma maior resistência aos fármacos (18, 20).

Embora o processo de adesão de microrganismos sobre a superfície da resina acrílica esteja definido (5, 10-12), especula-se que em um cenário de alta complexidade como o descrito anteriormente, as interações entre o substrato e o biofilme transcorram além do período de adesão inicial e podem promover alterações na arquitetura do biofilme com conseqüente comprometimento nas funções biológicas. Dessa forma considerando os poucos estudos para essa elucidação foi objetivo neste trabalho avaliar a influência da superfície de resinas de poli (metil metacrilato) na estrutura e bioatividade de biofilmes de *Candida albicans*.

2. CAPÍTULO 1

Improvement of XTT assay performance for studies involving *Candida albicans* biofilms

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Abstract

2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay has been used to study Candida biofilm formation. However, considering that the XTT reduction assay is dependent of cellular activity, its use for evaluating mature biofilms may lead to inaccuracies since biofilm bottom cells layers tends to be relatively quiescent at later stages of biofilm formation. The aim of this study was the improvement of the XTT reduction assay by using glucose supplements in the standard XTT formulation. Candida albicans ATCC 90028 was used to form 24, 48 and 72 hours biofilm. The oxidative activity at 90 180 and 270 minutes of incubation was evaluated. The standard XTT composition was modified by the addition of 50, 100 and 200 mM of glucose. The control comprised XTT formulation without glucose supplements.: The XTT assay with 200 mM glucose showed more accurate and consistent readings correlating with biofilm development at 24, 48 and 72 hours. Biofilm growth yield after 180 min incubation, when evaluated with the 200 mM glucose supplemented XTT produced the most consistent readings on repetitive testing. It can be concluded that glucose supplementation of XTT, could minimize variation and yield more accurate data for XTT assay.

Key words: *Candida albicans*, biofilm, XTT.

Running title: XTT assay improvement for C. *albicans* biofilm research

Introduction

Candida is a commensal fungus that inhabits half of human oral cavities. However under certain circumstances, particularly in compromised host population such as HIV/AIDS patients, organ transplant recipient, *Candida* can cause opportunistic infection ranging from superficial mucous membrane infection to life-threatening systemic disease (1).

A major virulent attribute of Candida is its ability to adhere and form surface attached microbial communities known as "biofilms". *Candida* biofilms are especially widespread and have been observed in most, medical devices such as stents, shunts, implants, endotracheal tubes, pacemakers, and various types of catheters (2). Denture wearers can also be named as surface substrata. Biofilm development can be divided into four sequential steps: first, adhesion of microorganisms to a surface, second, discrete colony formation, and organization of cells; third, secretion of extracellular polysaccharides (EPS) and maturation into a three-dimensional structure; and fourth, dissemination of progeny biofilm cells (3). Therefore, it is important to evaluate the dynamic process of formation and development of *Candida* biofilms for understanding the pathogenicity and treatment of this medically important fungus.

Development of biofilm on a surface can be better studied by quantifying the biofilm mass along different time points. Methods such as counting colony forming units (cfu), spectrophotometric analysis, and colorimetric assays such as 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay have been employed to quantify the *Candida* biofilms. However, each method carries its own limitations and drawbacks. For example, cfu count has been shown to under-represent the cell number. In spectrophotomeric analyses of cell density, all cells, both live or dead, contribute to the readings, which produces an over-estimation of cell number.

XTT reduction assay has been used as a routine tool for the quantification of *Candida* biofilms (4-7) because it measures cellular activity. The intracellular reduction of XTT releases a formozan compound which can be quantified by colorimetric estimation (4). At present, XTT reduction assay formula contains three components namely XTT,

menadione, and phosphate buffer saline (PBS). XTT reduction depends on cellular activity, instead of cell mass, however some researchers have questioned its accuracy in biofilm quantification (4, 6). Development of biofilm into complex spatial structures results in uneven distribution of nutrients across their multiple layers which can lead cell population to heterogeneous spectrum of metabolic activity. While nutrient rich upper layers exhibit a relatively higher metabolic activity, nutrient deficient basal layers have been shown to have lower metabolic activity (3, 5, 8, 9). Therefore, XTT reduction assay in mature biofilms can underestimate the cell activity of the biofilm.

Previous studies have attempted to improve the XTT assay by supplementing various ingredients to compensate for the inequalities of metabolic activity, increasing the accuracy of biofilm quantification. Carbohydrates are the main source of energy in *Candida* metabolism. Glucose is easily metabolized by *Candida* (1) and its reasonable low cost suggests its use as first choice. Therefore, the purpose of this study was to evaluate whether addition of glucose on XTT reduction assay can improve XTT reduction assay for the quantification of *Candida* biofilms. The tested null hypotheses was that the supplementation of XTT formulation with glucose can improve the reliability and reproducibility of the test by providing the sufficient nutrient to the *Candida* cells across the different biofilm layers.

Materials and methods

Candida albicans strain

A reference strain of *Candida albicans* (ATCC® 90028™) was selected for this study. The identity of isolate was confirmed by carbohydrate assimilation tests using a commercially available API 32C identification system (bioMérieux, Marcy l'Etoile, France).

Preparation of Candida albicans suspension

Prior to each experiment, the yeast strain was aerobically cultured at 37 °C for 18 h on Sabouraud Dextrose Agar (SDA) and a loopful of growth was inoculated in Yeast Nitrogen Base (YNB) broth (Difco Laboratories, Detroit, MI) supplemented with 50

mM glucose. After 18 h of incubation, the cells, which was in late exponential growth phase, were harvested, washed twice with PBS (pH 7.2) and resuspended in YNB supplemented with 100 mM glucose. Standard *Candida* suspensions were prepared to a concentration of 10^7 cells/mL by adjusting the optical density to according to the McFarland standards using a *Beckman DU530 UV/visible* spectrophotometer (Beckman Coulter Inc., Fulleton,CA USA). Previous studies have shown that this cell concentration is optimal for biofilm development of *C. albicans* (1).

Biofilm development

C. albicans biofilm formation was performed as described by Jin et al. (1) with some modifications. Briefly, biofilms were grown in commercially available pre-sterilized, polystyrene, flat-bottomed 96-well microtiter plates (IWAKI, Tokyo, Japan). Aliquots of 100 µL of standard cell suspensions of yeasts (10^7 cells/mL) were transferred into each well, and incubated for 1.5 h (*adhesion phase*) at 37 °C at 75 rpm in an orbital shaker (Lab-line Incubator Shaker, Elliott Bay Laboratory Services, USA). After the adhesion phase, the cell suspensions were gently aspirated and each well was washed twice with PBS to remove any remaining planktonic cell taking care not to disturb the adherent cells. In order to allow the growth of biofilm (*biofilm phase*), 200µL of freshly prepared YNB with supplemented with 100mM glucose was added to each well. The plates were incubated for 24, 48 or 72 h at 37 °C at 75 rpm in an orbital shaker. At 24 h of incubation, medium was aspirated and, biofilms were washed twice with PBS followed by addition of fresh 200 µL medium. At different time points biofilms were quantified using the XTT reduction assay. All assays were repeated six times in two separate occasions.

Oxidative activity assay

To prepare modified standard XTT, anhydrous D (+) glucose (Sigma-Aldrich, St Louis, MO, United States) was dissolved in sterile PBS to obtain final concentrations of 200, 100 or 50 mM and PBS without glucose was used to prepare standard XTT.

XTT reduction assay was performed as previously described. (1) XTT (Sigma-Aldrich, St Louis, MO) was dissolved in PBS at a final concentration of 1 mg/mL. The solution was filter sterilized and stored at −70°C, until being used. Menadione 0.4 mM (Sigma-Aldrich, St Louis, MO) solution was prepared immediately prior to each assay. For each assay, XTT solution was thawed on ice and mixed with menadione solution at a volume ratio of 20:1.

The biofilms were washed twice with 200μL of PBS to remove no adherent cells. Afterwards, 158 μL of PBS with or without glucose at different concentrations, 40 μL of XTT, and 2 μL of menadione were transferred to each well of 96-well plates. The plates were covered with aluminum foil and incubate in dark at 37 °C for 1.5 h, 3 h or 4.5 h. Thereafter, 100 μL of the solution was transferred to each well of new 96-well plates. The colorimetric changes were measured at 492 ηm using a microtiter plate reader (Spectra-MAX 340, Molecular Devices Ltd., Sunnyvale, CA).

Statistical analysis

The statistical analysis was carried out using the SAS 9.0 software (SAS Institute Inc., Cary, NC) with the significance at $\alpha = 0.05$. The dependent variables (time of incubation, glucose concentration and biofilm development) and the independent variable (Oxidative activity) were statistically analyzed. The assumptions of homogeneity of variances and normal distribution of errors were tested. As the data were not normally distributed, they were transformed by exponentiation ($x^{2.4}$). ANOVA was used to test the null hypothesis of no differences among the treatments. The Tukey HSD test was then used for post-ANOVA comparisons.

Results

Initial analysis for the three different XTT incubation times (90 min, 180 min, and 270 min) showed that the longest incubation time had the highest OD values ($p<0.001$). However, the use of 270-min incubation showed a high metabolic activity in 24-h biofilm, overestimating the OD value for this initial period. An incubation period of 180 min showed OD values consistent with the increased biomass and metabolic activity. Results of 90-min incubation showed a high variance of absorbance values, indicating a lack of the accuracy (Fig. 1). The incubation periods of 90 and 270 min were discontinued from further analysis due inaccuracy of values for reduced XTT absorbance. Thus, 180 min of XTT treatment was considered for all subsequent analyses.

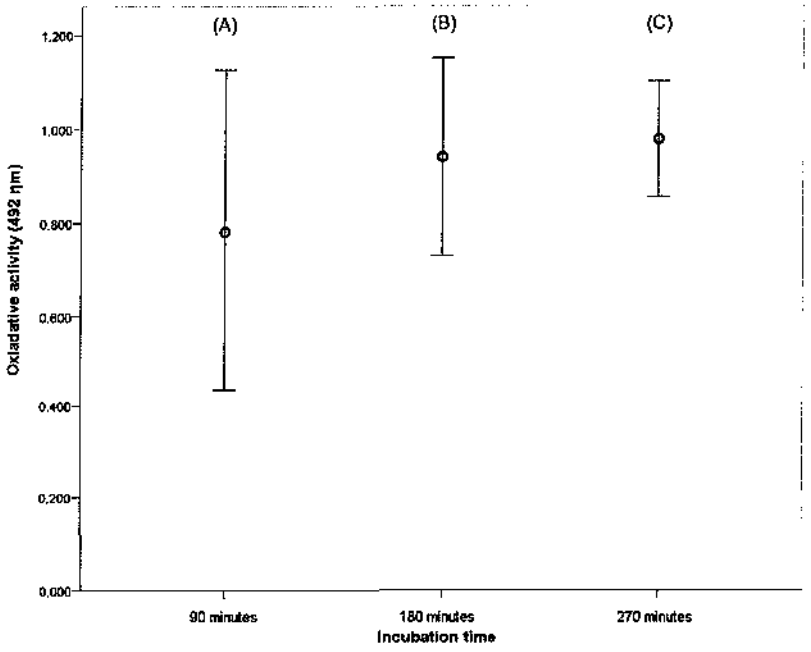

Fig. 1. Oxidative activity of biofilms for different incubation time.

Glucose concentration ($p<0.001$) and biofilm development ($p<0.001$) were able to interfere in oxidative activity of *Candida* biofilms. The readings of 180-min readings of XTT-incubated biofilms at each glucose concentration showed a significant difference from control ($p<0.001$). The addition of glucose was responsible for oxidative activity, indicating interference on metabolic activity and resulting in lower coefficients of

variation (control = 11.39 %; 50 mM = 8.81 %; 100 mM = 12.19% and 200 mM = 6.56%) (Fig. 2).

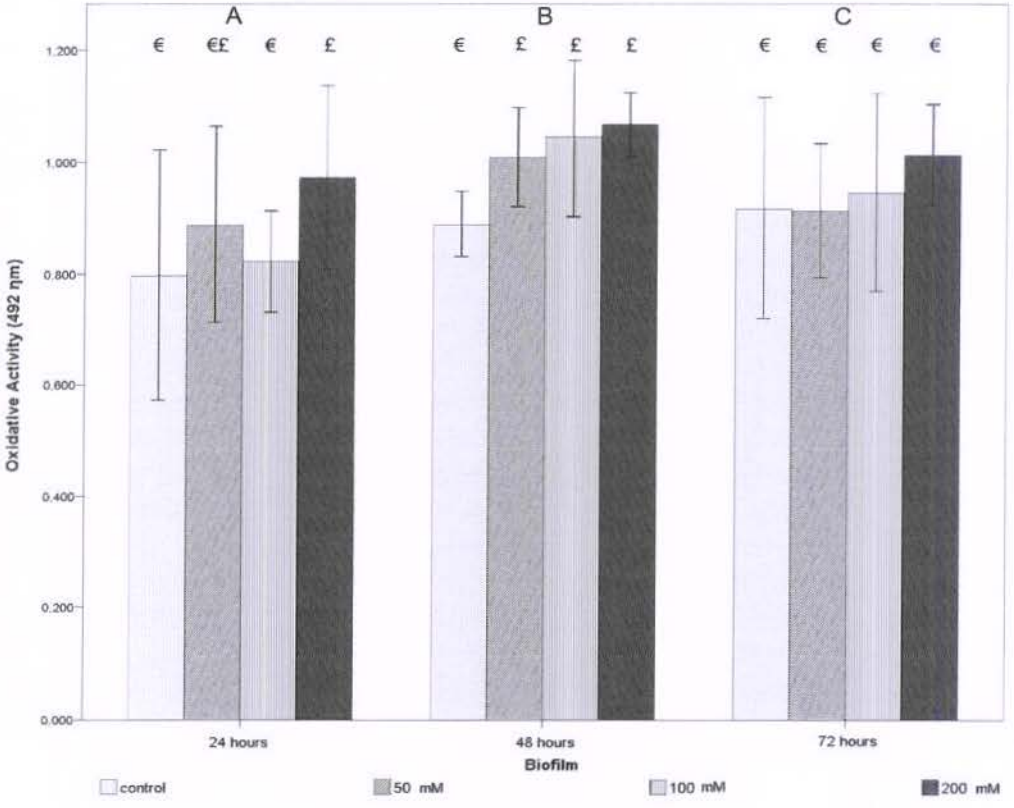

Fig. 2. Oxidative Activity of biofilms for different glucose concentration.

Distinct upper case letters show differences between oxidative activity for biofilms developed at 24, 48 and 72 hours; $P<0.0001$. Distinct symbols show differences between metabolic activity and glucose concentration ($P<0.001$).

Discussion

The present study was carried out in order to improve the reliability and reproducibility of XTT assay.

C. albicans biofilm can be considered in three distinct developmental layers. The basal layer of the *Candida* biofilm act as founder cells anchoring the developing biofilm on substrate. The middle layer is composed of hyphae and pseudohyphae, and the most superficial layer of the biofilm consists predominantly of a thicker and porous hyphal layer with abundant extracellular matrix (3, 5, 8, 9). During the growth phase, approximately 48 h after its formation, biofilm develops into metabolically active communities of cells that get interspersed with water channels. Biofilm structural complexity of the biofilm may create a gradient of environmental conditions in which the *C. albicans* cells enter distinct physiological states that is different from the planktonic counterparts (10).

The idea of using glucose to stimulate the mitochondrial activity of *C. albicans* biofilms derived from the hypothesis that cells in different layers undergo drastic differences concerning to oxygen and nutrient accessibility or oxidative/reductive stress. The tested hypothesis was that when not using any mitochondrial stimulus only the top layer cells, that remained exposed to outer environment, is in a position to reduce the XTT. Thus, an underestimation of the true cell content may occur. As it is well known that this yeast has a great affinity for carbohydrates (1) and that low-molar masses solutes may promptly achieve the bottom layers of biofilms (11, 12) flowing through the interstitial spaces (13) and diffusing from these points, it is reasonable to suppose that more cells may consume the carbohydrate and use the enzymatic mitochondrial machinery.

None of the XTT-reduction based protocols currently used for biomass evaluation, proposes the use of glucose to enhance the formazan detection. Recently, Knight and Dancis (14) proposed that the reduction of some tetrazolium salts by *C. albicans* in unbuffered conditions may be due to reductases and they used an assay buffer containing 277 mM glucose to assess the enzymatic activities. In the present study, the results showed that the addition of glucose increased the detection of XTT-reduced products, the formazans, compared to the controls without any addition. Moreover,

significant differences were not observed when different glucose amounts were added, thought 200 mM glucose was proven to reduce the coefficient of variation, wich is desirable to improve the accuracy of the assay. It may be suggested that by adding glucose the examiner can simultaneously detect the cytosolic reductases (14) and the mitochondrial succinoxidase and cytochrome P450 systems, as well as flavoprotein oxidases (6).

Two questions arose during the elaboration of this idea: Is it possible that after the addition of XTT-glucose the cells in top layers start to grow faster than those in basal layers, which could to an erroneous result? If the cells entrapped into the extra-polymeric matrix are under absence of oxygen, how they could active the mitochondrial enzymes that work in an aerobic pathway?

In fact, the first question sounds reasonable for those cells evaluated during the *adhesion phase*. However, it is believe that even these washed cells when received the XTT-glucose revealing solutions required a considerable time to pass trough the "lag phase" and to enter in "log phase". The assumption is based on the statements of Chen et al. (2004)(15) that this "lag phase" is usually attributed to the requirement for auto-stimulatory compounds that are released into the medium as the cells proliferate, but fall below some critical concentration when the cells are diluted into fresh medium. Moreover, it is opportune to consider that the XTT-glucose solutions are not *strictu sensu* culture broths.

In order to answer the second question, a previous review article (16) was revisited. The relative effective diffusion coefficient (D_e/D_{aq}) of oxygen in biofilms is near to 0.65×10^{-6} cm^2s^{-1}, which is much higher than the 0.30×10^{-6} cm^2s^{-1} for glucose. If is assumed that glucose may achieve the bottom layers, it is reasonable to propose that the oxygen also achieve those microenvironments. In addition, both conditions of incubations (presence or absence of glucose) were conduced in opened-air systems making it possible the diffusion of oxygen whose diffusion coefficient in water at 35.5 °C is 25.2×10^{-6} cm^2s^{-1} (17). On the other hand, it is possible that aerobic respiration compromise the entire XTT reduction. Recently, it was showed (14) that the metalloenzymes superoxide dismutases (SODs), when present at 30 $\mu g\ ml^{-1}$ may inhibit up to 36% of XTT reduction, although most XTT reduction is SOD-independent. Such enzymes are constitutively produced by

planktonic *C. albicans* cells (18, 19) and at least one isozymic form has its production increased upon the entry and during the stationary phase (20). To authors knowledge no data concerning to the production of SODs in *C. albicans* cells living in biofilm phase is available.

Conclusion

In conclusion, XTT supplemented with 200 mM of glucose and incubation time of 180-min were proven a promising modification for the reduction XTT assay. This XTT assay can be readily used to increasing reproducibility and reliability of the results up to 72-h developed *Candida* biofilms. Since XTT reduction assay is widely used for various applications of *Candida* biofilms, suggested novel XTT formulation can be useful for further studies in this research line.

Resumo

O teste de redução do 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) tem sido utilizado para mensurar o desenvolvimento de biofilmes de *Candida*. Contudo, a reação de XTT é dependente da atividade celular e o seu uso para biofilmes maduros pode ser questionado, considerando que diferentes camadas celulares têm atividade metabólica diferenciadas. O objetivo deste estudo foi avaliar se a adição de glicose à formula de XTT diminuiria a variabilidade na mensuração da atividade metabólica. Biofilmes de *Candida albicans* ATCC 90028 com tempos de crescimento de 24, 48 e 72 horas foram utilizados. Para avaliar o melhor tempo de incubação do XTT, este foi mantido a temperatura de 37 °C, em tempos de 90 180 e 270 minutos. A fórmula padrão do teste XTT (Controle) foi modificada com a adição de 50, 100 e 200 mM de glicose para os grupos experimentais. Os melhores resultados para a incubação foi observado com tempo de 180 minutos e para a suplementação de glicose à concentração de 200 mM ($p<0.001$). Concluiu-se que a incubação de 180 minutos utilizando a suplementação de 200 mM de glicose apresenta resultados de atividade metabólica celular com a menor variação para o estudo de biofilmes de *Candida albicans*.

Acknowledgement

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References

1. Jin Y, Samaranayake LP, Samaranayake Y, Yip HK. Biofilm formation of Candida albicans is variably affected by saliva and dietary sugars. Arch Oral Biol 2004;49:789-98.

2. Ramage G, Martinez JP, Lopez-Ribot JL. Candida biofilms on implanted biomaterials: a clinically significant problem. Fems Yeast Research 2006;6:979-86.

3. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. Biofilm formation by the fungal pathogen Candida albicans: development, architecture, and drug resistance. J Bacteriol 2001;183:5385-94.

4. Roehm NW, Rodgers GH, Hatfield SM, Glasebrook AL. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. J Immunol Methods 1991;142:257-65.

5. Ramage G, Vandewalle K, Wickes BL, Lopez-Ribot JL. Characteristics of biofilm formation by Candida albicans. Rev Iberoam Micol 2001;18:163-70.

6. Kuhn DM, Balkis M, Chandra J, Mukherjee PK, Ghannoum MA. Uses and limitations of the XTT assay in studies of Candida growth and metabolism. J Clin Microbiol 2003;41:506-8.

7. Jahn B, Martin E, Stueben A, Bhakdi S. Susceptibility testing of Candida albicans and Aspergillus species by a simple microtiter menadione-augmented 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. J Clin Microbiol 1995;33:661-7.

8. Baillie GS, Douglas LJ. Role of dimorphism in the development of Candida albicans biofilms. J Med Microbiol 1999;48:671-9.

9. Reese S, Guggenheim B. A novel TEM contrasting technique for extracellular polysaccharides in in vitro biofilms. Microsc Res Tech 2007;70:816-22.

10. Uppuluri P, Sarmah B, Chaffin WL. Candida albicans SNO1 and SNZ1 expressed in stationary-phase planktonic yeast cells and base of biofilm. Microbiology 2006;152:2031-8.

11. Thurnheer T, Gmur R, Shapiro S, Guggenheim B. Mass transport of macromolecules within an in vitro model of supragingival plaque. Appl Environ Microbiol 2003;69:1702-9.

12. Zhang T, Fang HH. Effective diffusion coefficients of glucose in artificial biofilms. Environ Technol 2005;26:155-60.

13. Samaranayake YH, Ye J, Yau JY, Cheung BP, Samaranayake LP. In vitro method to study antifungal perfusion in Candida biofilms. J Clin Microbiol 2005;43:818-25.
14. Knight SA, Dancis A. Reduction of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) is dependent on CaFRE10 ferric reductase for Candida albicans grown in unbuffered media. Microbiology 2006;152:2301-8.
15. Chen H, Fujita M, Feng Q, Clardy J, Fink GR. Tyrosol is a quorum-sensing molecule in Candida albicans. Proc Natl Acad Sci U S A 2004;101:5048-52.
16. Stewart PS. Diffusion in biofilms. J Bacteriol 2003;185:1485-91.
17. Han P, Bartels D. Temperature dependence of oxygen diffusion in H2O and D2O. J Phys Chem 1996;100:5597-602.
18. Rosa EA, Pereira CV, Rosa RT, Hofling JF. Grouping oral Candida species by multilocus enzyme electrophoresis. Int J Syst Evol Microbiol 2000;50 Pt 3:1343-9.
19. Rosa EA, Rosa RT, Pereira CV, Boriollo MF, Hofling JF. Analysis of parity between protein-based electrophoretic methods for the characterization of oral Candida species. Mem Inst Oswaldo Cruz 2000;95:801-6.
20. Lamarre C, LeMay JD, Deslauriers N, Bourbonnais Y. Candida albicans expresses an unusual cytoplasmic manganese-containing superoxide dismutase (SOD3 gene product) upon the entry and during the stationary phase. J Biol Chem 2001;276:43784-91.

3. CAPÍTULO 2

Characteristics of *Candida albicans* biofilm developed on poly (methyl methacrilate) resins surfaces

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Abstract

The aim of this study was to evaluate the characteristics of *Candida albicans* biofilm developed on the surface of two different poly (methyl methacrilate) resins (PMMA). Two different *Candida albicans* strains (ATCC 90028 and SC5314) were allowed to develop biofilm in two PMMA surfaces with different polymerization method: waterbath (WB) and microwave (MW) and at time points: adhesion, 24, 48 and 72 hours were compared. Surface roughness (SR) and surface free energy (SFE) of PMMA specimens were previously measured. Oxidative activity of biofilms was measured by modified XTT reduction assay. Confocal images were used to measure bio-volume, thickness, biofilm roughness, diffusion spaces and the live/dead cells proportion in the different biofilm development stages. Cell counts were used to estimate the number of cells in those biofilms. WB and MW showed no difference between SR and SFE means ($p>0.05$). Oxidative activity of biofilms showed different results for each specie ($p<0.001$). Cells counts and confocal images showed an increasing biomass at the evaluated time points. It can be concluded that the different polymerization method of PMMA are not able to interfere with biofilm development, however main differences detected were related to the characteristic of each *Candida* strain and biofilm development time.

Introduction

Chronic erythematous candidosis (CEC) is the most prevalent form of oral candidosis and it affects more than 65% of denture wearers, thus becoming a predisposing factor for CEC [1-3]. Generally it occurs in elderly denture users and can cause opportunistic infection ranging from superficial mucous membrane infection to life-threatening systemic disease [4]. A major virulent attribute of *Candida albicans* is its ability to adhere and form surface attached microbial communities known as "biofilms". The development of theses communities could be divided didactically into four sequential steps: adhesion of microorganisms to a surface, initial colony organization, secretion of extracellular polysaccharide with the development into a matrix structure and dissemination of progeny biofilm cells [5-7].

Although *Candida* biofilms are especially widespread and have been observed in most medical devices, such as, stents, shunts, implants, endotracheal tubes, pacemakers, and various types of catheters [8], many authors highlight its occurrence on denture base material and its consequence for removable denture wearers [9-11]. Therefore, it is important to evaluate this dynamic process of formation and development of *Candida* biofilms on prosthesis dental material for understanding the pathogencity and the treatment for this medically important yeast.

Studies involving development of *Candida* biofilms on different dental biomaterials surfaces had been conducted without considering the full complexity of these microbiological structures [5,11,12], thus information concerning the development stages of the biofilms structures, such as nutrients diffusion, biomass and thickness, remain undefined. Surface free energy (SFE) and surface roughness (SR) of poly (methyl metacrilate) (PMMA) had been claimed as the main responsible for regulating the adhesion of *Candida* cells on such surface [3,11,13]. However the effects of these characteristics on the development of biofilms structures remain unclear.

Thus, this study aimed to verify the characteristics of biofilm development on poly (methyl methacrylate) surface and its influence on the structure and bioactivity of the biofilm of two *Candida albicans* strains.

Materials and methods

Experimental design

Discs shaped measuring 10.0 x 2.0 mm were made of acrylic resin polymerized by water bath (WB) or microwave (MW) PMMA resins, according to manufacturer's directions. The resins surface were finished and polished and SR and SFE measurements were performed. Next, the samples were randomly divided into two groups, according to PMMA resin, and exposed for 90 min to one of the two *Candida albicans* (ATCC90028 or SC5314) inoculum (10^7 cells/mL) and then washed to eliminate non-adhered cells. New medium was added and biofilms were allowed to develop for 72 h. Oxidative activity of biofilms and cell counts were measured in four time points (adhesion phase, 24, 48 and 72 h). Scanning electronic microscopy (SEM), confocal scanning laser microscopy (CSLM) were used to evaluate the structure of the biofilm at the evaluated time points. The statistical analysis was made considering three factors – polymerization method, *Candida* strain and biofilm development period.

Preparation of acrylic samples

The PMMA resins used in this study were water bath (Lucitone, Denstply, De Trey, USA; WB) and microvable (Acron MC, GC. America, USA; MW). A wax matrix was flasked in Type III dental stone (Herodent Soli-Rock; Vigodent, Rio de Janeiro, Brazil) using a metal dental flask (Uraby; DLC, São Paulo, Brazil) or a plastic flask (BMF1; Artigos Odontológicos Clássico Ltd, São Paulo, Brazil) for water bath and microwave polymerization, respectively. After the gypsum set, wax was eliminated with boiling water. WB was then packed and the metal flasks were placed in a polymerizing unit (Termotron P-100; Termotron Equipamentos Ltd, Piracicaba, Brazil) filled with water at 74°C for 9 h. The plastic flasks with MW were placed in a microwave oven (AW-42 Continental Eletrodomésticos Ltd, Manaus, Brazil) and polymerized for 3 min at 500 W, according to manufacturer's instructions. All flasks were allowed to bench cooling for at least 3 h and the samples were removed.

Specimens were ground using progressively smoother aluminum oxide papers (grit 320, 400 and 600) in a horizontal polisher (model APL-4; Arotec, São Paulo,

Brazil). For mechanical polishing, a brush disc with pumice slurry and a felt cone with chalk powder were used. They were also ultrasound cleansed (Thornton T 740, Thornton-Inpec Eletrônica LTDA, Vinhedo, Brazil) for 20 min and then immersed in distilled water at 37°C for 12 h for residual monomer release. After these procedures, SR and SFE were measured.

Surface roughness

SR of the acrylic samples was measured using a profilometer (Surfcorder SE 1700 Kozaka Industry, Kozaka, Japan) with a 0.01 μm resolution, calibrated at sample length of 0.8 mm, 3.2 mm percussion of measure, and 0.5 mm/s. Three readings were taken for each sample and a mean value was calculated [14].

Surface free energy

SFE was calculated with the deposition of 15 μL of distilled water (in triplicate) on each acrylic resin specimen. The image of each sessile drop was captured using a digital camera (Mavica CD 350, Sony, Tokyo, Japan) immediately after its deposition and the mean value of contact angles were measured using Autocad 2005 (Auto Desk, Sankt Augustin, USA). Hence, SFE was calculated using cos Θ of contact angles, according to Silva *et al.* 2008 [14].

After making the surface roughness and surface free-energy measurements the contaminants of the acrylic resin specimens were removed by sonication for 20 minutes, washed with sterile distilled water, and sonicated again for 20 minutes[11]. Next, specimens were randomly divided according the PMMA resin, *C .albicans* strains and biofilm evaluation period.

Candida albicans strain

Candida albicans reference strain ATCC® 90028™ and a wild-type (SC5314) were selected for this study. The identity of both strains was reconfirmed by

carbohydrate assimilation tests using a commercially available API 32C identification system (bioMérieux, Marcy l'Etoile, France).

Preparation of Candida albicans suspension

Prior to each experiment, the yeasts strains were aerobically cultured at 37°C for 24 h on Sabouraud Dextrose Agar (SDA) and a loopful of growth was inoculated into Yeast Nitrogen Base (YNB) broth (Difco Laboratories, Detroit, MI) supplemented with 50 mM glucose. After 18 to 20 hours of incubation, cells were washed twice with PBS and suspended in YNB supplemented with 100 mM glucose. Standard *Candida albicans* suspensions were prepared to a concentration of 10^7 cells/mL [4].

Biofilm development

Biofilms were developed in PMMA surfaces placed inside pre sterilized flat-bottomed 24-well microtiter plates (IWAKI, Tokyo, Japan). Aliquots of 2.0 mL of standard cell suspensions of yeasts were transferred into each well with one disc, and incubated for 90 min at 37°C in an orbital shaker (Lab-line Incubator Shaker, Elliott Bay Laboratory Services, USA) at 75 rpm (adhesion phase). After the adhesion phase, the cell suspension were gently aspirated and each specimen was washed twice with PBS. For biofilm phase, 2.0 mL of freshly prepared YNB supplemented with 100 mM glucose was added to each well. The plates were incubated for 24, 48, or 72 h at 37°C at 75 rpm in an orbital shaker as described before. At the end of each 24 h incubation, medium was aspirated and specimens were washed with PBS followed by the addition of fresh 2.0 mL medium. At the different time points, cell count and bioactivity of biofilms were performed. SEM and CSLM were used to evaluate the structure of the biofilms. Oxidative activity of biofilms assays were carried out in five replicates in three different occasions. Cell counts were performed twice in three separate occasions. SEM and CSLM were performed once in two distinct occasions.

Oxidative activity of biofilms

For biofilm bioactivity measurement, XTT (Sigma-Aldrich, St Louis, MO) was dissolved in PBS at a final concentration of 1 mg/mL. The solution was filter sterilized and stored at −70°C, until being used. 0.4 mM menadione (Sigma-Aldrich, St Louis, MO) solution was prepared immediately prior to each assay[15]. For each assay, XTT solution was mixed with menadione solution at a volume ratio of 20:1.

The biofilms were washed twice with 1.0 mL of PBS to remove nonadherent cells. Afterwards, 1580 μL of PBS 200 mM glucose, 400 μL of XTT, and 20 μL of menadione were transferred into each well. The plate was covered with aluminum foil and incubated covered by aluminum foil at 37°C for 3 h. After, 1000 μL of the solution was centrifuged and transferred to a 96-well plate. The colorimetric changes were measured at 492 ηm using a microtiter plate reader (Spectra-MAX 340, Molecular Devices Ltd., Sunnyvale, CA).

Cell Counts

After each time point, specimens were washed twice with PBS and transferred into 1 ml of PBS followed by 3 min vortexed to remove the biofilm. Serial dilutions were prepared and inoculated on SDA using a spiral plate. Resultant number of cells was visually quantified after 48 h of incubation.

Scanning Electron Microscopy (SEM)

Specimens with attached biofilm were rinsed with sterile PBS and placed in 1% osmium tetraoxide for 1 h. Specimens were subsequently washed in distilled water, dehydrated in a series of ethanol washes (70% for 10 min, 95% for 10 min and 100% for 20 min) and air dried in a desiccator prior to sputter coating with gold. Next, specimens were mounted on aluminum stubs, with copper tape, coated with gold in a low-pressure atmosphere with an ion sputter coater (JEOL JFC1 100: JEOL, Tokyo, Japan). The topographic features of the biofilm were visualized with a SEM (Philip XL30CP) in high vacuum mode at 10 kV.

Confocal Scanning Laser Microscopy (CSLM)

Biofilms formed on PMMA surfaces were stained using the Molecular Probes' Live/Dead BacLight Viability kit comprising SYTO-9 and propidium iodide (PI) (Molecular Probes, Eugene, OR) and incubated during 20 min covered by aluminum foil at 37 °C before the CSLM examinations[16]. Images of stained biofilms were captured using a CSLM system (FLUOVIEW FV 1000, Olympus, Tokyo, Japan). A series of images were obtained at 1 μm intervals in the z section for a three-dimensional view of the biofilm. At least five representative optical fields were examined for each specimen.

COMSTAT software was used to analyze CSLM images[17]. The structural properties of biofilm analyzed by COMSTAT include bio-volume ($\mu m^3/\mu m^2$), average thickness (μm), diffusion distance (μm), roughness coefficient and surface-to-volume ratio ($\mu m^2 \cdot \mu m^{-3}$). COMSTAT was also used to calculate the biofilms live/dead cells ratio (%), subtracting the bio-volume of dead cells from the total biofilm bio-volume.

Statistical analysis

All data were analyzed using ANOVA, considering the PMMA, *Candida* strain and biofilm development at time points as dependent variables and oxidative activity, cell counts, bio-volume, average thickness, diffusion distance, roughness coefficient, surface-to-volume ratio and live/dead cells ratio as a independent variables. Data which did not fit the assumptions of normal distribution of errors and equality of variances were transformed [18]. Tukey HSD test was used for post-ANOVA comparisons. All analyses were performed using the SAS software (SAS Institute Inc., version 8.01, Cary, NC, USA), and a P-value of <0.05 was considered statistically significant.

Results

Surfaces characteristics of SR or SFE for the PMMA resins polymerized by water bath or by microwave did not show statistically significant differences (SR- WB: 0.22 ± 0.03; MW: 0.23 ± 0.05 μm; SFE -WB: 30.72 ± 1.86; MW: 31.27 ± 1.81 mJ.m-2).

With no substrate differences, main differences for biofilms properties were related to morphologic variations of both strains. Considering the XTT reduction assay,

significant difference between the strains were detected, and a higher activity was observed for SC5314 strain ($p<0.0001$) (table 1). At the evaluated time points, *Candida albicans* SC5314, also showed higher activity at 48 h ($p<0.0001$). As the biofilm development, an increasing on the cell counts means for both strains were detected. While *Candida albicans* SC5314 counts was higher and significantly different among adhesion phase, 24 and 48 h ($p<0.0001$), *Candida albicans* ATCC 90028 counts showed differences between 24 h and 48 h ($p<0.0001$) (table 2).

Table 1. Oxidative activity (mean ± s.d.) for *Candida albicans* strains biofilms developed on PMMA surface in the evaluated time points.

	Bio-activity (XTT O.D.)			
Strain	**Adhesion**	**24 h**	**48 h**	**72 h**
ATCC 90028	1.7±0.1 (A,a)	1.6±0.1 (A,a)	1.8±0.1(A,a)	1.8±0.1(A,a)
SC5314	2.0±0.1 (B,a)	1.7±0.1 (A,b)	2.1±0.2(B,a)	1.7±0.1 (A,b)

Distinct upper case letters show statistically significant difference between *Candida albicans* strain. Distinct lower case letters show statistically significant difference among biofilms time point within strain.

Table 2. Cell count (mean ± s.d.) for *Candida albicans* strains biofilms developed on PMMA surface in the evaluated time points.

	Cell count							
Strain	**Adhesion (a)**		**24 h (a)**		**48 h (b)**		**72 h (b)**	
ATCC 90028	3.94×10^5 (2.79×10^5)	(A,a)	7.43×10^5 (3.73×10^5)	(A,a)	1.97×10^6 (5.09×10^5)	(A,b)	1.42×10^6 (3.33×10^5)	(A,b)
SC5314	5.13×10^5 (4.50×10^5)	(A,a)	1.22×10^6 (5.18×10^5)	(A,b)	3.40×10^6 (1.12×10^6)	(B,c)	3.40×10^6 (9.24×10^5)	(B,c)

Distinct upper case letters show statistically significant difference between *Candida albicans* strain. Distinct lower case letters show statistically significant difference among biofilms time point within strain.

Biofilm development showed significant differences ($p<0.0001$) on the structure properties, and different results were detected for both strains (fig. 1). The strain ATCC 90028 showed higher bio-volume and also a thicker biofilm. However, the SC5314 produced irregular biofilms surfaces with higher roughness coefficient than ATCC 90028. With the development of the biofilm, an increase in the diffusion distances was observed for ATCC 90028. The biofilm live/dead cells ratio changed with biofilm development ($p<0.0001$).

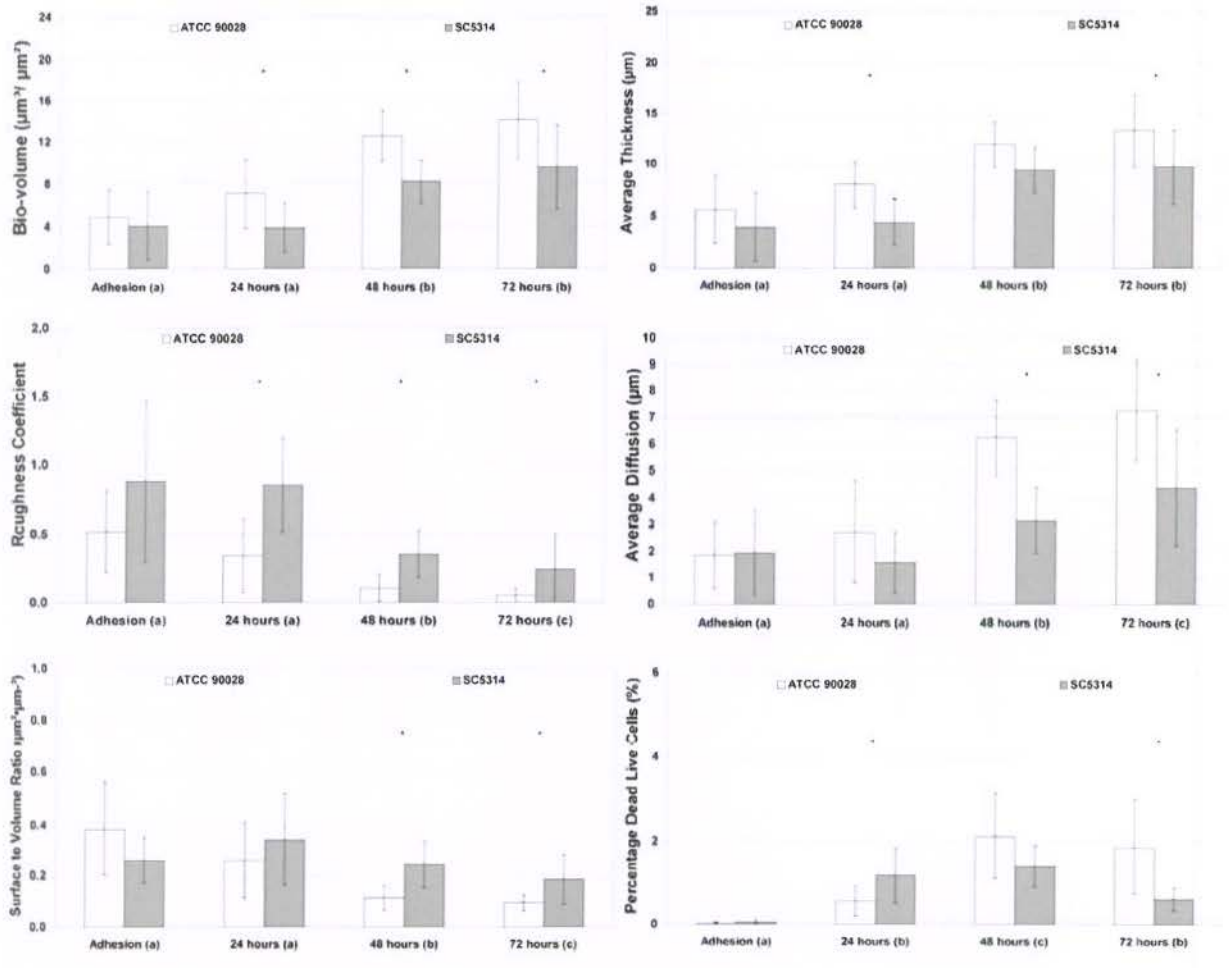

Fig. 1. Bio-volume, average thickness, roughness coefficient, average-diffusion, surface-to-volume ratio and dead/live cells (mean ± s.d.) for *Candida albicans* strains biofilms developed on PMMA surface in the evaluated time points.
Distinct lower case letters show statistically significant difference among biofilms time point. Significant difference between *Candida albicans* strains are illustrated by symbol.

Morphologic aspects for both strains were seen in CLSM and MEV microscopic techniques (fig 2 and 3). Both SEM and CLSM images exhibited a colonization pattern following the scratches and irregularities on PMMA surface. CSLM images (fig. 2) exhibited that the initial adherence of the reference strain ATCC 90028 is followed by the formation of small colonies. After 48 h voids were observed, probably

from extracellular polysaccharide matrix, in which the nutrients diffuse throughout the biofilm and after 72 h the full architecture of a biofilm was formed. SEM images (fig. 3) showed evident differences on the morphology of both strains. ATCC 90028 images were suggestive of a higher production of extracellular polysaccharide, resulting in a thicker biofilm surface with spaces for nutrient diffusion.

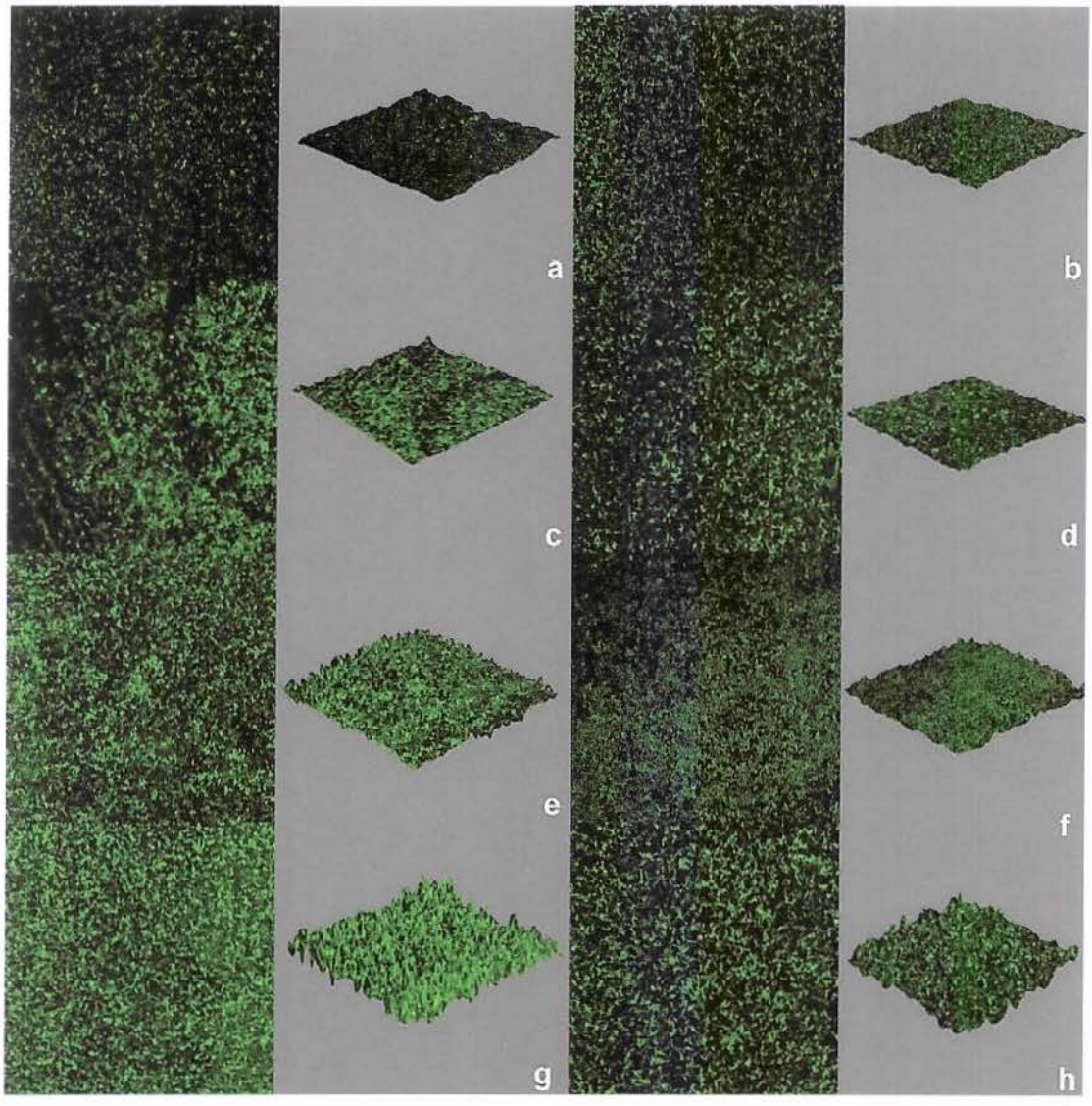

Fig. 2. CSLM images: Adhesion phase (a - b); 24 h (c - d); 48 h (e - f) and 72 h biofilm growth (g - h) with respective topography for *Candida albicans* ATCC 90028 (a,c,e,f) and *Candida albicans* SC5314 (b,d,f,h).

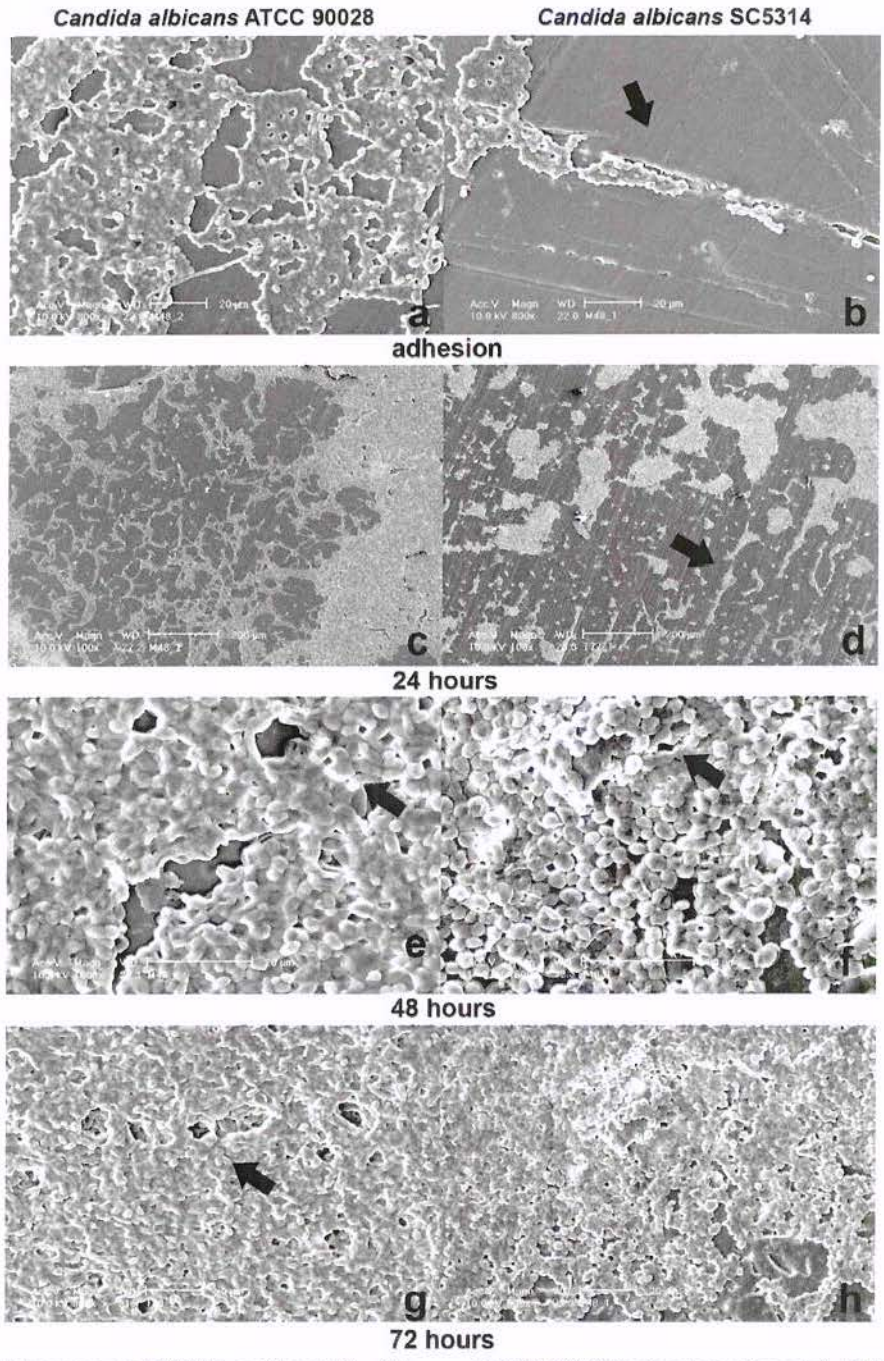

Fig. 3. SEM images on PMMA surface: *C. albicans* ATCC 90028 adhesion phase (a), 24 h (c), 48 h (e), 72 h (g). *C. albicans* SC5314 adhesion phase (b), 24 h (d), 48 h (f) and 72 h (h).
Arrows show the adhesion over to surface defects (b and d) and stratified multilayered structure with diffusion voids (e-g).

Discussion

To ensure clinical relevance, the adherence assay in this study was conducted on materials routinely used in prosthodontics procedures [13,19], which present similar chemical compositions, but vary on polymerization methods [20]. WB and MW resins were evaluated for SR and SFE before the adherence assay, and no significant differences were found between both PMMA resins. Probably, the similar SR values of PMMA resins may explain why the polymerization method did not interfere with strain adherence. It is known that surface roughness is a crucial factor in the entrapment of microorganisms and their protection from shear forces [13,19,21,22] (fig. 2 and 3). The SFE of PMMA resins was calculated by using deionized water and the results were similar to others [14,23-25]. However, due to using only one liquid method, it was not possible to calculate the other SFE elements, such as acidic and basic components [26], which can be considered a limitation of this study and could help to elucidate some interactions between cell wall and substratum.

Candida albicans biofilms can be considered in three distinct developmental layers. The basal layer of the *Candida* biofilm acts as founder cells, anchoring the developing biofilm to the substrate. The middle layer is composed of hyphae and pseudohyphae, and the most superficial layer of the biofilm consists predominantly of a thicker and porous hyphal layer with abundant extracellular matrix [5,27,28]. Approximately after 48 h, the biofilm developed into cells metabolically active communities, which get interspersed with extracellular polysaccharide matrix in which the nutrients diffuse throughout the biofilm (fig. 2 and 3). The structural complexity of the biofilm may create a gradient of environmental conditions in which the *Candida albicans* cells show distinct physiological states that is different from the planktonic counterparts [29]. With this complex scenario, results of the present study suggests that a global insight of *Candida albicans* biofilms formation could not be obtained by a single quantification method and this is evidenced by the results on tables 1 and 2.

Compared to conventional quantification methods, COMSTAT analysis carries the advantage to analyze the biofilm without disturbing its structure [17]. In previous studies, COMSTAT mathematical modeling has been successfully used for analyses of volumetric parameters of microbial biofilms architecture, such as bio-volume, average

thickness, diffusion distances, roughness coefficient and volume-to-surface ratio[17]. With the use of SYTO-9 and propidium iodide (PI) [16], the dead/live cells proportions of a biofilm can be also evaluated using COMSTAT tools.

Bio-volume represents the overall volume of the biofilm, and also provides an estimate to its biomass [17] and can be defined as the biomass volume divided by substratum area. In such way, ATCC 90028 strain showed higher biomass when compared to its wild type counterpart (fig. 1). SEM images (fig. 3) showed the morphologic aspect of biofilm, with the presence of extracellular polysaccharide matrix surrounding all biofilm. Morphologic differences in the cells size of both strains also corroborate to this finding.

Means of biofilm thickness provide a measurment of the spatial size of the biofilm [17]. The average thickness can provide information about the extension far upward of the biofilm. In overall, the strain ATCC 90028 showed thicker biofilms than SC5314 (fig. 1). Cell morphologic differences seem in ATCC 90028 SEM images (fig. 3) and bio-volume results (fig. 1) can explain the difference in this property.

Biofilm roughness coefficient provides a measure of how much the thickness of the biofilm varies and is an indicator of its heterogeneity [17]. The diffusion distance is the average of distances among all biomass pixels, suggesting as measures of the distances over which nutrients and other substrate components have to diffuse from the voids to the microorganism within micro-colonies [17]. Both properties are intrinsically related to biofilm morphology aspects, such as cell size and the presence of extracellular polysaccharide matrix. The absence of a specific extracellular polysaccharide matrix stain provides not so accurate results for these two properties. In SEM images is possible to see the interspaced channels among biofilm cells, but not in such frequency as suggests by CSLM images. The lack of specific stain for other biofilms structures rather than cells is a reasonable explanation. The decrease of roughness coefficient and the increase of diffusion distances can be associated to the development of biofilm structure, as seen in both CSLM and SEM images.

The surface to volume ratio reflects what fraction of the biofilm is in fact exposed to the nutrient flow, and thus may indicate how the biofilm adapts to the environment [17]. Organized biofilms with extracellular polysaccharide surrounding (48 and

72 hours) express lower surface volume ratio than their counterparts of adhesion and 24 hours.

Biofilm development results of this study are in accordance with previous studies [5,11]: initial adhesion, followed by multiplication of cells, resulting in the increasing of the biofilm density with the presence of nutrients diffusion voids. Then, with increased number of dead cells (fig. 1), biofilm architecture starts to disintegrate by 72 hours. In addition, the characteristic of the substrate surface may influence the structure of biofilm, however an appropriate study design should be used, modifying both SR with the use of different polishing techniques and SFE by formations of pellicle from saliva or humam serum.

Conclusion

Within the limitations of this study, it is possible to conclude that a biofilm evaluation using only one technique can provide lack of important information regards the full complexity of these microbial communities activity. Results showed that PMMA resins surfaces characteristics are not able to induce difference in biofilm structures; however an appropriate experimental design should be used to further explore this possibility.

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References

1. Arendorf TM, Walker DM. Denture stomatitis: a review. J Oral Rehabil 1987;14(3):217-27.
2. Quirynen M, Bollen CM. The influence of surface roughness and surface-free energy on supra- and subgingival plaque formation in man. A review of the literature. J Clin Periodontol 1995;22(1):1-14.
3. Radford DR, Challacombe SJ, Walter JD. Denture plaque and adherence of Candida albicans to denture-base materials in vivo and in vitro. Crit Rev Oral Biol Med 1999;10(1):99-116.
4. Jin Y, Samaranayake LP, Samaranayake Y, Yip HK. Biofilm formation of Candida albicans is variably affected by saliva and dietary sugars. Arch Oral Biol 2004;49(10):789-98.
5. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. Biofilm formation by the fungal pathogen Candida albicans: development, architecture, and drug resistance. J Bacteriol 2001;183(18):5385-94.
6. Sutherland IW. Exopolysaccharides in biofilms, flocs and related structures. Water Sci Technol 2001;43(6):77-86.
7. Chaffin WL. Candida albicans cell wall proteins. Microbiol Mol Biol Rev 2008;72(3):495-544.
8. Ramage G, Martinez JP, Lopez-Ribot JL. Candida biofilms on implanted biomaterials: a clinically significant problem. Fems Yeast Research 2006;6(7):979-986.
9. Dar-Odeh NS, Shehabi AA. Oral candidosis in patients with removable dentures. Mycoses 2003;46(5-6):187-91.
10. Ramage G, Tomsett K, Wickes BL, Lopez-Ribot JL, Redding SW. Denture stomatitis: a role for Candida biofilms. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004;98(1):53-9.
11. Moura JS, da Silva WJ, Pereira T, Del Bel Cury AA, Rodrigues Garcia RC. Influence of acrylic resin polymerization methods and saliva on the adherence of four Candida species. J Prosthet Dent 2006;96(3):205-11.

12. Kuhn DM, Chandra J, Mukherjee PK, Ghannoum MA. Comparison of biofilms formed by Candida albicans and Candida parapsilosis on bioprosthetic surfaces. Infect Immun 2002;70(2):878-88.

13. Radford DR, Sweet SP, Challacombe SJ, Walter JD. Adherence of Candida albicans to denture-base materials with different surface finishes. J Dent 1998;26(7):577-83.

14. Silva WJ, Rached RN, Rosalen PL, Del Bel Cury AA. Effects of Nystatin, Fluconazole and Propolis on Poly(Methyl Methacrylate) Resin Surface. Braz Dent J 2008;19(3):190-6.

15. Silva WJ, Seneviratne J, Parahitiyawa N, Rosa EAR, Samaranayake LP, Cury AADB. Improvement of XTT assay performance for studies involving Candida albicans biofilms. Braz Dent J in press.

16. Jin Y, Zhang T, Samaranayake YH, Fang HH, Yip HK, Samaranayake LP. The use of new probes and stains for improved assessment of cell viability and extracellular polymeric substances in Candida albicans biofilms. Mycopathologia 2005;159(3):353-60.

17. Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersboll BK, Molin S. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology 2000;146 (Pt 10):2395-407.

18. Box GEP, Hunter WG, Hunter JS. Statistics for experimenters. New York: John Wiley &Sons Inc; 1978.

19. Vasilas A, Molina L, Hoffman M, Haidaris CG. The influence of morphological variation on Candida albicans adhesion to denture acrylic in vitro. Arch Oral Biol 1992;37(8):613-22.

20. Lai CP, Tsai MH, Chen M, Chang HS, Tay HH. Morphology and properties of denture acrylic resins cured by microwave energy and conventional water bath. Dent Mater 2004;20(2):133-41.

21. Bollen CM, Lambrechts P, Quirynen M. Comparison of surface roughness of oral hard materials to the threshold surface roughness for bacterial plaque retention: a review of the literature. Dent Mater 1997;13(4):258-69.

22. Quirynen M, Marechal M, Busscher HJ, Weerkamp AH, Darius PL, van Steenberghe D. The influence of surface free energy and surface roughness on early plaque formation. An in vivo study in man. J Clin Periodontol 1990;17(3):138-44.
23. Schakenraad JM, Busscher HJ, Wildevuur CR, Arends J. The influence of substratum surface free energy on growth and spreading of human fibroblasts in the presence and absence of serum proteins. J Biomed Mater Res 1986;20(6):773-84.
24. Busscher H, Pelt Av, Jong Hd, Arends J. Effect of spreading pressure on surface free enrgy determinations by means of contact angle measurements. J Colloid Interface Sci 1983;95(1):23-27.
25. van der Valk P, van Pelt AW, Busscher HJ, de Jong HP, Wildevuur CR, Arends J. Interaction of fibroblasts and polymer surfaces: relationship between surface free energy and fibroblast spreading. J Biomed Mater Res 1983;17(5):807-17.
26. Gindl M, Sinn G, Gindl W, Reiterer A, Tschegg S. A comparison of different methods to calculate the surface free energy of wood using contact angle measurements. Colloids and Surfaces A: Physicochemical and Engineering Aspects 2001; 181 (1-3):279–287.
27. Baillie GS, Douglas LJ. Role of dimorphism in the development of Candida albicans biofilms. J Med Microbiol 1999;48(7):671-9.
28. Ramage G, Vandewalle K, Wickes BL, Lopez-Ribot JL. Characteristics of biofilm formation by Candida albicans. Rev Iberoam Micol 2001;18(4):163-70.
29. Uppuluri P, Sarmah B, Chaffin WL. Candida albicans SNO1 and SNZ1 expressed in stationary-phase planktonic yeast cells and base of biofilm. Microbiology 2006;152(Pt 7):2031-8.

3. DISCUSSÃO GERAL

Na candidose, ou estomatite induzida por prótese, a *Candida albicans* atua como o patógeno mais importante e virulento (5, 13, 21) e sua associação como principal patógeno está diretamente relacionada a sua eficiência de aderir e colonizar superfícies de próteses dentais removíveis (22, 23). Desta maneira, é importante conhecer o processo de colonização e desenvolvimento de biofilmes de *Candida albicans*.

O processo de adesão inicial sobre a superfície da resina a base de poli (metil metacrilato) inicia-se com a interação entre a parede celular da *Candida albicans* e a superfície do substrato (5). Após essa interação com a superfície, este microrganismo utiliza filamentos extracelulares para se fixar. Nesse momento se dá o início da fase de colonização, onde ocorre a formação de micro-colônias e, em condições favoráveis, a posterior formação de biofilme (5). O desenvolvimento do biofilme inicia-se a partir das micro-colônias aderidas, onde as células basais multiplicam-se formando uma firme estrutura que a mantém aderida ao substrato (18). A formação destas colônias com várias camadas celulares ocorre concomitantemente à liberação de polissacarídeos extracelular, formando uma matriz que é moldada em função do fluxo de nutrientes do meio em que se encontra o biofilme (14-16). Nesta complexa estrutura, após atingir o ápice do desenvolvimento em aproximadamente 72 h e uma vez detectada a escassez de nutrientes, moléculas químicas ligadas ao "*quorum sensing*" são liberadas pelas células das camadas intermediárias e basais, fazendo com que as células das camadas superficiais do biofilme de *Candida albicans* desprendam-se e, uma vez lançadas ao meio, tem a função de gerar novas colônias e o desenvolvimento de novos biofilmes (18).

A avaliação de biofilmes de *Candida albicans* tem sido realizada com os métodos de análise espectrofotométrica, corantes de estruturas celulares e técnicas colorimétricas. Contudo, cada técnica apresenta desvantagem inerente a sua metodologia. A restrição a técnica de análise espectrofotométrica decorre da participação de todas as células na leitura, independente se viáveis ou não. O mesmo ocorre com corantes de estruturas celulares. No que tange a técnica colorimétrica, o uso do teste de redução do XTT (19, 24-27) é o mais comum. Este teste consiste na redução do XTT pelas mitocôndrias, devolvendo ao meio um elemento de cor marrom conhecido como

"formazan". A leitura deste composto em um espectrofotômetro é utilizada para se determinar a atividade metabólica do biofilme avaliado, e não sua biomassa (25, 26, 28). Contudo, em função de ser diretamente dependente da atividade metabólica, este método apresenta como limitação o fato das camadas celulares que compõem um biofilme estarem em diferentes estados metabólicos, uma vez as camadas superiores do biofilme são ativas em função da grande riqueza de nutrientes, o mesmo não ocorre com as camadas basais, que se encontram em um estado de latência (17-19), considerando que a presença da matriz de polissacarídeos extracelular dificulta a difusão de nutrientes ao longo do biofilme (17,19).

Diante do exposto, a avaliação de biofilme de *Candida albicans* deve ir além da avaliação da atividade metabólica, pois a arquitetura do biofilme é importante na determinação de sua virulência. Dessa forma, a utilização da microscopia de varredura confocal a laser possibilita a observação do biofilme de *Candida* em toda sua extensão mantendo-o intacto sem alterar suas características estruturais e o Microscópio eletrônico de varredura possibilita a análise da superfície do biofilme.

No presente trabalho buscou-se no primeiro estudo propor uma modificação na metodologia do uso do XTT com a adição de glicose em diferentes concentrações à formulação básica e a escolha desse carboidrato decorreu de sua fácil assimilação pela *Candida albicans* (29). Os resultados deste estudo mostraram uma menor variação nos resultados quando contrapostos à formulação sem o emprego da glicose.

No segundo estudo foi avaliado o processo de colonização da resina acrílica por *Candida albicans*. Esse processo é regulado pelas características físicas da resina a base de poli (metil metacrilato) tais como energia livre e rugosidade de superfície. Diante do conhecimento do processo de adesão e colonização do biofilme de *Candida albicans* e a maneira como a superfície da resina de poli (metil metacrilato) influência neste processo, foi delineado o segundo estudo que consistiu na avaliação da morfologia e atividade metabólica do biofilme de *Candida albicans* desenvolvidos sobre a superfície de resina acrílica a base de poli (metil metacrilato).A escolha da resina a base poli (metil metacrilato) com duas técnicas de ativação (banho de água e energia de microondas) ocorreu pois em trabalho anterior (12) apenas a fase de adesão de *Candida albicans* sobre estes substratos

foi realizada e a evidência da influência do substrato sobre o desenvolvimento do biofilme não foi elucidada. Assim, no estudo atual, as características de superfície das duas resinas possibilitaram eliminar a influência de outros fatores, que não a do substrato.

Pode ser observado que os biofilmes de ambas as cepas de *Candida* tiveram o ápice da atividade metabólica em 48 horas e que houve diferenças entre as cepas estudadas. Essa diferença de comportamento entre as cepas pode ser explicada pela arquitetura do biofilme formado uma vez que as imagens de MEV sugerem que a cepa ATCC 90028 produziu uma maior quantidade de polissacarídeos extracelular dificultando a difusão de nutrientes e atingindo a fase de latência anteriormente quando comparada a cepa SC5314.

4. CONCLUSÃO

De acordo com os resultados obtidos e considerando as limitações deste trabalho, é possível concluir que a estrutura do biofilme de *Candida albicans* não foi afetada em função das propriedades de superfície da resina a base de poli (metil metacrilato) e sim em função de fatores inerentes ao isolado de *Candida* tais como a morfologia e a matriz de polissacarídeos extracelular formada.

REFERÊNCIAS

1. Cheng MF, Yang YL, Yao TJ, Lin CY, Liu JS, Tang RB, et al. Risk factors for fatal candidemia caused by Candida albicans and non-albicans Candida species. BMC Infect Dis. 2005;5(1):22.

2. Nucci M, Marr KA. Emerging fungal diseases. Clin Infect Dis. 2005 Aug 15;41(4):521-6.

3. Sachdeo A, Haffajee AD, Socransky SS. Biofilms in the Edentulous Oral Cavity. J Prosthodont. 2008 Mar 17.

4. Fueki K, Kimoto K, Ogawa T, Garrett NR. Effect of implant-supported or retained dentures on masticatory performance: a systematic review. J Prosthet Dent. 2007 Dec;98(6):470-7.

5. Radford DR, Challacombe SJ, Walter JD. Denture plaque and adherence of Candida albicans to denture-base materials in vivo and in vitro. Crit Rev Oral Biol Med. 1999;10(1):99-116.

6. Arendorf TM, Walker DM. Denture stomatitis: a review. J Oral Rehabil. 1987 May;14(3):217-27.

7. Minagi S, Miyake Y, Inagaki K, Tsuru H, Suginaka H. Hydrophobic interaction in Candida albicans and Candida tropicalis adherence to various denture base resin materials. Infect Immun. 1985 Jan;47(1):11-4.

8. Sipahi C, Anil N, Bayramli E. The effect of acquired salivary pellicle on the surface free energy and wettability of different denture base materials. J Dent. 2001 Mar;29(3):197-204.

9. Canuto MM, Gutierrez Rodero F. Antifungal drug resistance to azoles and polyenes. Lancet Infect Dis. 2002 Sep;2(9):550-63.

10. Bollen CM, Lambrechts P, Quirynen M. Comparison of surface roughness of oral hard materials to the threshold surface roughness for bacterial plaque retention: a review of the literature. Dent Mater. 1997 Jul;13(4):258-69.

11. Quirynen M, Marechal M, Busscher HJ, Weerkamp AH, Darius PL, van Steenberghe D. The influence of surface free energy and surface roughness on early plaque formation. An in vivo study in man. J Clin Periodontol. 1990 Mar;17(3):138-44.

12. Moura JS, da Silva WJ, Pereira T, Del Bel Cury AA, Rodrigues Garcia RC. Influence of acrylic resin polymerization methods and saliva on the adherence of four Candida species. J Prosthet Dent. 2006 Sep;96(3):205-11.

13. Egusa H, Ellepola AN, Nikawa H, Hamada T, Samaranayake LP. Exposure to subtherapeutic concentrations of polyene antifungals suppresses the adherence of Candida species to denture acrylic. Chemotherapy. 2000 Jul-Aug;46(4):267-74.

14. Sutherland IW. The biofilm matrix--an immobilized but dynamic microbial environment. Trends Microbiol. 2001 May;9(5):222-7.

15. Sutherland IW. Exopolysaccharides in biofilms, flocs and related structures. Water Sci Technol. 2001;43(6):77-86.

16. Chaffin WL. Candida albicans cell wall proteins. Microbiol Mol Biol Rev. 2008 Sep;72(3):495-544.

17. Baillie GS, Douglas LJ. Role of dimorphism in the development of Candida albicans biofilms. J Med Microbiol. 1999 Jul;48(7):671-9.

18. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. Biofilm formation by the fungal pathogen Candida albicans: development, architecture, and drug resistance. J Bacteriol. 2001 Sep;183(18):5385-94.

19. Ramage G, Vandewalle K, Wickes BL, Lopez-Ribot JL. Characteristics of biofilm formation by Candida albicans. Rev Iberoam Micol. 2001 Dec;18(4):163-70.

20. Reese S, Guggenheim B. A novel TEM contrasting technique for extracellular polysaccharides in in vitro biofilms. Microsc Res Tech. 2007 Sep;70(9):816-22.

21. Nikawa H, Jin C, Hamada T, Makihira S, Kumagai H, Murata H. Interactions between thermal cycled resilient denture lining materials, salivary and serum pellicles and Candida albicans in vitro. Part II. Effects on fungal colonization. J Oral Rehabil. 2000 Feb;27(2):124-30.

22. Chaffin WL, Lopez-Ribot JL, Casanova M, Gozalbo D, Martinez JP. Cell wall and secreted proteins of Candida albicans: identification, function, and expression. Microbiol Mol Biol Rev. 1998 Mar;62(1):130-80.

23. Kumamoto CA, Vinces MD. Alternative Candida albicans Lifestyles: Growth on Surfaces. Annu Rev Microbiol. 2004 Mar 16.

24. Thein ZM, Samaranayake YH, Samaranayake LP. In vitro biofilm formation of Candida albicans and non-albicans Candida species under dynamic and anaerobic conditions. Arch Oral Biol. 2007 Aug;52(8):761-7.

25. Roehm NW, Rodgers GH, Hatfield SM, Glasebrook AL. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. J Immunol Methods. 1991 Sep 13;142(2):257-65.

26. Kuhn DM, Balkis M, Chandra J, Mukherjee PK, Ghannoum MA. Uses and limitations of the XTT assay in studies of Candida growth and metabolism. J Clin Microbiol. 2003 Jan;41(1):506-8.

27. Jahn B, Martin E, Stueben A, Bhakdi S. Susceptibility testing of Candida albicans and Aspergillus species by a simple microtiter menadione-augmented 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. J Clin Microbiol. 1995 Mar;33(3):661-7.

28. Honraet K, Goetghebeur E, Nelis HJ. Comparison of three assays for the quantification of Candida biomass in suspension and CDC reactor grown biofilms. J Microbiol Methods. 2005 Dec;63(3):287-95.

29. Jin Y, Samaranayake LP, Samaranayake Y, Yip HK. Biofilm formation of Candida albicans is variably affected by saliva and dietary sugars. Arch Oral Biol. 2004 Oct;49(10):789-98.

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Prezado Professor,

O trabalho BDJ 1264 **Improvement of XTT assay performance for studies involving Candida albicans biofilms** dos autores Wander José da Silva, Jayampath Seneviratne, Nipuna Parahitiyawa, Edvaldo Antonio Ribeiro Rosa, Lakshman Perera Samaranayake, Altair Antoninha Del Bel Cury, foi aceito no mérito científico para publicação.

Atenciosamente,

Prof. Dr. Manoel D. Sousa Neto ***Prof. Dr. Jesus Djalma Pécora***

Editor ***Editor***

ANEXO 2 – Comprovante de submissão de artigo

From: pmiller@orthogencorp.com (pmiller@orthogencorp.com)
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Dear Mr. Silva:
We are pleased to receive your manuscript entitled "Characteristics of Candida albicans biofilm developed on poly (methyl methacrilate) resins surfaces" by Silva, Wander; Seneviratne, Jayampath; Samaranayake, Lakshman; Del Bel Cury, Altair. We will be sending it out for review shortly.
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