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Faculdade de Odontologia de Piracicaba

ALINE ARAUJO SAMPAIO

POTENCIAL CARIOGÊNICO DA *CANDIDA ALBICANS*

CARIOGENIC POTENTIAL OF CANDIDA ALBICANS

PIRACICABA

2016

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Tese de doutorado 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 requirement for the degree of Doctor in Clinical Dentistry, Dental Prosthetics area.

Orientador: Prof. Dr. Jaime Aparecido Cury

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA ALINE ARAUJO SAMPAIO, E ORIENTADA PELO PROF. DR. JAIME APARECIDO CURY.

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PROF. DR. ANTÔNIO PEDRO RICOMINI FILHO

A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

Ata da Defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

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RESUMO

A cárie radicular é uma preocupação atual devido ao aumento da longevidade das populações e a conservação de dentes naturais por mais tempo na cavidade bucal com exposição radicular. O *Streptococcus mutans* é considerada a bactéria bucal mais cariogênica mas tem sido sugerido que a *Candida albicans* poderia aumentar a sua cariogenicidade. Como o efeito desta combinação na cárie de dentina é desconhecido, o objetivo deste estudo foi avaliar se *C. albicans* aumentaria a capacidade do biofilme de *S. mutans* em desmineralizar dentina. Neste estudo experimental *in vitro* e cego, biofilmes de *C. albicans* ATCC 90028, *S. mutans* UA 159 e da combinação *C. albicans* + *S. mutans* foram crescidos sobre blocos de dentina radicular bovina de dureza de superfície (DS) conhecida (n=12/grupo). Os biofilmes foram crescidos em meio à base de triptona e extrato de levedura contendo 0,1 mM de glicose durante 96 h, a 37ºC e 10% de CO_2. Os biofilmes foram expostos 8x/dia durante 3 min à sacarose a 10%. O meio de cultura foi trocado 2x/dia após os 8 desafios cariogênicos e após o período noturno de jejum, e alíquotas foram analizadas para determinação do pH e concentração de ácido lático. Após 96 h, os biofilmes foram coletados para determinação do peso úmido, da contagem de unidades formadoras de colônia (UFC), da quantidade de polissacarídeos intracelulares e dos extracelulares (solúveis e insolúveis). Na dentina foi determinada a perda de dureza de superfície (% PDS), como indicador de desmineralização. A arquitetura dos biofilmes foi analisada qualitativamente por microscopia confocal de varredura a laser. O biofilme *C. albicans* + *S. mutans* foi também analisado por microscopia eletrônica de transmissão (MET). Os dados foram analisados por ANOVA seguido pelo teste de Tukey ($\alpha = 0,05$). O biofilme de *C. albicans* + *S. mutans* mostrou maior acidogenicidade que os biofilmes de *S. mutans* e *C. albicans* nos tempos de crescimento de 56 e 80 h ($p < 0,05$). Os grupos diferiram (*C. albicans* < *S. mutans* < *C. albicans* + *S. mutans*) com relação as análises da %PDS, UFC e quantidade de polissacarídeos ($p < 0,05$). As imagens por confocal sugerem que o biofilme de *C. albicans* + *S. mutans* é mais volumoso que os demais. As imagens por MET mostram que as células de *S. mutans* interagem com a *C. albicans* através de polissacarídeos da matriz do biofilme formado. Concluiu-se que embora a *C. albicans* nas condições estudadas tenha mostrado um baixo potencial cariogênico, ela foi capaz de aumentar o potencial cariogênico de biofilme de *S. mutans*, provocando maior desmineralização da dentina.

Palavras-chave: Biofilme dental, *Streptococcus mutans, Candida albicans*, cárie dentária, desmineralização, sacarose.

ABSTRACT

Root caries is a current concern due to the increased population longevity and the preservation of natural teeth in the oral cavity with root exposure for a longer time. *Streptococcus mutans* are considered the most cariogenic bacteria and it has been suggested that *Candida albicans* could increase their cariogenicity. However, the effect of this combination on dentin caries is unknown. The aim of this study was to evaluate whether *C. albicans* biofilms would increase the capacity of *S. mutans* in demineralize dentin. In this *in vitro* and blind experimental study, biofilms of *C. albicans* ATCC 90028, *S. mutans* UA 159 and the combination of *C. albicans* + *S. mutans* were grown on bovine root dentine blocks of known surface hardness (SH) (n = 12 / group). Biofilms were grown in ultra-filtered tryptone and yeast extract based medium containing 0.1 mM glucose, for 96 h under 37 °C and 10% CO_2. Biofilms were exposed 8x/day for 3 min to 10% sucrose. The medium was changed 2x/day, after the 8 cariogenic challenges and after the night period of famine, and aliquots were analyzed to determinate the pH, and concentration of lactic acid. After 96 h, biofilms were collected to determine the wet weight, colony forming units (CFU), the amount of intracellular and extracellular polysaccharides (soluble and insoluble). Dentine demineralization was assessed by surface hardness loss (% SHL). The architecture of the biofilms was qualitatively analyzed by confocal laser scanning. The structure of *C. albicans* + *S. mutans* biofilm was also analyzed by transmission electron microscopy (TEM). Data were analyzed by ANOVA followed by Tukey's test ($\alpha = 0.05$). *C. albicans* + *S. mutans* biofilm showed higher acidogenicity than the biofilms of S. mutans and C. albicans in the times of 56 and 80 h of growth ($p < 0.05$). The groups differed (*C. albicans* <*S. mutans* <*C. albicans* + *S. mutans*) regarding the analysis of SHL%, CFU and amount of polysaccharides ($p < 0.05$). Confocal images suggested that *C. albicans* + *S. mutans* biofilm is more volumous than the others biofilms. The TEM images suggested that *S. mutans* cells interact with *C. albicans* through matrix biofilm polysaccharides. According to the studied conditions, it was concluded that although *C. albicans* has shown a low cariogenic potential, it was able to increase the cariogenic potential of *S. mutans* biofilms, causing an increased demineralization of the dentin.

Key-words: Dental plaque, *Streptococcus mutans, Candida albicans*, dental caries, demineralization, sucrose.

SUMÁRIO

1 INTRODUÇÃO

A cárie dentária é considerada um problema de saúde pública em muitas partes do mundo devido à sua alta prevalência, impacto na economia e na qualidade de vida dos indivíduos afetados (Petersen & Yamamoto, 2005; Kassebaum et al., 2015). No contexto da população idosa, o aumento da expectativa de vida das populações e a manutenção de dentes naturais por um período maior na cavidade bucal e consequente possibilidade de superfícies radiculares expostas, tornam a cárie radicular um problema atual e com tendência de crescimento no futuro (Jones, 1995; Tan et al., 2010; Walls e Meurman, 2012).

A cárie dentária é uma doença reconhecidamente bacteriana, mas dependente da exposição a açúcares da dieta, que se manifesta pela desmineralização progressiva da estrutura mineral dos dentes (Fejerskov, 2004). Entretanto, espécies do fungo Candida, como *C. albicans*, comumente encontradas na cavidade bucal de indivíduos saudáveis e com implicação em doenças da mucosa bucal, tem sido também associadas a cárie dentária (Carvalho et al., 2006; Ugun Can et al., 2007; Klinke et al., 2011; Charone et al., 2013).

C. albicans tem sido relacionada com cárie precoce da primeira infância pois há associação entre maior contagem deste microrganismo na cavidade bucal das crianças com lesões de cárie (Mòalic et al., 2001; Raja et al., 2010; Yang et al., 2012). Em acréscimo, isolados de *C. albicans* de crianças HIV positivas ou com anemia falciforme mostraram maior potencial cariogênico quando comparados aos isolados de indivíduos saudáveis (Charone et al., 2013; Brighenti et al., 2014). Entretanto, esse fungo também tem sido associado com cárie radicular pois ele está presente em maior quantidade na saliva e em biofilmes dentais de idosos acometidos por cárie radicular (Beighton et al., 1991; Beighton e Lynch, 1993; Shen et al., 2002; Shen et al., 2005; Zaremba et al., 2006). Além disso, uma maior frequência de *C. albicans* na saliva da população idosa com cárie radicular mostrou estar relacionada ao maior número de raízes expostas (Beighton et al., 1991; Shen *et al.,* 2002). Em acréscimo, lesões de cárie mais próximas da gengiva marginal livre e tendo maior severidade apresentaram maior contagem deste microorganismo (Beighton & Lynch, 1993). Entretanto, apesar deste fungo ter sido associado a cárie em crianças (esmalte) e em idosos (dentina radicular), os trabalhos não permitem concluir que a *C. albicans* por si só é cariogênica ou atuaria aumentando a virulência das bactérias presentes nos biofilmes.

Assim, estudos experimentais in vitro tem sido feitos para dar suporte a essa casualidade. *C. albicans* é capaz de aderir a pérolas de hidroxiapatita (HA) (Cannon et al., 1995; Nikawa et al., 1998) o que também poderia ocorrer com a superfície dental. Essa adesão do fungo à HA é facilitada pelos glucanos sintetizados pela enzima glicosiltransferase de *Streptococcus mutans* quando da exposição à sacarose (Gregoire et al., 2011). No entanto, a literatura diverge quanto a habilidade de *C. albicans* formar biofilmes tendo como substrato de aderência discos de HA. Enquanto alguns autores mostram que este fungo forma biofilmes sobre estes discos (Nikawa et al., 2003; Pereira-Cenci et al., 2008), outros afirmam que este fungo por si só apresenta dificuldade em colonizar HA (Falsetta et al., 2014; Willems et al., 2016). Entretanto, há consenso que *S. mutans* favorece a formação de biofilme desse fungo sobre HA (Pereira-Cenci et al., 2008; Falsetta et al., 2014; Willems et al., 2016). No entanto, nessas pesquisas não foram usados substratos dentais (esmalte ou dentina) e o efeito na dissolução da HA não foi quantificado.

Nikawa et al. (2003) e Willems et al. (2016) avaliaram quimicamente o efeito de biofilmes de *C. albicans* e *S. mutans* na desmineralização da HA, determinando cálcio liberado para o meio de cultura. Nikawa et al. (2003) observaram que os biofilmes de *C. albicans* provocam maior desmineralização da HA que biofilmes de *S. mutans*. Entretanto, Willems et al. (2016) mostraram resultados contrários, o biofilme de *C. albicans* não sendo capaz de provocar significante desmineralização da HA quando comparada ao biofilme de *S. mutans*. Somente Willems et al. (2016) avaliaram o efeito da associação da *C. albicans* ao biofilme de *S. mutans* na desmineralização da HA, encontrando que a combinação desses microorganismos foi capaz de provocar menor liberação de cálcio para o meio que os biofilmes de *S. mutans*. Entretanto, nos estudos citados não foi avaliada a formação de biofilmes sobre a superfície de estruturas dentais (esmalte ou dentina) e obviamente não foi avaliada a consequente desmineralização ocorrida.

Especificamente com relação ao esmalte dental, o potencial cariogênico da *C. albicans* e sua combinação com *S. mutans* tem sido estudado usando o modelo de cárie experimental em animal. Klinke et al. (2011) investigaram a incidência de cárie em ratos infectados com *C. albicans*, *S. mutans* e sua combinação quando tratados com sacarose, glicose ou sacarose + glicose. Os autores observaram que *C.albicans* foi capaz de provocar cárie oclusal severa (atingindo dentina) nos ratos, independente dos açúcares administrados na dieta, sacarose ou glicose. Entretanto, a *C. albicans*

não foi capaz de causar cárie de superfície lisa nos dentes dos ratos. Em contrapartida, *S.mutans* causou cárie de superfície lisa e, a associação do fungo com esta bactéria não teve efeito somatório na severidade das lesões de cárie oclusal e de superfície lisa. Por outro lado, Falsetta et al. (2014) encontraram que os biofilmes de *Candida albicans* são capazes de gerar pouca lesão de cárie nos dentes dos ratos, porém os biofilmes com *C.albicans* e *S. mutans* causaram lesão de cárie severa.

Com relação a dentina radicular, não há na literatura estudos avaliando o efeito dos biofilmes de *C. albicans* na desmineralização dentinária. Makihira et al. (2002) investigaram a habilidade das células deste microorganismo e de *S. mutans* de aderirem a membranas de nitrocelulose impregnadas com colágeno do tipo I, íntegro e desnaturado, demonstrando que as células de *C. albicans* apresentaram maior capacidade de adesão a esses substratos quando comparada ao *S. mutans*. Adicionalmente, a capacidade da *C. albicans* em colonizar dentina e penetrar em túbulos dentinários foi comprovada somente por análises qualitativas por microscopia eletrônica de varredura (Sen et al., 1997; Sen et al., 1997) ou por microscopia de luz (Waltimo et al., 2000), porém essas metodologias não são válidas para estimar desmineralização dentinária.

Assim, apesar dos dados experimentais darem suporte à associação entre *C. albicans* e cárie em esmalte, o papel desempenhado por este microrganismo e sua combinação com *S. mutans* sobre cárie radicular não foi avaliada experimentalmente. Portanto, o objetivo deste estudo foi avaliar o efeito de *C. albicans* biofilme na desmineralização da dentina quando combinado com *S. mutans.*

2 ARTIGO

Artigo submetido ao periódico Biofouling (ANEXO 2)

Candida albicans increases dentin demineralization provoked by *S.mutans* biofilm

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ABSTRACT

Streptococcus mutans are considered the most cariogenic bacteria but it has been suggested that *Candida albicans* could increase their cariogenicity. However, the effect of this combination on dentin caries is unknown. Biofilms of *C. albicans*, *S. mutans* and *C. albicans* + *S. mutans* (n=12/biofilm) were grown for 96 h on root dentine slabs and they were exposed 8x/day for 3 min to 10% sucrose. The duospecie *C. albicans* + *S. mutans* biofilm provoked highest dentine demineralization than the monospecies biofilms ($p < 0.05$). This greatest demineralization found was supported by results of biofilm acidogenicity, viable microrganisms and amount of intra and extracellular polysaccharides in the *C. albicans* + *S. mutans* biofilm formed ($p < 0.05$). *C. albicans* biofilm presented low cariogenicity. The findings show that *C. albicans* enhances the cariogenic potential of *S. mutans* biofilm provoking an increased demineralization in dentin.

Key-words: Dental plaque, *Streptococcus mutans, Candida albicans*, dental caries, demineralization, sucrose

Introduction

Dental caries is considered a public-health problem in many parts of the globe due to its high prevalence, impact on economy and in the quality of life of affected individuals (Petersen et al. 2008; Kassebaum et al. 2015). The increased life expectancy of different populations and the maintenance of natural teeth for a longer period in the oral cavity with a consequent increase in the incidence of exposed root surfaces, makes root caries an important health issue worldwide (Jones 1995; Tan et al. 2010; Walls and Meurman 2012).

Although *Streptococcus mutans* are considered the most cariogenic bacteria (Bowen and Koo., 2011), there has been a growing body of evidence on the importance of *C. albicans* to dental caries development (Carvalho et al. 2006; Ugun Can et al. 2007; Raja et al. 2010; Klinke et al. 2011; Falsetta et al. 2014). Association studies showed a positive relationship between *C. albicans* and tooth decay, also demonstrating a correlation of higher counts of this fungus in saliva and dental biofilms of individuals with a greater incidence of root caries (Beighton et al. 1991; Beighton e Lynch 1993; Shen et al. 2002; Shen et al. 2005; Zaremba et al. 2006). However, the exact role played by *C. albicans* in the progression of root dentin caries is still obscure (Carvalho et al. 2006; Maijala et al. 2007; Willems et al. 2016). Moreover, from these studies it is not possible to state whether *C. albicans* alone is cariogenic or it would increase the virulence of cariogenic bacteria found in biofilms.

Available in vitro evidences show that *C. albicans* is able to adhere to hydroxyapatite (HA) beads (Cannon et al. 1995; Nikawa et al. 1998; Gregoire et al. 2011) and to the tooth surface (Sen et al. 1997; Metwalli et al. 2013). However, the literature diverges on the ability of *C. albicans* to form biofilms (Nikawa et al. 2003; Pereira-Cenci et al, 2008; Falsetta et al, 2014; Willems et al, 2016) when using HA disc as adhesion substrate (Falsetta et al, 2014; Willems et al., 2016), even though some authors have demonstrated that *S. mutans* favor the *C. albicans* biofilm formation on HA (Pereira-Cenci et al. 2008; Falsetta et al. 2014; Willems et al. 2016).

Nikawa et al. 2003 and Willems et al. 2016 evaluated the effect of *C. albicans* and *S. mutans* biofilms on the demineralization of HA, determining the concentration of calcium released in the culture medium. Nikawa et al. 2003 reported that *C. albicans* biofilm caused higher demineralization of HA than *S. mutans* biofilm, whereas Willems

et al. 2016 did not found that *C. albicans* biofilm was able to cause any significant demineralization when compared to *S. mutans* biofilm. Willems et al. 2016 also evaluated the combination effect of the *C. albicans* + S*. mutans* biofilm on HA demineralization, that the duospecie biofilm provoked lower HA dissolution than *S. mutans* biofilm. Besides discrepants, these studies did not evaluate the formation of biofilms on dental structures (enamel or dentin) and their demineralization.

Similarly, studies made with rats about *C. albicans* biofilm ability to cause caries lesion and its potential synergistic with *S. mutans* in the development of cariogenic biofilms are also conflictings (Klinke et al. 2011; Falsetta et al. 2014). Klinke et al. 2011 investigated the incidence of caries in rats infected with *C. albicans, S. mutans* and their combination when treated with sucrose, glucose, or sucrose + glucose. The authors found that *C. albicans* was able to cause severe occlusal caries (affecting the dentin) independent of the carbohydrate used, glucose or sucrose. However, *C. albicans* was not able to cause smooth surface lesions. In contrast, *S. mutans* was strongly associated with smooth surface caries, but the association of both microorganisms did not increase the severity of occlusal and smooth surface caries. On the other hand, Falsetta et al. 2014 found that *C. albicans* biofilms were slightly cariogenic to rats but severe caries lesion were found the animals were infected with the fungus and *S. mutans*.

Regarding root caries, there seems to be no study in the literature evaluating the effect of *C. albicans* biofilm in dentin demineralization. Makihira et al. 2002 investigated the ability of this microorganism and of *S. mutans* to adhere to intact and denatured type I collagen-impregnated nitrocellulose membranes, demonstrating that *C. albicans* cells showed a significantly higher adhesion to this substrate when compared to *S. mutans*. Additionally, the ability of *C. albicans* to colonize dentin and to penetrate dentinal tubules was evidenced by scanning electron microscopy (Sen et al. 1997; Sen et al. 1997) and by light microscopy (Waltimo et al. 2000), but these methodologies are not suitable to estimate the potential of this fungus to provoke dentin demineralization.

Hence, despite of the experimental data giving support to the association between *C. albicans* and enamel caries, the role played by this microorganism and its combination with *S. mutans* on root caries has not been experimentally evaluated.

Therefore, the aim of this study was to evaluate the effect of *C. albicans* biofilm on dentin demineralization when combined with *S. mutans*.

Material and Methods

Experimental design and ethical issues

This randomized (regarding dentine slabs allocation) and blinded (regarding %SHL analysis) in vitro study was repeated three times in quadruplicate (final n =12/type of biofilm). The factor under study was the type of biofilm formed on dentine slabs: Biofilm of *C. albicans* (ATCC 90028) or *S. mutans* (UA159) or the combination of *C. albicans* + *S. mutans*. The biofilms were formed on bovine root dentin slabs, whose surface hardness (SH) was predetermined. The slabs were coated by saliva and the biofilms were grown in ultrafiltered tryptone yeast extract broth (UTYEB) culture medium, being exposed 8x/day to 10% sucrose solution. The medium was changed twice daily. The pH of the medium and the concentration of lactic acid were determined as indicators of biofilm acidogenicity. After 96 h of growth, the biofilms were collected to evaluate biofilm wet weight (biomass), counting viable microrganisms and amount of polysaccharides. Dentine SH was again determined and the percentage of loss was calculated (%SHL) as indicator of demineralization. An independent study was conducted (n=2) to visualize the architecture of the biofilms, that were analyzed by confocal laser scanning and transmission electron microscopy (TEM). The hypothesis under study was that *C. albicans* would increase the capacity of *S. mutans* biofilm demineralize dentin. The data were statistically analyzed by ANOVA followed by Tukey's considering dentin slab/biofilm as a statistical block (n=12). To create the biofilm models used in this research, pilot tests were done to determine the most appropriate inoculum concentration, type of culture medium, pH of the culture medium, type of carbohydrate on adhesion period, adhesion time and position of the substrate.

This study was approved by the local Research and Ethics Committee of FOP-UNICAMP (Protocol no. 075/2015).

Bovine root dentin slab preparation

Root-dentin slabs (7 x 4 x 1 mm) were obtained from bovine incisors teeth (Hara et al. 2003). Baseline surface hardness (SH) of the slabs flattened and polished was measured using a Knoop microhardness tester coupled to FM-ARS 900 software

(Future-Tech FM, Kawasaki, Kanagawa, Japan). Three indentations spaced 100 μm from each other were made using a 5 g load for 5 s. Slabs with 36.6 ± 3.5 kg/mm^2 (mean ± SD) were selected and randomness allocated to the groups under study. The slabs were vertically positioned on metallic holders (Ccahuana-Vásquez & Cury 2010) and placed individually in each well of a 24-well plate (Techno Plastic Products, Zellkultur-Plastik, Trasadingen, Switzerland), and sterilized with ethylene oxide.

Saliva collection and acquired pellicle formation

Stimulated human whole saliva was collected on ice from two healthy volunteers (27 years old), who chewed Paraffin film (American Can Co., Greenwich, CT, USA). The saliva was mixed with adsorption buffer [1:1 (v/v)] and 0.1 M phenylmethylsulfonylfluoride [PMSF; 1:100 (v/v)], and centrifuged at 3,800 *g* for 10 min at 4 °C, ultrafiltered and used immediately (Koo et al. 2003). Salivary acquired pellicle was formed on dentine slabs as described elsewhere (Koo et al. 2003; Ccahuana-Vásquez and Cury 2010). For pellicle formation, slabs were maintained in a 24-well plate and incubated with filtered saliva in an orbital shaker at 60 rpm and 37°C for 30 min.

Biofilm development (adhesion and growth phases)

Microorganisms adhesion to dentine slabs

Frozen *C. albicans* ATCC 90028 and *S. mutans* UA159 strains were reactivated on Blood agar plates and incubated for 24 h, at 37°C, in 10% CO_2. To inoculum preparation, colonies were transferred to UTYE medium supplemented with 1% glucose and incubated for 20 h at 37°C, in 10% CO_2 (Gregoire et al. 2011) to achieve a log phase, as previously standardized from growth curves for both microorganisms. The cells were centrifuged and resuspended in fresh UTYEB to obtain 10^6 cells/mL for *C. albicans* (OD 0.25 at 520 nm) (Jin et al. 2004) and 10^8 cells/mL for *S. mutans* (OD 0.70 at 600 nm) (Cavalcanti et al. 2014). These suspensions were used to prepare the inoculum for the adhesion of the microorganisms to dentine slabs. They were diluted 1:20 (v/v) in UTYEB supplemented with 1% sucrose. For the adhesion phase of biofilm development, the UTYEB medium was strongly buffered (10x higher than the usual phosphate concentration) to avoid pH fall and dentin demineralization during this phase. In the pilot test, it was proven that microbial adhesion on substrate was not altered by using a strongly buffered UTYEB medium (data not shown).

The volume of 2.0 ml of inoculum of *C. albicans, S. mutans* or the combination of these microorganisms were distributed in 24-wells plate. Dentine slabs saliva coated were individually positioned in a new 24-well plate containing the inoculum as described by Ccahuana-Vásquez and Cury (2010). The plates were incubated for 8 h, at 37°C, in 10% CO_2 (Koo et al, 2003). After adhesion, the slabs were then transferred to plates containing fresh UTYEB supplemented with 0.1 mM glucose (salivary basal concentration) and incubated for an overnight period at 37°C, in 10% CO_2 (Ccahuana-Vásquez and Cury 2010).

Biofilm growth

Following the overnight incubation period, the biofilms were submitted to feast and famine episodes (Ccahuana- Vásquez and Cury 2010), consisting of 8 exposures daily to 10% sucrose solution for 3 minutes, to simulate the intermittent cariogenic challenges with dietary sugars that occur daily in the oral cavity. The cariogenic challenges were made for 3 days at predetermined times (8:00 am, 9:30 am, 11:00 am, 12:00 am, 1:30 pm, 3:00 pm, 4:00 pm, and 5:30 pm). After each cariogenic challenge, biofilms were washed 3 times in 0.9% NaCl and replaced to the same medium. The culture medium was changed twice, once after the first challenge (8:00 am) and once after the last (5:30 pm) challenge with sucrose on each day (Ccahuana-Vásquez and Cury 2010). After the last cariogenic challenge, the biofilms attached to dentin slabs were washed three times in 0.9% NaCl and transferred to fresh UTYEB supplemented with 0.1 mM glucose, simulating the night starvation that occurs in the real life. In the next day, the medium was again replaced to fresh one before starting the treatments with sucrose. The pH of the media (after the daily cariogenic challenge and after the starvation) were determined and the media were stored in microcentrifuge tubes at -80 °C for further determination of lactic acid concentration (Figure S1).

Biofilm acidogenicity (medium pH and lactic acid concentration)

Medium pH (n=12/biofilm/time/condition) and lactic acid concentrations (n=6) were determined as indicators of biofilm acidogenicity. The pH was determined with a microelectrode (Accumet; Cole-Parmer, Vernon Hills, Ill., USA) coupled to a pH meter (Procyon SA-720, Olímpia, Brazil). Lactic acid concentration was evaluated by High Performance Liquid Chromatography (Alliance 2795, Waters, USA) using a refractive

index detector and an Aminex HP-87H column (Bio-Rad Laboratories, USA) (Carvalho-Netto et al. 2015).

Biofilm collection

After 96 h of growth, dentine slabs containing biofilms on theirs surface were removed from the culture medium, washed three times with NaCl 0.9% solution and individually transferred to microcentrifuge tubes containing 1 ml of NaCl 0.9% solution. The tubes were sonicated at 7 W for 30 s (Branson, Sonifer 50, Danbury, CT, USA) to detach the biofilms on the slabs (Ccahuana-Vásquez and Cury 2010). Aliquots of sonicated biofilm suspension were used to determine biofilm wet weight, colony forming units, and polysaccharides production. The dentine slabs were saved for the demineralization analysis.

Biofilm analyses

For biomass determination (biofilm wet weight), 400 µl of the cell suspensions was transferred to preweighed microcentrifuge tubes and centrifuged at 10,000 *g*, for 5 min, at 4°C. The supernatant was carefully removed and the biofilm wet weight determined after volume normalization and subtraction of the microcentrifuge tube weight.

To determine microorganisms viability, cell suspensions were serially diluted in NaCl 0.9% solution and inoculated in triplicate using the drop-counting technique in the following culture media: Sabouraud Dextrose Agar for *C. albicans* (Jin et al. 2004), Mitis Salivarius Agar plus 0.2 units bacitracin per ml and 15% sucrose (MSB) for *S. mutans* (Gold et al. 1973), for quantification these microorganisms in the single and duo species biofilms. The inocula were incubated at 37°C, for 48 h, in 10% CO_2 and the colony forming units (CFUs) were counted under a stereoscopic microscope.

An aliquot of 400 µL of the sonicated biofilm suspension was used for the extraction and quantification of distinct polysaccharides found in biofilm (IPS-intracellular polysaccharides, soluble and insoluble EPS) as decribed by Aires et al. (2008).

Dentin demineralization assessment (%SHL)

The SH was again determined by three adjacent indentations spaced 100 µm from the three baseline measurements, using the same parameters above described.

The percentage of surface hardness loss (%SHL) was calculated (Cury et al. 2000) and used as indicator of mineral loss because there is a high correlation between SH and transverse microradiography (TMR) (Vale et al. 2011).

Confocal laser scanning microscopy (CLSM)

An independent experiment (n=2/group of biofilm) was conducted in the same conditions above described to assess the biofilm organization and composition (*C. albicans* + *S. mutans* biofilm). *C. albicans* and *S. mutans* biofilms were stained with SYTO-9 green fluorescent nucleic acid (Excitation 485 nm/ Emission 498 nm; Thermo Scientific, USA) as describe by Klein et al. 2009. For the duo-specie *C. albicans* + *S. mutans* biofilm, the microrganisms were visualized by FISH technique. The biofilms were fixed with 4% paraformaldehyde, treated with lysozyme (70,000 UI ml^{-1}) and fluorescent probes specific for 16S rRNA gene sequences (EUK 532 for *C. albicans* cells and MUT 647 for *S. mutans* cells) were used (Thurnheer et al. 2004). Images were obtained with a CLSM (Leica Microsystems CMS, Mannheim, Germany) at distance of 1 µm of Z-axis and they were three-dimensionally analyzed using the software Image J (Hartig 2013).

Transmission electron microscopy (TEM)

Another independent experiment (n=2/biofilm) was idealized to evaluate by TEM the interaction between *C. albicans* and *S. mutans* in the duospecie biofilm. The mono-specie and duo-specie biofilms were formed in the same conditions above described but on 7x3x1 mm resin specimens (Dr. Spurr, Electron Microscopy Sciences), not dentine. The biofilms were analyzed according to Reese and Guggenheim (2007) that used sodium periodate to break polysaccharides in their glycosidic linkages α (1-4) and α (1-6), allowing the subsequent labeling by osmium tetroxide. Ultrathin sections were viewed on JEOL JEM 1400 transmission electron microscopic (TEM) at 80 kV in the Microscopy and Image Center of the Piracicaba Dental School - University of Campinas.

Statistical Analysis

The assumptions of homogeneity of variances and normal distribution of errors were checked by the Kolmogorov-Smirnov test and the dependent variable CFU and

IEPS were transformed to log_{10}. All data were analyzed by ANOVA-one-way, followed by Tukey's test. The software SAS 8.01 (SAS Institute Inc., Cary, N.C., USA) was used for the analysis and the significance level was set at 5%.

Results

The duo-specie biofilm, *C. albicans* + *S. mutans*, showed the highest ($p < 0.05$) amount of polysaccharides, and the *S. mutans* biofilm presented higher values than those formed by C. albicans (Table 1). Regarding the biofilms wet weight and values of viable microorganisms, *C. albicans* + *S. mutans* and *S. mutans* did not differ ($p > 0.05$). The weight of *C. albicans* biofilm formed on a single dentine slab was not possible to be determined with accuracy.

Table 1. Biofilms wet weight (biomass), number of viable microorganisms (CFU) and amount of polysaccharides, according to the biofilms formed on dentine slabs (mean ± SD, n=12).

Groups of biofilms	Biomass (mg/biofilm)	Total viable microorganisms (CFULog10/biofilm)	Polysaccharides		
			SEPS (µg/biofilm)	IEPS (µg/biofilm)	IPS (µg/biofilm)
C. albicans	-	5.84 ± 0.19[a]	1.87 ± 0.97[a]	0.66 ± 0.48[a]	2.83 ± 0.92[a]
S.mutans	9.8 ± 4.2[a]	8.98 ± 0.18[b]	4.00 ± 0.94[b]	18.86 ± 5.92[b]	4.53 ± 1.73[b]
C.a + *S.m*	9.1 ± 2.3[a]	8.97 ± 0.17[b]	6.39 ± 2.14[c]	24.89 ± 9.22[c]	6.62 ± 1.43[c]

-: Below the detection limit of the balance

SEPS, soluble extracellular polysaccharides; IEPS, insoluble extracellular polysaccharides; IPS, intracellular polysaccharides

Groups followed by distinct letters (within columns) differ statistically by Tukey's test ($p<0.05$)

The CLSM images shows that the *C. albicans* + *S. mutans* biofilm are more volumous than the single-specie biofilms (Fig 1). FISH analysis shows the contact between *S. mutans* cells organized in microcolonies (green) and *C. albicans* in phenotypic form of yeast and hyphae (red). TEM images show the structure of *C. albicans* + *S. mutans* biofilm and the interaction between microorganisms surrounded by polysaccharide matrix. The *S. mutans* cells interact with the *C. albicans* cell wall by polysaccharides (Fig 2).

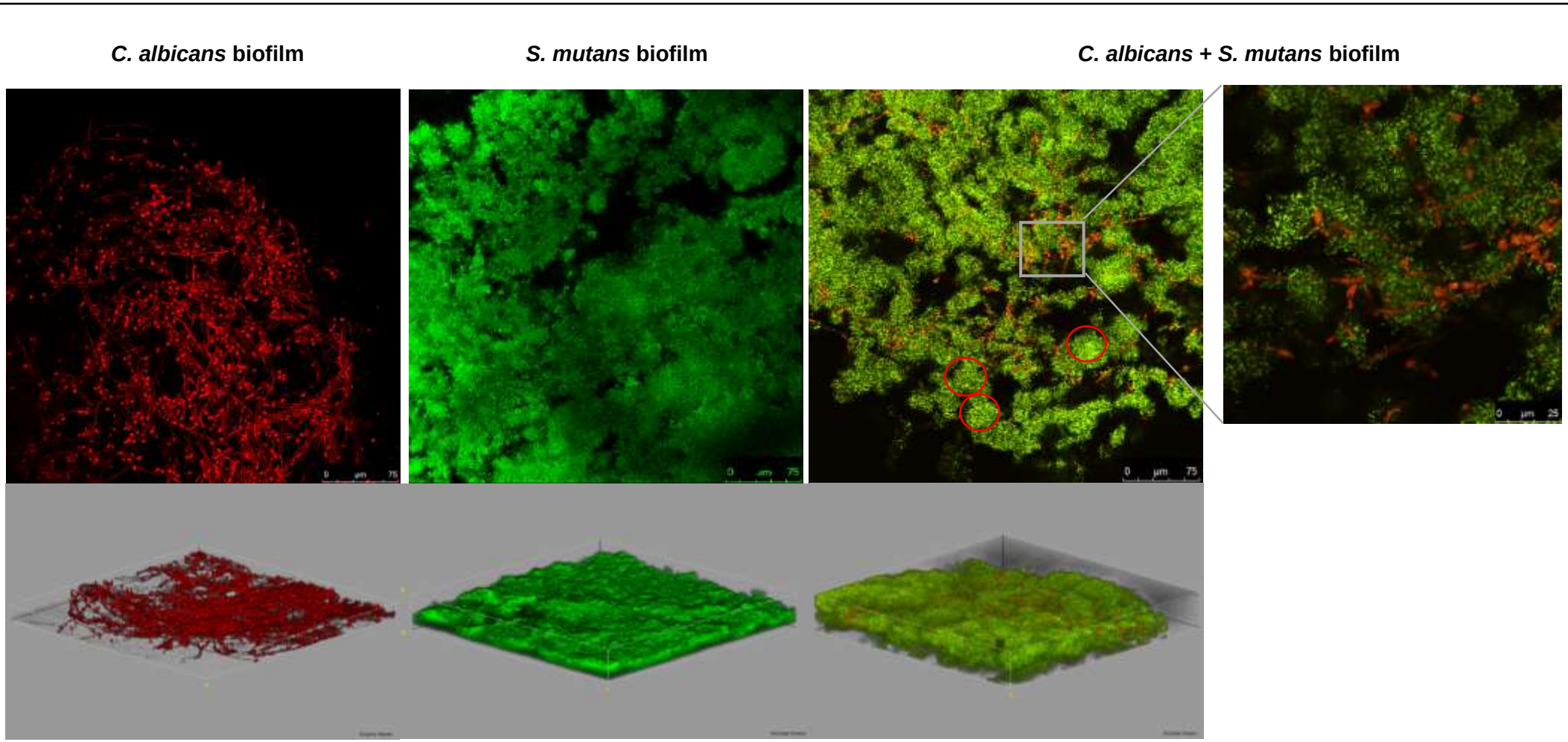

Fig 1. CLSM images from the *C. albicans* biofilm (left) and *S. mutans* biofilm (middle) assessed by cells stained with SYTO9 and *C. albicans* + *S. mutans* biofilm assessed by FISH (rigth) analysis after 96 h. CLSM image showing the presence of *S. mutans* microcolonies (green, red circle) and yeast and hyphae of *C. albicans* (red, white arrow) in *C. albicans* + *S. mutans* images. Oil immersion objective of 40x (numeric aperture 1.25).

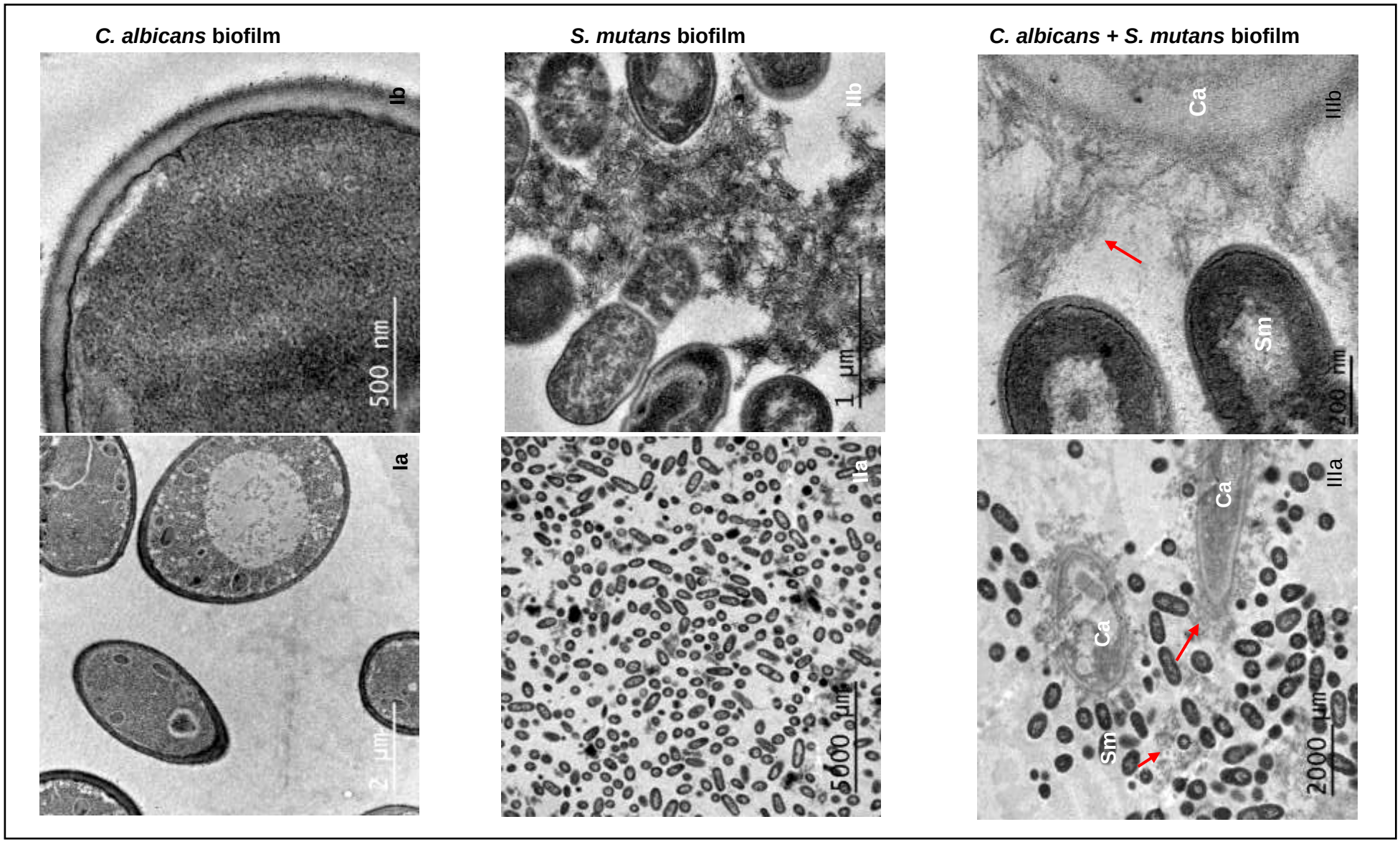

Figure 2. TEM images exhibiting organization of *C. albicans* biofilm (Ia, Ib), *S. mutans* biofilm (IIa, IIb) and *C. albicans* + *S. mutans* biofilm (IIIa, IIIb). *C. albicans* biofilm image shows the absence of polysaccharides between fungus cells (Ia) and its cell wall (Ib). *S. mutans* biofilm image shows the presence of a large amount of polysaccharides (arrow) between bacterial cells (IIa, IIb). The discernable morphological differences which allow the identification of the microorganisms comprising the biofilm: *C. albicans* (C.a) surrounded by *S. mutans* (S.m) (IIIa). The intimate contact with fungus and bacteria is mediated by polysaccharides (arrow) (IIIa, IIIb).

Figure 3 shows pH changes of the culture media provoked by the biofilms after the adhesion phase (8 h), the daily exposures to sucrose (times of 32, 56 and 80 h) and after the starvation (times of 48, 72 and 96 h). The *C. albicans* + *S. mutans* biofilm showed the highest acidogenicity at the times of 56 and 80 h and *C. albicans* presented the lowest ($p < 0.05$).

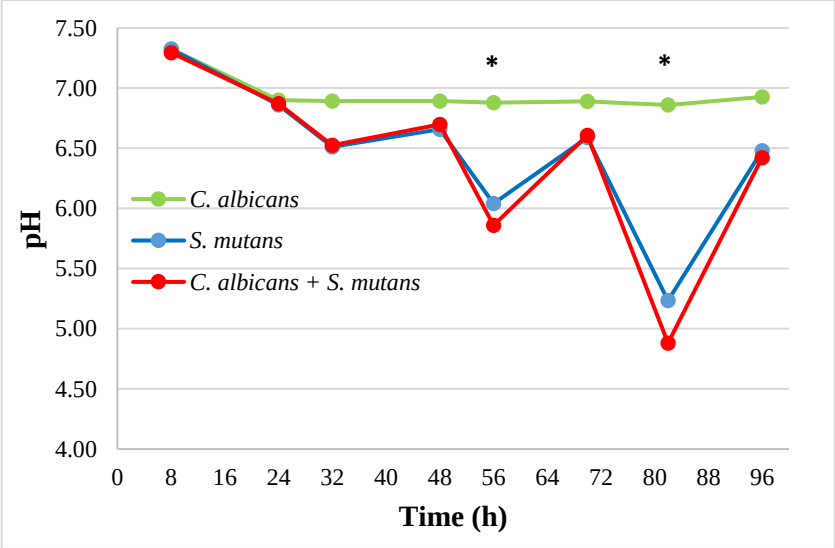

Figure 3. Culture medium pH according to the type of biofilms and time (h) of growth (Means; SD; n = 12).*Times that all biofilms differed between them ($p < 0.05$).

Regarding lactic acid released to the culture media (Fig. 4), the concentration for the duospecie biofilm was the highest at 32, 56 and 80 h and the lowest was found for *C. albicans* biofilm ($p < 0.05$).

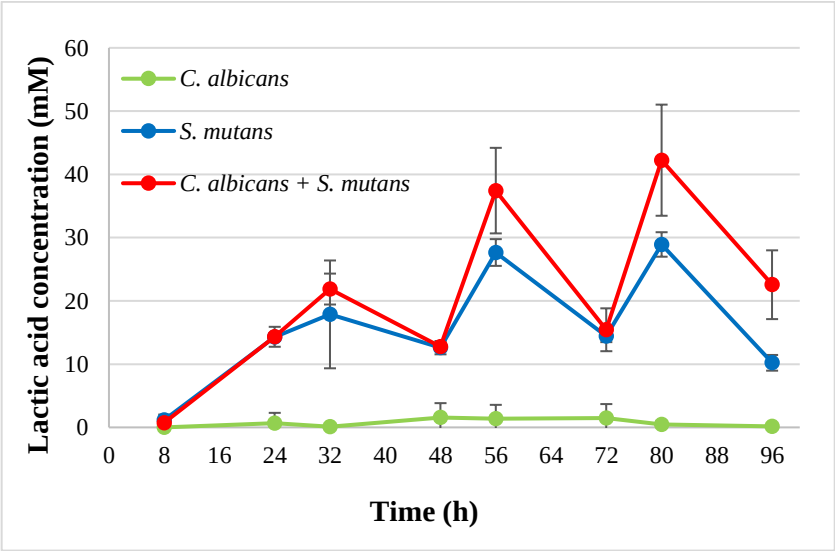

Figure 4. Lactic acid concentration (mM) in the culture medium according to the type of biofilms and time (h) of growth (Mean; SD; n = 6).

In terms of dentin demineralization (Fig. 5), highest %SHL was observed in the slabs of *C. albicas* + *S. mutans* biofilm group and *C. albicans* biofilm provoked the lowest demineralization ($p<0.05$).

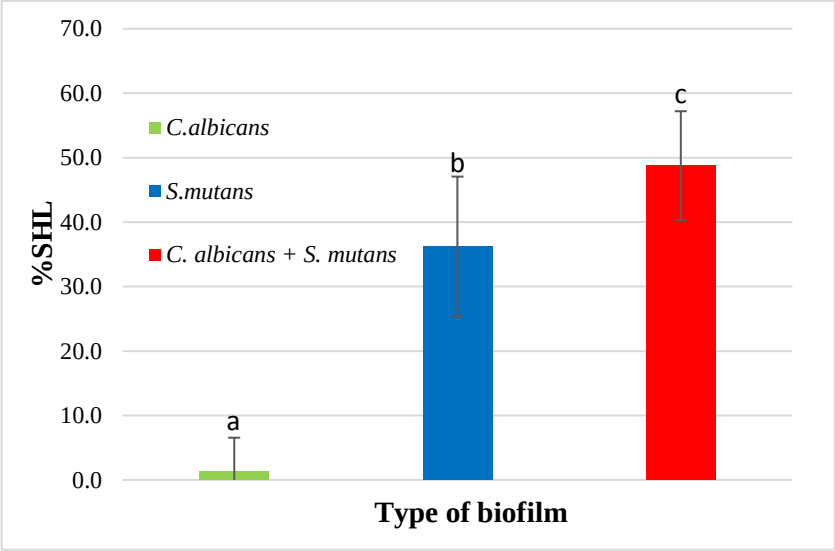

Figure 5. Dentin surface hardness loss (%SHL) according to the type of biofilms (Mean ± SD; n = 12). All groups differed statistically between them ($p<0.05$).

Discussion

Previous association studies have demonstrated that *C. albicans* is involved on root caries in elderly population (Beighton et al.1991, Beighton and Lynch 1993 Shen et al. 2002, Shen et al. 2005, Zaremba et al. 2006), and according to a recent experimental study made in rats the interaction with *C. albicans* and *S. mutans* may lead to a more cariogenic biofilm (Falsetta et al. 2014). The cariogenic potential of *C. albicans* biofilm and its combination with *S. mutans* to cause dentin demineralization is unkown. This uncertainty led to the development of this study that investigated the hypothesis that *C. albicans* would increase the capacity of *S. mutans* biofilm to demineralize dentin. For that, it was used a validated biofilm caries model of single-specie (Ccahuana - Vásquez and Cury 2010) modified to evaluate a dual-species biofilm.

According to the results obtained, although *C. albicans* biofilms were not able to demineralize dentin, the results clearly show that this fungus increased dentin demineralization provoked by *S. mutans* biofilm ($p <0.05$). These data are supported by the higher acidogenicity (lower pH and increased production of lactic acid) found in the culture medium of *C. albicans* + *S. mutans* biofilm after the second and third days

of treatment with sucrose when compared with the *S. mutans* and *C. albicans* single-specie biofilms (Figure 3). The lower pH found for *C. albicans* biofilm ($p< 0.05$) supports the hypothesis that *C. albicans* by itself has no ability to cause dental demineralization (data not shown). Apparently, *C. albicans* cells have limited capacity to adhere to dentin slabs, what has also been demonstrated by Falsetta et al. 2014 and Willems et al. 2016 by using hydroxyapatite discs as substrate.

The salivary pellicle used in this biofilm model to better simulate the oral conditions, could at a first glance be implicated as the reason for causing this limited adherence of *C. albicans* due to the antifungal and antibacterial effect of some salivary peptides commonly present in the pellicle (Lee et al. 2013). However, the use of sucrose as a source of carbohydrate and the consequent polysaccharides production by microorganisms overrides the effect of the pellicle peptides in the biofilm formation.

The lower pH values for *C. albicans* + *S. mutans* biofilm in this study cannot be explained by the biofilm biomass or amount of microorganism (Table 1). The counting of *S. mutans* was not significantly different in *C. albicans* + *S. mutans* biofilm compared to *S. mutans* biofilm ($p >0.05$) which shows that the lower pH found in *C. albicans* + *S. mutans* biofilm is better attributed to changes in matrix composition and biofilm structure (Cury et al. 2001). The results also demonstrated that *C. albicans* cells, even by representing the minor component of the mature *C. albicans* + *S. mutans* biofilm, participated in the increased amount of all types of polysaccharides in the duo specie biofilm ($p<0.05$) (Table 1). There was a higher production of the insoluble extracellular polysaccharides (IEPS), responsible for biofilm structural organization, therefore being a virulence determinant factor of cariogenic biofilm (Koo et al. 2013) (Table 1). Although the percentage of *C. albicans* in duo specie biofilm was of approximately 1%, which is consistent with clinical findings that illustrate the lower prevalence of this fungus in the oral cavity of healthy individuals, this study showed that the fungus was able to modify the biofilm architecture, giving rise to a more cariogenic biofilm.

The presence of *C. albicans* in *S. mutans* biofilm caused an increase in the production of polysaccharides by this biofilm when compared to single-specie biofilms ($p< 0.05$) (Table 1). The presence, amount and characteristics of polysaccharides determine the structural characteristics of the biofilm, influencing the potential for dental caries development (Rölla 1989; Cury et al. 2000; Koo et al. 2013). Moreover, the CLSM images obtained suggest the presence of a higher porosity and volume in

the *C. albicans* + *S. mutans* biofilm than in single-specie biofilms, what is also consequence of the increased production of polysaccharides (Figure 1). More porous biofilms favor the sucrose diffusion to inside the biofilm, consequently, the cells metabolize this sugar into acid that is retained near to the tooth surface for a longer time, leading to a higher demineralization and making it more difficult to be removed by salivary flow (Xiao et al. 2012). It is relevant to consider that in the context of the elderly population, low salivary flow and the use of dentures are predisposing factors to increase fungal counts in the oral cavity (Sen et al. 1997; Altarawneh et al. 2013).

In this study, the confocal images suggest the predominance of *C. albicans* in its hyphae form in the single-specie biofilm, possibly as a consequence of the neutral pH in which the biofilm grew, with a preponderance of yeasts present in the *C. albicans* + *S. mutans* biofilm, possibly due to a more acidic pH in the culture medium (Figure 1) (Konno et al. 2006). This finding may also be supported by the effect of diffusible molecules CSP secreted by *S. mutans* to inhibit germ tube of *C. albicans* in co-cultures (Jarosz et al., 2009). However, it is currently unclear if *C. albicans* morphological presentation influences its ability to modify the biofilm and its cariogenic capacity, what remain to be further addressed in future studies. The methodology used in this study simulated the cycling pH that occur in the oral cavity as a consequence of the intermittent sugar exposure in biofilm, avoiding the oxidative stress of the biofilm cells, which could result in distant conclusions of what would happen in real life.

Polysaccharides also played a strong role in the interaction between *C. albicans* and *S. mutans* in the duo-species biofilm, which is demonstrated in the TEM images that show *S. mutans* cells in close contact with *C. albicans* cell wall (Figure 2). In agreement with these findings, previous studies reported that cross-kingdom interaction is mediated by the bacterial enzyme glicosyltransferase B (GtfB) that easily binds to the surface of the yeast cell (Gregoire et al. 2011; Falsetta et al. 2014), posteriorly causing the glucan-rich matrix production by *C. albicans* that facilitate the accumulation of *S. mutans* on the fungal cell wall (Gregoire et al. 2011). TEM images of *C. albicans* biofilm and *S. mutans* biofilm reinforce the relevance of the interaction between fungus and bacteria mediated by polysaccharides in the cariogenic duo specie biofilm. *C. albicans* grown isolate did not present exopolysaccharides, demonstrating only the mannoproteins layer (Chaffin 2008). *S. mutans* TEM images demonstrate the presence of a lot of amount of exopolysaccharides throughout the

biofilm. Whereas the fungal cell wall appears totally different in the duo-biofilm, the wall thickening and presence prolongations in close contact with *S. mutans* cells can be explained by the interaction mediated by the bacterial enzyme glycosyltransferase B. This extraordinary relationship between *C. albicans* and *S. mutans* is responsible for the formation of water channels in the biofilms, which facilitate sugar diffusion, acids retained within the micro colonies for a longer period and close to the tooth surface, what would be responsible for the increased demineralization caused by duo-specie biofilm. Therefore, the biofilm environment created by the fungus-bacteria interaction is the direct responsible for the lower pH found.

As a limitation of the current study, the *C. albicans* biofilm cariogenicity is questionable because of the impossibility of achieving a satisfactory adhesion of cells to dentin slabs and the consequent formation of a complex biofilm.

In conclusion, the results obtained indicate that *C. albicans* was able to increase the cariogenic potential of *S. mutans* biofilms, causing an increased demineralization of dentin. Moreover, the present results also support previous clinical data suggesting that *C. albicans* increases the virulence of dental biofilm, what may have important implications in future investigations about the mechanisms involved in this synergic interaction of these microorganisms and in the design of anti-biofilm therapies.

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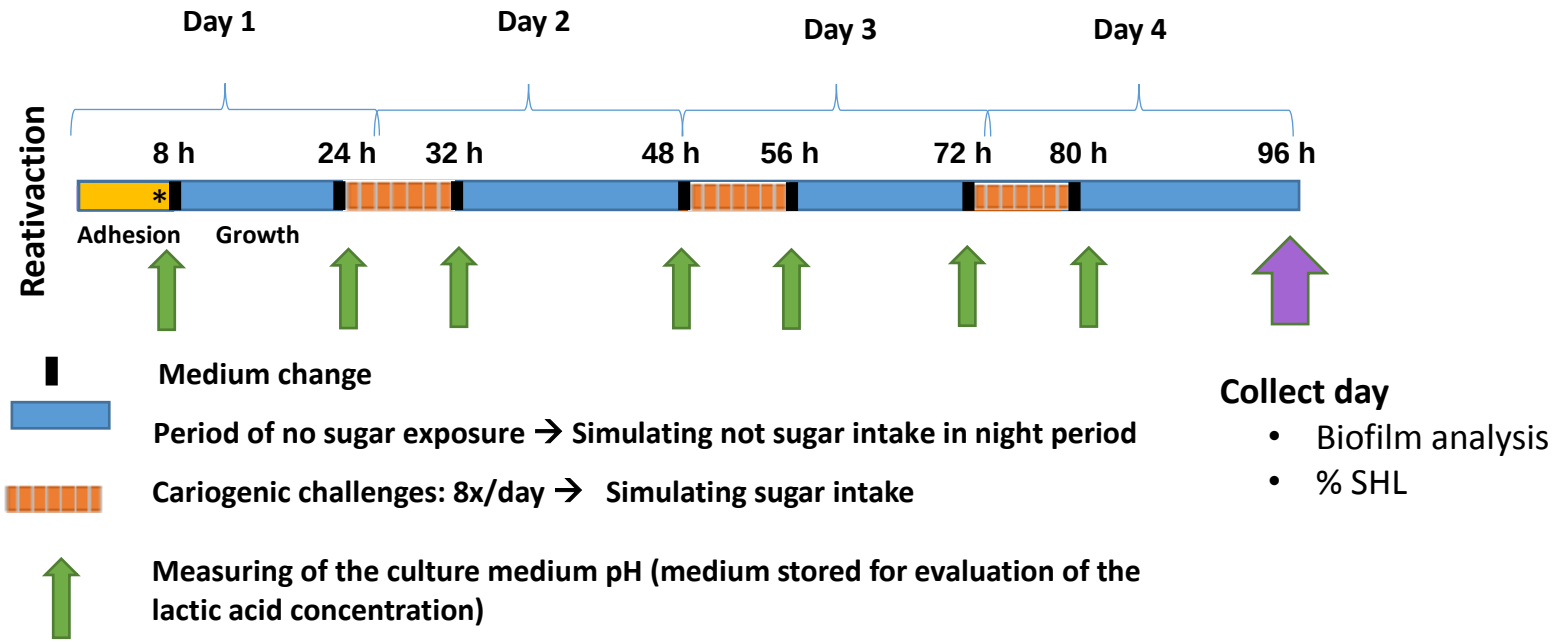
Supplement

Figure S1: Experimental Flowchart



3 CONCLUSÃO

Os resultados deste trabalho permitem concluir que embora a *C. albicans* tenha mostrado um baixo potencial cariogênico, ela foi capaz de aumentar o potencial cariogênico de *S. mutans*, provocando maior desmineralização da dentina.

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* De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors - Vancouver Group. Abreviatura dos periódicos em conformidade com o PubMed.

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ANEXO 1: *Certificado do Comitê de Ética em Pesquisa da FOP-UNICAMP*

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

CERTIFICADO

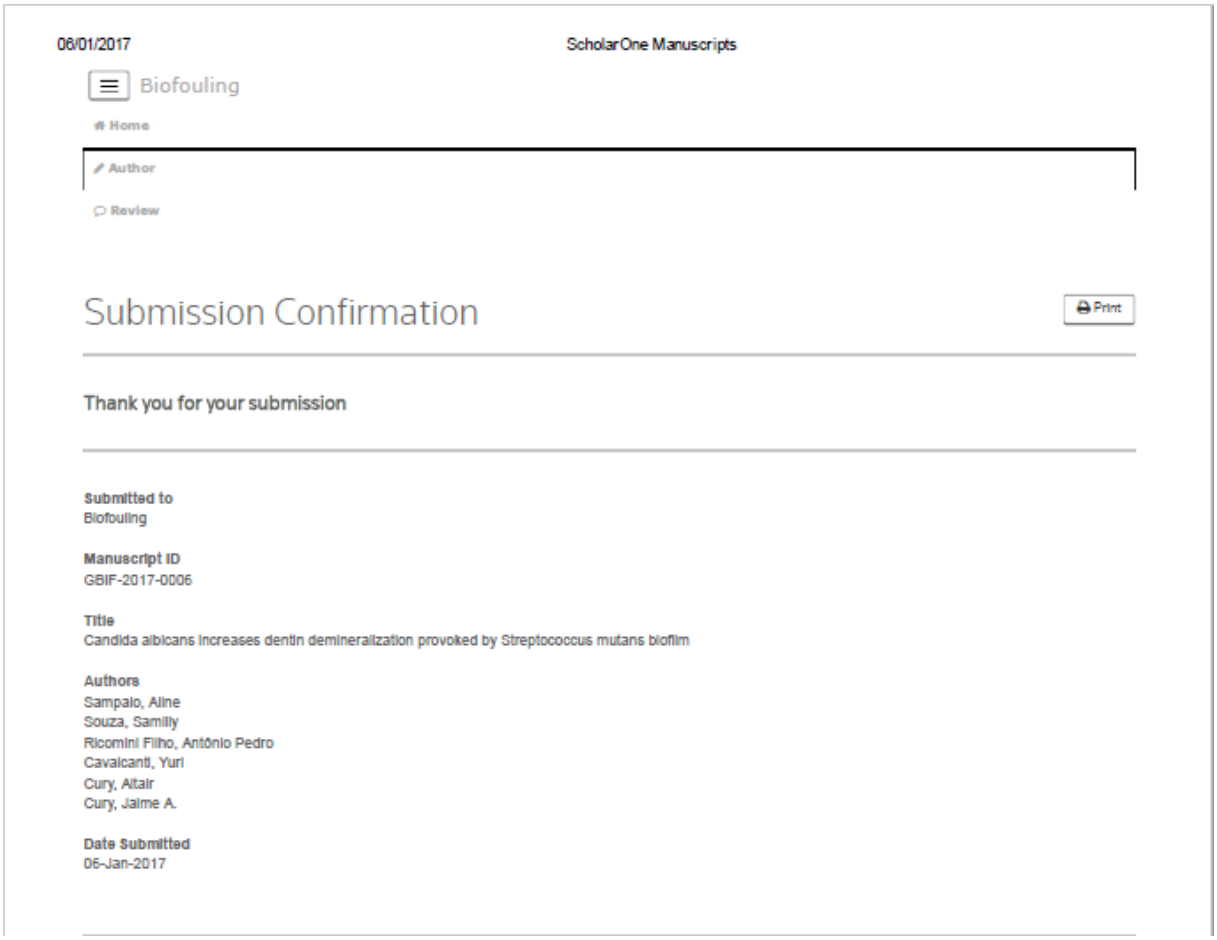
O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "***Modelo de biofilme para avaliar o potencial cariogênico da* Candida albicans**", protocolo nº **075/2015**, dos pesquisadores **ALINE ARAÚJO SAMPAIO, JAIME APARECIDO CURY, SAMILLY EVANGELISTA SOUZA e ALTAIR ANTONINHA DEL BEL CURY**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 29/07/2015.

The Ethics Committee in Research of the Piracicaba Dental School, University of Campinas, certify that the project "***Biofilm model to evaluate the cariogenic potential of* Candida albicans**", register number **075/2015**, of **ALINE ARAÚJO SAMPAIO, JAIME APARECIDO CURY, SAMILLY EVANGELISTA SOUZA and ALTAIR ANTONINHA DEL BEL CURY** 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 on July 29, 2015.

Prof. 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 ao periódico Biofouling



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