UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA

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PAPEL DA *CANDIDA ALBICANS* NA DESMINERALIZAÇÃO DO ESMALTE DENTAL E NO POTENCIAL ACIDOGÊNICO DE BIOFILMES DE *STREPTOCOCCUS MUTANS*: ESTUDO *IN VITRO*

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Papel da *Candida albicans* na desmineralização do esmalte dental e no potencial acidogênico de biofilmes de *Streptococcus mutans*: estudo *in vitro*

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RESUMO

Papel da *Candida albicans* na desmineralização do esmalte dental e no potencial acidogênico de biofilmes de *Streptococcus mutans*: estudo *in vitro*

Objetivos: avaliar o papel da *Candida albicans* (ATCC 90028) no potencial cariogênico e acidogênico de biofilmes *in vitro* de *Streptococcus mutans* (UA159). **Métodos:** biofilmes de dupla-espécie (*C. albicans* + *S. mutans*) e de espécie única (*S. mutans* ou *C. albicans*) foram cultivados sobre a superfície hígida de blocos de esmalte bovino com dureza inicial conhecida na presença de meio de cultura suplementado com glicose 20 mM e sacarose 3 mM por 24, 48 e 72 horas. O meio foi substituído diariamente e seu pH foi registrado. Blocos de esmalte foram então coletados ao final de cada período experimental e contagens de células viáveis foram realizadas. A desmineralização foi avaliada através da porcentagem de alteração de microdureza superficial (% SMC) e análise de microradiografia transversal (perda mineral integrada e profundidade de lesão). **Resultados:** o pH do meio de cultura permaneceu constante nos biofilmes de espécie única de *C. albicans* causando quase nenhuma desmineralização no esmalte. Não houve diferença no potencial acidogênico dos biofilmes de espécie única de *S. mutans* ao longo dos períodos experimentais sendo estes valores de pH sempre menores quando comparados ao pH relacionado aos demais biofilmes ($p < 0.01$) A % SMC dos biofilmes de espécie única de *S. mutans* aumentou significativamente com o tempo. Além disso, lesões de cárie após 72 horas de crescimento de biofilme bacteriano apresentaram maior perda mineral integrada e foram mais profundas do que lesões de cárie relacionadas a biofilmes de dupla-espécie ($p < 0.01$). O pH do meio de cultura relacionado a biofilmes de dupla-espécie foi baixo no primeiro dia, mas aumentou no decorrer do tempo para valores acima do pH crítico para desmineralização do esmalte. Em consequência, a % SMC permaneceu a mesma depois de 48 horas de crescimento de biofilmes de dupla-espécie e foi significantemente menor do que os valores relacionados a *S. mutans* em espécie única ($p < 0.01$). **Conclusões:** os presentes dados sugerem que a *C. albicans* apresenta um baixo potencial de desmineralização de esmalte e esse micro-organismo parece reduzir tanto o potencial cariogênico quanto acidogênico de biofilmes de *S. mutans* pela modulação do pH extracelular durante o crescimento *in vitro* de biofilmes de dupla-espécie.

Palavras-chaves: *Candida albicans*; *Streptococcus mutans*; biofilmes; cárie dentária; esmalte dentário.

ABSTRACT

Role of *Candida albicans* on enamel demineralization and on acidogenic potential of *Streptococcus mutans in vitro* biofilms

Aim: evaluate the role of *Candida albicans* (ATCC 90028) on the cariogenic and acidogenic potentials of *Streptococcus mutans* (UA159) *in vitro* biofilms. **Methods:** dual-species (*C. albicans* + *S. mutans*) and single-species (*S. mutans* or *C. albicans*) biofilms were grown on the surface of sound bovine enamel slabs with known baseline hardness in the presence of culture medium supplemented with 20 mM glucose and 3 mM sucrose for 24, 48 and 72 hours. The medium was replaced daily and its pH was recorded. Enamel slabs were collected at the end of each experimental period and counts of viable cells were performed. Demineralization was evaluated through percentage of surface microhardness change (% SMC) and transversal microradiography analysis (integrated mineral loss and lesion depth). **Results:** spent medium pH remained neutral in *C. albicans* single-species biofilms causing almost no enamel demineralization. No difference in acidogenic potential of *S. mutans* single-species biofilms was found among experimental periods and its pH was always lower than pH related to other biofilms ($p < 0.01$). The % SMC in *S. mutans* single-species biofilms significantly increased with time. In addition, caries lesions after 72 hours of *S. mutans* biofilm growth had higher integrated mineral loss and were deeper than caries lesions related to dual-species biofilms ($p < 0.01$). Spent medium pH related to dual-species biofilms was low in the first day, but increased with time to values above critical pH for enamel demineralization. As a consequence, the % SMC remained the same after 48 hours of dual-species biofilm growth and was significantly lower than *S. mutans* single-species related values ($p < 0.01$). **Conclusions:** the present data suggest that *C. albicans* has low enamel demineralization potential and may decrease both the cariogenic and acidogenic potentials of *S. mutans* by modulating extracellular pH during *in vitro* dual-species biofilm growth.

Keywords: *Candida albicans*; *Streptococcus mutans*; biofilms; dental caries; dental enamel.

SUMÁRIO

1 CONSIDERAÇÕES INICIAIS

Pesquisas envolvendo biofilmes formados sobre os dentes, também chamados de placa dentária, iniciaram já no século XVII com as observações de Antonie van Leeuwenhoek. Ao longo do tempo, as complexas relações entre o microbioma e o hospedeiro, ou em outras palavras, entre o conjunto de micro-organismos e o organismo humano, foram sendo parcialmente explicadas por diferentes hipóteses de desenvolvimento de doenças relacionadas a biofilmes (Marsh *et al.*, 2011).

A Hipótese da Placa não-específica sugere que qualquer placa dentária tem o mesmo potencial de causar doença (Miller, 1890). Como consequência, a remoção não-específica de biofilme pela escovação dentária seria a melhor forma de controlar a doença. Apesar do abandono dessa teoria após o desenvolvimento da identificação de micro-organismos nos meados do século XX, a remoção mecânica de placa dentária ainda é um dos mais eficientes meios de manutenção da saúde oral (Rosier *et al.*, 2014).

Outro conceito de desenvolvimento de doença vem da Hipótese da Placa específica, onde determinados micro-organismos estariam em maior número em condições de doença e seriam responsáveis por ela. Ou ainda, da Hipótese da Placa de espécies "chave", onde diferentemente da teoria da placa específica, certos patógenos microbianos poderiam desencadear alterações relacionadas à doença, mesmo que presentes em pequena proporção na microbiota oral (Rosier *et al.*, 2014). Em outras palavras, de acordo com essa última teoria, espécies "chave" constituiriam um componente menor da comunidade microbiana que seria capaz de modular a resposta do hospedeiro e remodelar a microbiota comensal, causando uma disbiose, que por sua vez, poderia levar à doença (Hajishengallis *et al.*, 2012). Consequência de tais teorias é que os tratamentos poderiam ser focados em um número limitado de micro-organismos e possivelmente seriam mais eficientes justamente por atuarem sobre micro-organismos específicos (Hajishengallis *et al.*, 2012; Rosier *et al.*, 2014).

Contudo, a teoria mais aceita atualmente assume que doenças mediadas por biofilme resultem de uma mudança na microbiota, de um estado de simbiose para um de disbiose. De acordo com a Teoria da Placa ecológica, esse desequilíbrio microbiano não seria causado pelo efeito direto dos micro-organismos em si, mas sim por fortes pressões seletivas, que por sua vez, seriam promovidas por alterações ecológicas (Marsh, 2003). Um fator comum capaz de quebrar a homeostase intrínseca das comunidades microbianas e seu equilíbrio com o hospedeiro é a mudança no perfil nutricional de um habitat. Por exemplo, a introdução de novos substratos pode alterar a expressão gênica de alguns micro-organismos, levando a um estado de

maior competitividade, onde espécies associadas com doença podem prevalecer sobre espécies normalmente encontradas em saúde (Marsh, 2003; Marsh e Zaura, 2017).

Em acordo com a Teoria da Placa ecológica, evidências mostram que o catabolismo de carboidratos em um biofilme com uma matriz extracelular rica em substâncias poliméricas promove a formação de áreas com maior concentração ácida. Tais microambientes favorecem a seleção de micro-organismos acidúricos, ou em outras palavras, com tolerância ácida. Uma vez que muitas dessas áreas se localizam próximas à superfície dentária, o resultado da mudança ecológica inicial nesse caso é a desmineralização do dente associada a biofilmes, ou simplesmente, o desenvolvimento da cárie dentária (Xiao *et al.*, 2012).

Verdade é que os mecanismos exatos que promovem as doenças mediadas por biofilmes ainda não são completamente entendidos. Porém, novas tecnologias têm proporcionado avanços significativos na análise das comunidades microbianas como um todo. Tal visão holística da ecologia oral deve em breve providenciar evidências que suportem ou descartem teorias que atualmente tentam explicar as transições entre o que entendemos por saúde e doença oral (Rosier *et al.*, 2014).

Nesse contexto, um estudo envolvendo placa dentária mineralizada mostrou que a composição da microbiota permaneceu com uma certa constância e era substancialmente mais diversa entre os períodos neolítico e medieval do que é hoje em dia. Transições nutricionais e culturais parecem ter afetado o ecossistema oral nas recentes centenas de anos que nos precedem (Adler *et al.*, 2013). Tal evidência é suportada pelo achado de que uma população ameríndia, que vivia ainda sob costumes primitivos, tinha uma microbiota muito mais diversa do que a encontrada em indivíduos com estilo de vida ocidental (Clemente *et al.*, 2015).

É interessante observar que a saúde oral está associada a um microbioma diverso e ecologicamente equilibrado. No caso da cárie dentária, a diversidade de uma comunidade microbiana sofre uma drástica redução de um local saudável, onde há equilíbrio ecológico, para um com doença, onde tal equilíbrio é quebrado (Simón-Soro *et al.*, 2013). O equilíbrio de um ecossistema, por sua vez, resulta de todas as complexas interações entre seus membros e o seu ambiente (Marsh *et al.*, 2011; Krom *et al.*, 2014).

Técnicas moleculares indicam que tal complexidade de sistemas polimicrobianos tem sido subestimada (Thein *et al.*, 2009; Filoche *et al.*, 2010). Como visto, a maior parte das doenças orais está relacionada a biofilmes polimicrobianos e advém da perturbação de toda comunidade microbiana (Thein *et al.*, 2009; Clemente *et al.*, 2012; Siqueira *et al.*, 2012; Wade, 2013). Portanto, o entendimento das interações não somente entre espécies, mas também entre

diferentes reinos de micro-organismos presentes nos biofilmes é crucial para o desenvolvimento de estratégias de saúde (Diaz *et al.*, 2014).

Entretanto, a maior parte dos estudos microbiológicos têm focado somente nas espécies bacterianas e pouco se sabe a respeito das interações dos fungos nas comunidades dos biofilmes orais (Shirtliff *et al.*, 2009). Porém o perfil do componente fúngico oral está longe de ser simples (Ghannoum *et al.*, 2010). De fato, a micobiota da cavidade oral tem mais diversidade e complexidade do que qualquer outra do corpo humano (Xu e Dongari-Bagtzoglou, 2015).

De acordo com um estudo que avaliou o componente fúngico do microbioma, conhecido especificamente como micobioma, na saliva humana estão presentes os seguintes gêneros de fungos: *Alternaria / Lewia, Aspergillus / Emericella / Eurotium, Aureobasidium, Candida / Pichia, Cladosporium / Davidiella, Cryptococcus / Filobasidiella, Cytospora / Valsa, Fusarium / Gibberella, Irpex, Lenzites / Trametes, Malassezia and Sporobolomyces / Sporidiobolus.* Além de *Epicoccum, Phoma e Saccharomyces*, que são possíveis candidatos a serem incluídos se dados adicionais forem coletados no futuro (Dupuy *et al.*, 2014).

Dentre tais fungos a espécie mais frequentemente encontrada no ambiente bucal e que raramente é encontrada vivendo independente na natureza é a *Candida albicans* (*C. albicans*) (Di Menna, 1954). Esse fungo polimórfico comum pode ser visto inicialmente como um comensal do organismo humano que sob alguns incidentes pode assumir uma forma patogênica invasiva (D' Enfert, 2009). A grande quantidade de portadores saudáveis de *C. albicans* corrobora para essa teoria (Polke *et al.*, 2015). Não obstante, a fisiologia e morfogênese da *C. albicans* pode ser modificada por diferentes espécies bacterianas (Thein *et al.*, 2006). Logo, o fungo *C. albicans* desempenha um importante papel em diversas doenças orais polimicrobianas (O'donnell *et al.*, 2015).

O papel da *C. albicans* na etiopatogenia da cárie dentária vem sendo estudado e tem alta relevância para o melhor entendimento dessa importante doença (Pereira *et al.*, 2017). Afinal, mesmo sabendo que as medidas modernas de higiene oral podem remover os biofilmes excessivos e controlar doenças como gengivite, periodontite e cárie dentária (Janus *et al.*, 2015), esta última é uma das doenças biofilme-dependentes mais prevalente mundialmente (Islam *et al.*, 2007). E, como inicialmente discutido, ela não é causada por um único patógeno e está associada com a microbiota comensal (Wade, 2013). Sendo que a presença de espécies específicas de fungos, especialmente altas contagens salivares de *C. albicans*, parece estar relacionada à severidade da experiencia de cárie (Moraga *et al.*, 2016).

Mesmo assim, espécies bacterianas como *Streptococcus mutans* (*S. mutans*) e *Lactobacillus casei* têm sido historicamente relacionadas com sua etiopatogenia por serem

acidogênicos e ácido tolerantes (Loesche, 1986). *C. albicans* tem uma alta tolerância ácida e é capaz de produzir ácidos mesmo em condições de baixo pH. Características que podem favorecer esse fungo durante as alterações microbiológicas associadas com a cárie dentária (Klinke *et al.*, 2009). De fato, como visto, a produção de ácidos é um importante fator ambiental que promove adaptação e seleção de micro-organismos em biofilmes cariogênicos (Takahashi e Nyvad, 2011).

A fermentação de carboidratos da dieta é o que dá origem a maior parte dos ácidos que causam a desmineralização dentária (Roella *et al.*, 1985). Portanto, biofilmes cariogênicos acumulam micro-organismos Gram-positivos e sacarolíticos (Janus *et al.*, 2015). Em outras palavras, carboidratos, especialmente a sacarose, mediam a formação de biofilme e são determinantes para as alterações ecológicas que estão relacionadas ao desenvolvimento da cárie dentária (Roella *et al.*, 1985; Sheiham e James, 2015). Contudo, não podemos esquecer que somos onívoros e que o consumo de açúcar não é o único determinante do processo carioso (Gupta *et al.*, 2013; Giacaman, 2017). Mesmo assim, um melhor entendimento de sua relação com diferentes micro-organismos é importante (Klein *et al.*, 2009).

Um dos principais fatores de virulência de biofilmes cariogênicos é a presença de matriz extracelular, principalmente os polissacarídeos extracelulares (PEC) insolúveis sintetizados na presença de sacarose. Tais substâncias, além de aumentarem a co-agregação microbiana, também agem como barreira física, promovendo a criação de microambientes e aumentando a heterogeneidade dos biofilmes (Koo *et al.*, 2013; Koo e Bowen, 2014). Apesar dos *S. mutans* serem considerados os principais micro-organismos responsáveis pela síntese desses polissacarídeos, a enzima glicosiltransferase B, sintetizada pelos *S. mutans*, pode se adsorver à superfície de outros micro-organismos tornando-os aptos à produzirem PEC insolúvel (Xiao *et al.*, 2012; Klein *et al.*, 2015).

Existe evidência de que o fungo *C. albicans* pode modular a síntese de matriz extracelular e também aumentar a presença de *S. mutans* nos biofilmes (Gregoire *et al.*, 2011; Hwang *et al.*, 2017). Nesse sentido, uma associação entre a presença de *C. albicans* e o desenvolvimento de biofilmes cariogênicos pode existir. Especialmente em condições severas como a cárie precoce de infância (Li *et al.*, 2014; Qiu *et al.*, 2015). De fato, a frequência com que este fungo é isolado é significativamente superior em biofilmes associados com dentes cariados (De Carvalho *et al.*, 2006; Yang *et al.*, 2012; Fragkou *et al.*, 2016; Moraga *et al.*, 2016; Naidu e Reginald, 2016).

Alguns trabalhos mostram um aumentado potencial cariogênico da associação entre *C. albicans* e *S. mutans* em biofilmes *in vitro* e *in vivo* (Falsetta *et al.*, 2014; Kim *et al.*, 2017).

Contudo, resultados inconclusivos também são apontados e até mesmo efeitos de redução do potencial cariogênico são vistos na literatura (Klinke *et al.*, 2011; Willems *et al.*, 2016).

Dessa forma, fica evidente que o conhecimento das inter-relações microbianas é importante para um melhor entendimento e controle do processo carioso (O'donnell *et al.*, 2015). Portanto, estudos microbiológicos da cárie dentária deveriam incluir fungos nas suas investigações e o papel da *C. albicans* precisa ser melhor avaliado. O objetivo do presente estudo é avaliar o efeito do fungo *C. albicans*, associado ou não com a bactéria *S. mutans*, na desmineralização em esmalte e no potencial acidogênico de biofilmes *in vitro.*

2 ARTIGO CIENTÍFICO

TITLE

"Role of *Candida albicans* on enamel demineralization and on acidogenic potential of *Streptococcus mutans in vitro* biofilms."

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ABSTRACT

This study aimed to evaluate the role of *Candida albicans* (ATCC 90028) on the cariogenic and acidogenic potentials of *Streptococcus mutans* (UA159) *in vitro* biofilms. Dual-species (*C. albicans* + *S. mutans*) and single-species (*S. mutans* or *C. albicans*) biofilms were grown on the surface of sound bovine enamel slabs with known baseline hardness in the presence of culture medium supplemented with 20 mM glucose and 3 mM sucrose for 24, 48 and 72 hours. The medium was replaced daily and its pH was recorded. Enamel slabs were collected at the end of each experimental period and counts of viable cells were accessed. Demineralization was evaluated through percentage of surface microhardness change (% SMC) and transversal microradiography analysis (integrated mineral loss and lesion depth). Spent medium pH remained neutral in *C. albicans* single-species biofilms causing almost no enamel demineralization. No difference in acidogenic potential of *S. mutans* single-species biofilms was found among experimental periods and its pH was always lower than pH related to other biofilms ($p < 0.01$). The % SMC in *S. mutans* single-species biofilms significantly increased with time. In addition, caries lesions after 72 hours of *S. mutans* biofilm growth had higher integrated mineral loss (IML) and were deeper than caries lesions related to dual-species biofilms ($p < 0.01$). Spent medium pH related to dual-species biofilms was low in the first day, but increased with time to values above critical pH for enamel demineralization. As a consequence, % SMC remained the same after 48 hours of dual-species biofilm growth and was significantly lower than *S. mutans* single-species related values ($p < 0.01$). The present data suggest that *C. albicans* has low enamel demineralization potential and may decrease both the cariogenic and acidogenic potentials of *S. mutans* biofilms by modulating extracellular pH during *in vitro* dual-species biofilm growth.

INTRODUCTION

Despite billions of years of coexistence between bacteria and fungi, most of the biofilm studies have focused mainly on bacterial species and far less is known about the fungal interactions within biofilm communities. [1, 2] Fungi represent however, a small but significant component of the oral microbiome. [3-6]

Candida albicans (*C. albicans*) is the most frequent orally carried fungus and it is rarely found living free in nature. [7] This common polymorphic fungus is generally a commensal microorganism in humans, but under some circumstances, it may assume a pathogenic invasive form. [8] The high number of healthy individuals carrying *C. albicans* supports this hypothesis. [9] Nevertheless, the physiology and morphogenesis of *C. albicans* can be modified by different bacterial species. [10] Thus, *C. albicans* plays an important role in many oral polymicrobial diseases. [11]

Oral health is related to an ecologically balanced and diverse microbiome, being a natural result of all interactions between its members and their environment. [12, 13] Yet sometimes, by changing an environmental factor, a shift in the balance of the resident microbiota can lead to a disease-associated dysbiosis. [14] Dietary habits, poor oral hygiene, compromised immune system and genetics are the major factors usually related to these ecological shifts. [15] Among all factors, strong evidences suggest that dietary habits, especially the frequent consumption of sugared-food containing highly fermentable carbohydrates, have been directly related to microbiological changes on dental biofilms and to the onset and progression of dental caries. [14, 16]

Acid production, through fermentation of carbohydrates by a selective group of microorganisms, results in pH drops within biofilms. This acidification is directly related to the dissolution of tooth tissues that might occur if the balance between demineralization and remineralization is disturbed over an extended period of time. [17] So, in dental caries, frequent sugar intake results in a dysbiotic change in dental plaque that is accompanied by a breakdown of the host-microbial mutualism, where the acidophilic and acid-tolerant microorganisms prevail over the less acid-tolerant ones in response to biofilm acidification. [18]

C. albicans has a high acid tolerance and is capable of producing acids even under low pH conditions. Characteristics that may favor the fungus in the microbial shifts associated with dental caries. [19] In fact, the frequency of isolation of *C. albicans* is significantly higher in biofilms associated with carious teeth. [20-24] However, one of the microorganisms most frequently associated with dental caries is the bacteria *Streptococcus mutans* (*S. mutans*). Despite being not alone in this task, *S. mutans* has a high capacity of acid production. In

addition, it has the unique ability of converting sucrose into extracellular insoluble glucans that constitute the main component of the biofilm matrix. The latter acts as a barrier to the buffering effects of saliva. Thus, it can promote the establishment of acidic microenvironments and thereby the formation of selective ecological niches and demineralization sites. [25-27]

There is growing evidence that *C. albicans* can modulate the extracellular matrix formation and also increase the *S. mutans* carriage. [28-30] In addition, *S. mutans* also augments *C. albicans* accumulation in mixed biofilms, suggesting a synergistic cross-kingdom relationship. [31-33] Therefore, a positive association between the presence of *C. albicans* and the development of cariogenic biofilms may exist, especially in severe conditions such as early childhood caries (ECC). [34, 35]

Synergistic cross-kingdom interactions and enhanced dental caries severity are supported by some *in vitro* and *in vivo* studies, providing further evidence of the influence of *C. albicans* on the cariogenic potential of biofilms. [36, 37] Nevertheless, antagonistic effects are also observed in some studies and even caries reduction interactions of *C. albicans* and *S. mutans* are possible. [36, 38] Therefore, data is inconclusive and the cariogenic role of the interaction between bacteria and fungi needs to be further analysed. [39]

This way, the aim of this study was to evaluate the effect of *Candida albicans* on the cariogenic and acidogenic potentials of *Streptococcus mutans* biofilms using an *in vitro* dental enamel model with caries lesions assessment in an effort to better clarify the role played by this interaction on caries development. The null hypothesis was that there were no differences on enamel demineralization nor on acidogenic potential between biofilms.

MATERIALS AND METHODS

Experimental design

Dual-species (MIX; *C. albicans* + *S. mutans; n* = 24) and single-species (*C. albicans; n* = 24 or *S. mutans; n* = 24) biofilms were grown on the surface of sound bovine enamel slabs in the presence of culture medium supplemented with 20 mM glucose and 3 mM sucrose for 24, 48 and 72 hours (n=8 for each biofilm experimental period). The medium was refreshed daily and the pH of the spent medium was recorded. Biofilms were harvested and viable cell counts were performed at the end of each experimental period. Enamel demineralization was evaluated through the determination of percentage of surface hardness change, via hardness analysis, and integrated mineral loss and lesion depth, via transversal microradiography. The experiments were done in triplicate.

Enamel slabs preparation

Enamel slabs were cut from sound bovine anterior teeth, which were previously disinfected in 10% formaldehyde solution [40], using a drill bench (Schulz S.A., Joinville, SC, Brazil) coupled with a ¼ inch diamond grit hole saw (DeWALT, Baltimore, MD, USA). The resulting 6.4 mm diameter enamel slabs were then flattened and polished with a grinding and polishing machine (Arotec S/A, Cotia, SP, Brazil). A 1 mm nail varnish strip was made on the side of each slab to create an unexposed (control) area. Slabs with cracks, scratches, exposed dentin or with mean enamel baseline hardness out of the range from 320 to 360 Vickers microhardness (VMH) were discarded. Baseline hardness determination is described below. Slabs were then randomized and balanced into experimental groups by mean VMH values. Finally, the slabs were mounted on individual nylon cylinders attached to the lid of 24-well plates [41] and were sterilized by hydrogen peroxide.

Inoculum preparation

Candida albicans (ATCC 90028) and *Streptococcus mutans* (UA 159) were cultivated from frozen stocks on Brain Heart Infusion Agar (BHI; Kasvi, São José dos Pinhais, PR, Brazil). Colony-forming units (CFU) were then inoculated into tubes containing Tryptic Soy Broth (TSB; HiMedia, Mumbai, MH, India) supplemented with 12.5 mM sucrose. After 24 h of incubation, aliquots of each suspension were transferred into fresh medium and were further incubated for 24 h at 37 °C. Optical density was adjusted independently for each microorganism to 0.5 ± 0.1 at 550 nm with a spectrophotometer (Milton Roy Co., Ivyland, PA, USA). Dual-species consortium was obtained by combining equal amounts of the density-adjusted cultures. [41]

In vitro biofilm growth

The modified lids with the slabs were put onto 24-well plates with 0.4 mL inoculum (containing approximately 10^7 CFU/mL of each strain) and 1 mL TSB (supplemented with 20 mM glucose and 3 mM sucrose; TSBGS) on each well and kept at 37 ^{o}C. After 8 h of incubation, the slabs were transferred to new plates containing 1.4 mL of TSBGS medium and incubated at 37 °C overnight. Biofilms were then transferred every 24 hours to new 24-well plates containing fresh TSBGS medium and kept at 37 ^{o}C. [41] The pH values of the spent medium (at 8, 24, 48 and 72 h) were individually measured for each well using a pH meter (Digimed, São Paulo, SP, Brazil) previously calibrated with pH 7.0 and pH 4.0 standards.

Biofilm harvesting and counts of viable cells

At the end of each experimental period (after 24, 48 and 72 h of biofilm growth), enamel slabs (n = 8) were aseptically removed from the lids and individually transferred to tubes

containing 1 mL of sterile 0.9% NaCl and 4 sterile glass beads. Tubes were vortexed for 30 s to disperse the biofilms. Aliquots of the microbial suspensions were serially diluted and plated on BHI agar. Plates were incubated at 37 °C under microaerophilic conditions for 24 h. CFU were then counted under a stereomicroscope and the results expressed as CFU/mL. Enamel slabs were gently cleaned and stored in pre-codified vials at 4 ºC under humid environment until further assessment of caries lesion development.

Superficial microhardness

Baseline enamel surface hardness was determined with a microhardness tester (ISH-TDV2000; Insize Co. Ltd, Suzhou, JS, China) by making five Vickers indentations, spaced 100 µm from each other, with a load of 200 gf for 10 seconds. After each experimental period of biofilm growth, five indentations were placed 100 µm at the right side of the baseline indentations following the same parameters described above. The mean Vickers microhardness (VMH) values of baseline (B) and post-biofilm (P) VMH were averaged and the percentage of surface microhardness change (% SMC) was calculated as $\%\,SMC = [(P - B) \div B] \times 100$. [41]

Transversal microradiography (TMR)

After post-biofilm hardness analysis, half of the specimens were sectioned through the center with a low-speed diamond saw (Isomer; Buehler Ltd., Lake Bluff, IL, USA), perpendicularly to the surface and transversally to the varnish strip, in order to obtain slices approximately 150 µm thick representing both exposed and unexposed (sound) enamel surfaces. Slices were then hand polished plane-parallel from both cut sides with 600 and 1200 grit sandpapers to a thickness of approximately 100 µm. Specimens were mounted in a custom-made sample-holder with an aluminium calibration step wedge with 14 steps. Microradiographs were taken with an X-ray generator (Softex Co. Ltd., Ebina, Japan) on a high precision glass plate (Konica Minolta Inc., Tokyo, Japan) at a distance of 42 cm using 20 kV and 20 mA for 13 minutes. Plates were developed for 5 min, rinsed with deionized water and fixed for 8 min in a dark room at 20 °C. All plates were then washed in running water for 10 min and air-dried. Microradiographs were examined using a microscope (Carl Zeiss Microscopy GmbH, Jena, TH, Germany) in conjunction with a camera (Canon, Tokyo, Japan) and a computer running data-acquisition and calculation software programs (Inspektor Research Inc., Amsterdam, NH, Netherlands). The lesion depth (LD) was calculated using a threshold of 95% of the mineral content of sound enamel. Integrated mineral loss (IML; vol% mineral x µm) was also calculated. [42] All analyses were performed by a blinded examiner.

Statistical analysis

The mean and standard deviation of all outcomes (counts of viable cells, pH of spent medium, % SMC, LD and IML) were calculated for each tested condition. The assumptions of homogeneity of variances and normality of the distribution were checked. Counts of viable cells were log transformed to satisfy these assumptions. Two-way analysis of variance (ANOVA) was used to determine the effects and interactions of the tested conditions (biofilm microbial composition and biofilm age) on response variables. Bonferroni adjusted comparisons were used when two-way ANOVA indicated statistically significant effects. Correlation between pH and counts of fungal viable cells on dual-species biofilms was tested using Pearson's coefficient, while correlation between % SMC and transversal microradiography outcomes was tested with Spearman's rank coefficient. All analyses were performed on SPSS Statistics for Windows, version 22.0 (IBM Corp. in Armonk, NY, USA) with a significance level set as 1%.

RESULTS

An interaction effect between biofilm microbial composition and biofilm age was found for the total counts of viable cells, a variable that takes into account both fungal and bacterial CFU. Statistically higher numbers of CFU were found on *S. mutans* and dual-species biofilms in relation to *C. albicans* biofilms at each time point. In addition, *C. albicans* single-species biofilms presented a slight increase in counts of viable cells (expressed as Log CFU/mL) from 24 to 72 hours, whereas no difference on counts of total viable cells were found overtime for *S. mutans* single-species and dual-species biofilms (Table 1). When counts of viable fungal cells are compared between single-species and dual-species biofilms, isolated effects of biofilm microbial composition and biofilm age were found. This way, counts of viable fungal cells increased overtime and they were statistically lower in the presence of dual-species biofilms. For counts of viable *S. mutans* cells, no difference was found among the tested conditions (Table 1).

Statistically significant interaction effects between biofilm microbial composition and biofilm age were also found for the following response variables: spent medium pH, percentage of surface microhardness change (% SMC), integrated mineral loss (IML) and lesion depth (LD). Spent medium pH related to *S. mutans* biofilms was significantly lower than for other biofilms at each time point and remained constant overtime while *C. albicans* biofilms presented the highest pH values among the different biofilms (from 6.5 to 7.8), which increased slightly with time. The pH related to dual-species biofilms increased significantly overtime being statistically different at each time point. (Table 1). Figure 1 represents the mean of pH

according to experimental conditions. A moderate correlation between fungi CFU and spent medium pH ($r = 0.559$) was found in dual-species biofilms (Figure 2).

In relation to carious lesion development, higher % SMC was found in the presence of *S. mutans* single-species biofilms than for other biofilms at each time point. In addition, *S. mutans* related demineralization increased with time, being statistically different among the different time points. On the other hand, carious lesion development in the presence of *C. albicans* single-species biofilms was statistically lower than in the presence of other biofilms and it remained constant overtime. Additionally, the % SMC in the presence of dual-species biofilms at 48 h of biofilm formation was statistically higher than at 24 h, but it was not statistically different compared to enamel demineralization after 72 h of biofilm growth (Table 2).

At 24 h of biofilm growth, no statistical difference was found on IML and LD among the different biofilms, but enamel slabs exposed to *S. mutans* single-species biofilms presented IML and LD higher than slabs exposed to *C. albicans* single-species biofilms at 48 and 72 h and higher than dual-species biofilms at 72 h of biofilm growth. Furthermore, IML of enamel slabs exposed to *S. mutans* biofilms increased overtime, being statistically different at each time point, whereas LD at 72 h was statistically higher than that found at 24 h of biofilm growth. Moreover, IML of enamel slabs exposed to *C. albicans* and to dual-species biofilms remained constant overtime. This same behaviour was observed in relation to LD (Table 2).

Figure 3 shows representative transversal microradiographs of enamel slabs according to the experimental conditions where it can be easily seen that all carious lesions were sub-superficial. Yet, Figure 4 shows the average mineral profile of enamel slabs according to the experimental conditions where it can be seen that lesions formed in the presence of *S. mutans* biofilms were deeper and had lower mineral content compared to the other biofilms. Overall, a positive correlation was found between % SMC and IML ($\rho = 0.808$) as well as between % SMC and LD ($\rho = 0.760$) (Figure 5).

Table 1 – Viable cell counts and pH (mean ± SD) according to biofilm age and biofilm microbial composition.

Variable	Biofilm age	Biofilm microbial composition			Total
		C. albicans (n = 24)	**MIX** (n = 24)	***S. mutans*** (n = 24)	
Total (Log CFU/mL)	**24h**	6.5 ± 0.3 **A a**	7.8 ± 0.3 **A b**	8.0 ± 0.3 **A b**	7.4 ± 0.8
	48h	6.8 ± 0.5 **AB a**	7.7 ± 0.5 **A b**	7.8 ± 0.6 **A b**	7.4 ± 0.7
	72h	7.0 ± 0.6 **B a**	7.9 ± 0.4 **A b**	7.8 ± 0.5 **A b**	7.6 ± 0.6
p1 = 0.202 p2 < 0.001 p3 = 0.009	**Total**	6.7 ± 0.5	7.8 ± 0.4	7.9 ± 0.5	
Fungi (Log CFU/mL)	**24h**	6.5 ± 0.3	6.3 ± 0.4	-	6.4 ± 0.4 **A**
	48h	6.8 ± 0.5	6.6 ± 0.3	-	6.6 ± 0.4 **B**
	72h	7.0 ± 0.6	6.8 ± 0.3	-	6.9 ± 0.5 **C**
p1 < 0.001 p2 = 0.003 p3 = 0.734	**Total**	6.7 ± 0.5 **a** (n = 72)	6.5 ± 0.4 **b** (n = 72)		(n = 48)
Bacteria (Log CFU/mL)	**24h**	-	7.8 ± 0.4	8.0 ± 0.3	7.9 ± 0.3
	48h	-	7.7 ± 0.6	7.8 ± 0.6	7.7 ± 0.6
	72h	-	7.8 ± 0.5	7.8 ± 0.5	7.8 ± 0.5
p1 = 0.177 p2 = 0.098 p3 = 0.794	**Total**		7.7 ± 0.5	7.9 ± 0.5	
pH	**24h**	6.9 ± 0.3 **A a**	4.7 ± 0.2 **A b**	4.4 ± 0.1 **A c**	5.3 ± 1.1
	48h	7.2 ± 0.2 **B a**	5.1 ± 0.2 **B b**	4.4 ± 0.1 **A c**	5.6 ± 1.2
	72h	7.5 ± 0.2 **C a**	6.1 ± 0.6 **C b**	4.5 ± 0.1 **A c**	6.0 ± 1.3
p1 < 0.001 p2 < 0.001 p3 < 0.001	**Total**	7.2 ± 0.2	5.3 ± 0.7	4.4 ± 0.1	

Different uppercase letters show a significant difference between biofilm age and different lowercase letters show a significant difference between biofilm microbial composition by two-way ANOVA followed by Bonferroni test ($p < 0.01$). p1: biofilm age; p2: biofilm microbial composition; p3: biofilm age x biofilm microbial composition.

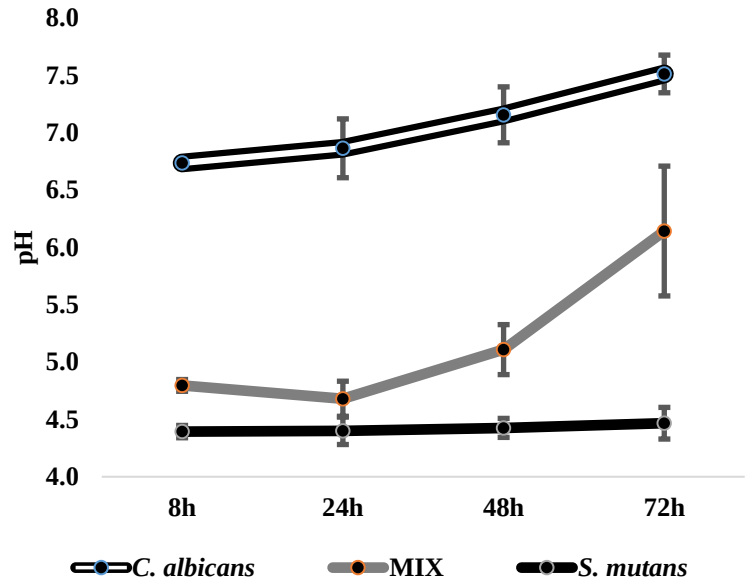

Fig. 1. pH of spent medium according to experimental periods of biofilm growth. Values are mean ± SD; *n* = 24 for each tested condition.

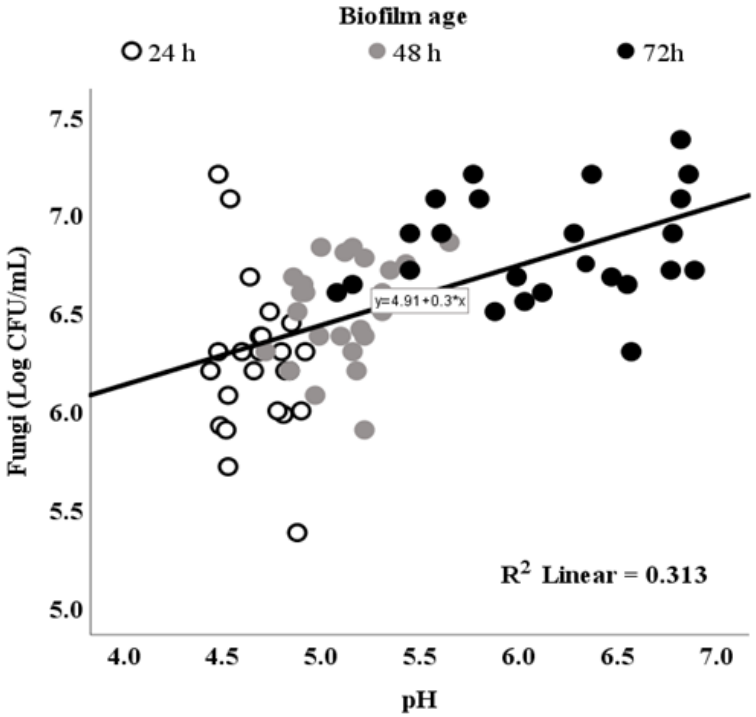

Fig. 2. Scatterplot showing moderate Pearson linear correlation between spent medium pH (r = 0.559; p < 0.01) and counts of fungi viable cells (Log CFU/mL). Note that biofilm age is displayed by color-coded dots. Only dual-species biofilm cases were selected (*n* = 72).

Table 2 – percentage of Surface Microhardness Change (% SMC), Integrated Mineral Loss (IML) and Lesion Depth (LD) (mean ± SD) according to biofilm age and biofilm microbial composition.

Variable	Biofilm age	Biofilm microbial composition			Total
		C. albicans (n = 24)	**MIX** (n = 24)	***S. mutans*** (n = 24)	
% SMC	**24h**	7.3 ± 3.3 **A a**	35.9 ± 9.1 **A b**	47.0 ± 7.3 **A c**	30.1 ± 18.2
	48h	7.1 ± 6.4 **A a**	47.2 ± 9.5 **B b**	66.3 ± 8.3 **B c**	40.2 ± 26.1
	72h	6.6 ± 3.6 **A a**	47.6 ± 9.5 **B b**	75.4 ± 3.9 **C c**	43.2 ± 29.1
p1 < 0.001 p2 < 0.001 p3 < 0.001	**Total**	7.0 ± 4.6	43.6 ± 10.7	62.9 ± 13.7	
IML (Vol% x µm)	**24h**	211.4 ± 60.9 **A a** (n = 7)	310.0 ± 140.8 **A a** (n = 7)	391.7 ± 146.5 **A a** (n = 6)	300.0 ± 136.6
	48h	276.7 ± 155.1 **A a** (n = 9)	667.5 ± 182.7 **A ab** (n = 8)	1172.9 ± 478.3 **B b** (n = 7)	668.3 ± 464.5
	72h	238.6 ± 130.6 **A a** (n = 7)	596.3 ± 199.0 **A a** (n = 8)	2025.6 ± 862.9 **C b** (n = 9)	1027.9 ± 958.8
p1 < 0.001 p2 < 0.001 p3 < 0.001	**Total**	245.2 ± 123.3	533.9 ± 229.1	1308.6 ± 906.0	
LD (µm)	**24h**	11.4 ± 6.0 **A a** (n = 7)	15.1 ± 5.7 **A a** (n = 7)	19.6 ± 7.2 **A a** (n = 6)	15.2 ± 6.8
	48h	18.9 ± 16.8 **A a** (n = 9)	31.9 ± 9.5 **A ab** (n = 8)	67.9 ± 60.2 **AB b** (n = 7)	37.6 ± 38.7
	72h	13.3 ± 7.7 **A a** (n = 7)	32.0 ± 11.8 **A a** (n = 8)	103.0 ± 56.7 **B b** (n = 9)	53.2 ± 52.8
p1 = 0.001 p2 < 0.001 p3 = 0.008	**Total**	14.9 ± 11.8	26.8 ± 12.0	69.1 ± 58.9	

Different uppercase letters show a significant difference between biofilm age and different lowercase letters show a significant difference between biofilm microbial composition by two-way ANOVA followed by Bonferroni test ($p < 0.01$). p1: biofilm age; p2: biofilm microbial composition; p3: biofilm age x biofilm microbial composition.

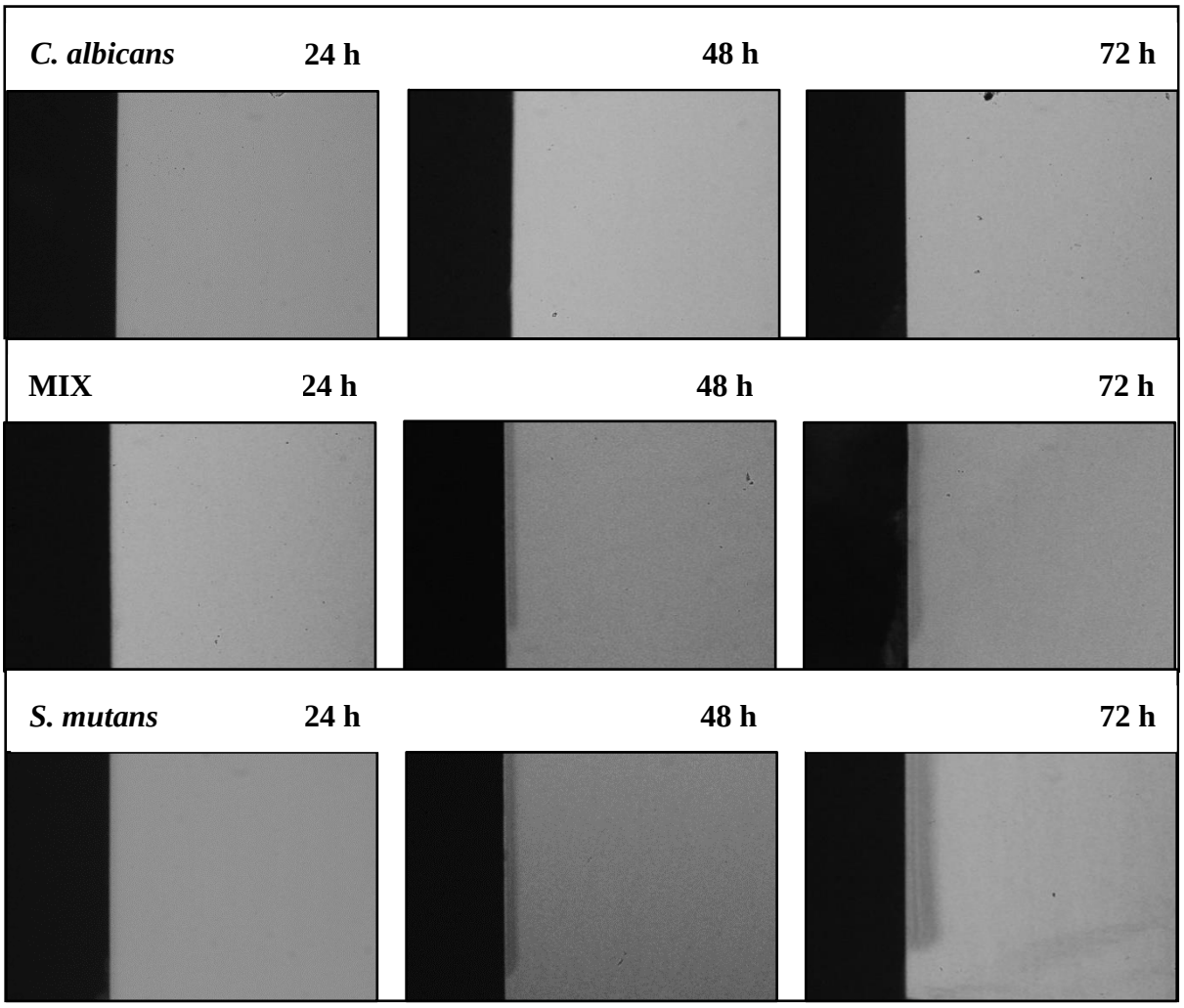

Fig. 3. Representative microradiographs after 24, 48 and 72 h of biofilm growth.

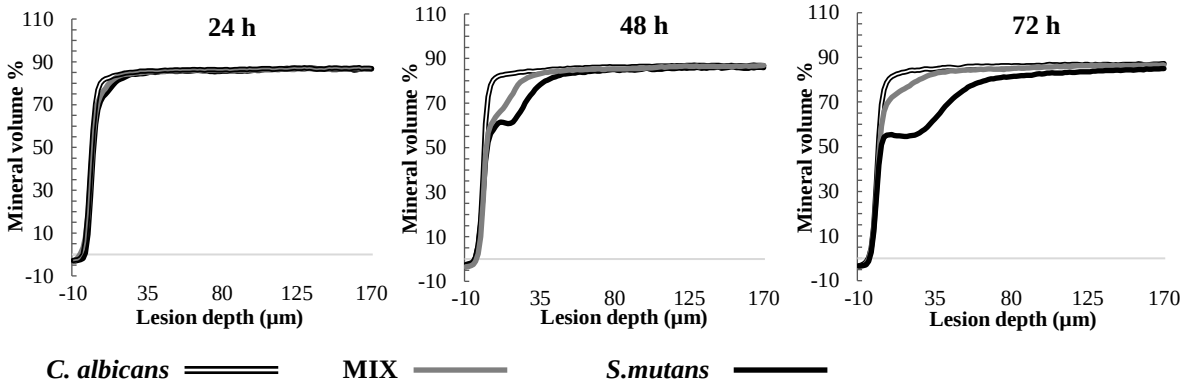

Fig. 4. Mineral profile of enamel slabs collected after 24, 48 and 72 hours of biofilm growth. Different coloured lines represent biofilm microbial composition. Lines based on mean values.

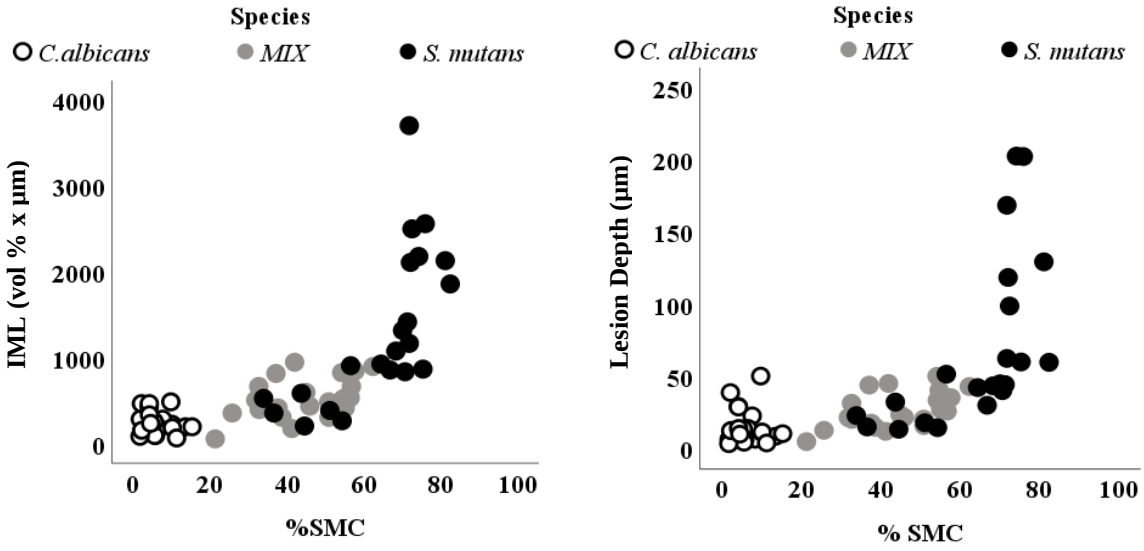

Fig. 5. Scatterplots showing high Spearman correlation between % SMC and IML ($\rho = 0.808$; $p < 0.01$) and between % SMC and LD ($\rho = 0.760$; $p < 0.01$). Note that biofilm species are displayed by color-coded dots. Only slabs where lesions were assessed via TMR were selected for this correlation analysis ($n = 68$).

DISCUSSION

The direct comparison of CFU numbers between bacteria and fungi is biologically fragile having in mind that their cellular size is different, being the fungal biomass far higher. [19] Even so, CFU counts were performed in the present study. Interestingly, the association of *C. albicans* and *S. mutans* in dual-species biofilms had no positive effect on counts of viable cells, but growth was not suppressed either (Table 1). This was not the case of other *in vitro* and *in vivo* studies that showed increased counts of microbial cells by the co-association of these microorganisms. [32, 36, 37]

The spent medium pH analysis showed that *S. mutans* can efficiently use the carbohydrates present in culture medium and metabolize them into acids. In addition, this well-known cariogenic bacterium can produce exoenzymes, known as glucosyltransferases (Gtfs), that can synthesize polysaccharides from sucrose, an essential factor associated to the cariogenic potential of biofilms. [25, 27, 43] So, as expected, enamel slabs exposed to *S. mutans* single-species biofilms presented an increased mineral loss over time, taking both superficial microhardness and transversal microradiography outcomes into consideration (Table 2, Figures 3 and 4). In fact, these variables were highly correlated (Fig. 5). It is important to discuss though that some outliers could be identified in the analysis of microradiography, leading to heterogeneity of variances. However, we decided not to exclude these outliers having in mind that the results of the analysis would not differ statistically.

Despite the fact that *C. albicans* is able to secrete a variety of acids, such as pyruvate and acetate, as a result from the catabolism of glucose, this fungus has a more complex acidogenicity profile than lactic acid-producing bacteria. [19] Similar to the findings of another study [38], almost no spent medium acidification was found for *C. albicans* single-species biofilms, even with the presence of glucose in culture medium. Indeed, the chemical characterization of *C. albicans* single-species related spent medium, as shown in another study, revealed minimum carbohydrate utilization and a poor ability of the fungus in metabolizing sucrose when growing alone. [33]

Regarding cariogenic potential of *C. albicans*, an *in vitro* study showed that this fungus can adhere to hydroxyapatite surfaces through electrostatic interactions and cause mineral dissolution at high rates. [44] Also in root dentin, *C. albicans* is able to release calcium after 4 days of *in vitro* growth. [45] Other *in vitro* studies showed that biofilms of *C. albicans* strains isolated from HIV+ children can cause enamel demineralization after 5 days of biofilm growth. [46, 47] In addition, studies in rats, showed that *C. albicans*-infected animals developed caries lesions. [36, 37] In the present study however, no acidification was found and consequently, enamel slabs exposed to *C. albicans* single-species biofilms showed very little demineralization (Table 2, Figures 3 and 4). This could be attributed to the differences on virulence of strains used in different studies, in addition to different biofilm growing conditions and/or experimental conditions. Nonetheless, it seems that the cariogenic potential of *C. albicans* in relation to enamel is very low.

Carbon source has a direct influence on biofilm morphogenesis. [48-50] It is important to discuss that, when *C. albicans* and *S. mutans* are growing together in mixed biofilms, they may compete for fermentable sugars. Therefore, enhanced sugar metabolism of dual-species biofilms can result in lower carbohydrate concentrations in the spent medium and that can promote adaptations in catabolic pathways of these microorganisms. [32, 51]

Under carbohydrate privation, *C. albicans* can use a variety of other carbon sources, such as amino acids, fatty acids and carboxylic acids. [52] Breakdown of exogenous amino acids can lead to extrusion of ammonia, a highly basic compound. As a consequence, in a glucose-limited milieu, *C. albicans* can actively modulate extracellular pH by alkalinisation of acidic environments. [53] Another distinct mechanism of extracellular alkalinisation takes place when *C. albicans* consumes carboxylic acids, such as pyruvate and lactate, as a carbon source. Here, there is no ammonia release, but neutralization of acidic environments rapidly occurs, maybe by the consumption of organic acids itself. [52]

No metabolic tests to verify ammonia extrusion nor consumption profile of carbohydrates or carboxylic acids were run. However, pH of the spent medium arose from 4.6 at 24 h of biofilm growth to 6.1 at 72 h of dual-species biofilm growth (Fig. 1). Whether this increase on pH reflects a consumption of acidic compounds or the production of buffer-related compounds by *C. albicans* is not possible to be answered by the experimental conditions of the present study. One could argue though that the increase on pH would be the result of a reduction on viable cells counts overtime. It is important to emphasize that, although the statistical analysis had indicated some differences on counts of viable cells among the tested conditions, they do not present any relevance since differences were too small. This way, considering that the viability of fungal and bacterial cells is not reduced overtime (Table 1), the change on pH could be directly attributed to changes on biofilm metabolism possibly induced by *C. albicans*.

It is well known that pH has a high importance in the physiology of microorganisms. Indeed, morphogenesis of *C. albicans* responds to the external pH, where neutral environments favour filamentation while acidic conditions favour yeast-form growth. So, pH may influence the virulence of this fungus, at least in relation to soft tissue invasion, which is normally related to hyphal formation. [54] The fact that *C. albicans* can effectively neutralize acidic medium and shift to the hyphal form may be an example of its adaptation to survive in the host. [52, 53] This autoinduction mechanism may explain the correlation that we found between fungal CFU and spent medium pH, especially in dual-species biofilms.

These findings corroborate to the results of other *in vitro* study, where lactic acid production and growth of *S. mutans* were not supressed, but pH was modulated by *C. albicans* resulting in values above critical pH for enamel demineralization after 72 hours of mixed biofilm growth. [38] In addition, these findings explain the demineralization results for dual-species biofilms, where enamel slabs showed an initial caries lesion development, but no significant mineral loss could be observed after 48 hours (Fig. 3).

Regarding cariogenic potential of the association between *C. albicans* and *S. mutans*, data is not conclusive. Under some experimental conditions, downregulation of bacterial exoenzymes can occur in the presence of *C. albicans* or dual-species spent medium, leading to lack of extracellular polymeric substances (EPS) production by *S. mutans*. [32] Interestingly, the same *in vivo* study that showed an association of *C. albicans* with advanced occlusal caries lesions, showed no difference from the controls in the number of advanced lesions when rats were coinfected with both *C. albicans* and *S. mutans*. [36]

On the other hand, that was not the case of other *in vivo* study, where coinfected-animals developed much more severe lesions than animals infected with only one type of

microorganism. [37] This can be due to a complex synergistic cross-kingdom interaction that may lead to a richer biofilm matrix and enhanced microcolony formation. Its mechanism relies on the production of glucans by bacterial exoenzymes (especially glucosyltransferase B; GtfB) that are mainly adhered to mannans on the outer layer of the fungal cell-wall. [30] Furthermore, quorum sensing molecules produced by *C. albicans*, such as farnesol, can stimulate the GtfB activity when at low concentrations (25-50µL), but can also inhibit *S. mutans* growth at higher concentrations (>100µL). [33]

The results of the present study support the results of Willems *et al.*, 2016, demonstrating through analysis of surface microhardness and transversal microradiography of dental enamel, that the association of these microorganisms can have a reduction effect on cariogenic potential when compared to *S. mutans* single-species biofilms. In addition, no synergistic cross-kingdom interactions that may explain findings of other studies on the augmented cariogenic potential of the association between *C. albicans* and *S. mutans* were found. However, these results must be interpreted with care taking all the limitations *in vitro* studies into consideration.

The present study supports the idea that *C. albicans* has a low enamel demineralization potential, but the association of this common fungus with oral bacteria, such as *S. mutans*, can enhance its cariogenic potential. However, after 48 hours of biofilm growth, *C. albicans* can actively modulate extracellular pH, promoting alkalinisation of the environment and thus, reducing the cariogenic potential of dual-species biofilms, when compared to *S. mutans* single-species biofilms. However, it is important to discuss that it could not be valid for advanced caries lesions or in a root caries scenario, because the critical pH for dentin is higher than for dental enamel. [55]

Finally, it is clear that the role of oral fungi in dental caries is complex and not unidimensional. In fact, *C. albicans* is much more than a commensal fungus that in some cases can invade human tissues. It is a fascinating microorganism that has a bouquet of survival strategies in the host and can interact with other members of the oral microbiome in very surprising ways.

CONCLUSION

The data of the present study suggests that the presence of *C. albicans* can reduce both the acidogenic and cariogenic potentials of *S. mutans* biofilms. Further studies are necessary to investigate the role of acidic conditions on fungal metabolism and its consequence in relation to the cariogenic potential of biofilms formed by *C. albicans*.

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3 CONSIDERAÇÕES FINAIS

O objetivo do presente estudo foi avaliar o papel do fungo *C. albicans* em biofilmes de espécie única e em biofilmes de dupla-espécie, em co-associação com a bactéria *S. mutans*, na desmineralização do esmalte e no potencial acidogênico de biofilmes *in vitro.* Mesmo com as limitações inerentes aos estudos *in vitro,* foram encontradas diferenças significativas entre os diferentes tipos de biofilmes em relação às variáveis estudadas, como pH, contagem de células viáveis, porcentagem de alteração de microdureza superficial (% SMC), perda mineral integrada (IML) e profundidade de lesão (LD).

Nossos resultados suportam os achados de outro estudo recente onde biofilmes *in vitro* de *C. albicans* e *S. mutans* demonstram diferenças claras no potencial de produção de ácido lático, liberação de cálcio e alteração do pH quando comparados a biofilmes de uma única espécie. Surpreendentemente, a presença do fungo nesse consórcio pareceu reduzir o potencial cariogênico dos biofilmes, apesar do fato de que a produção de ácidos e o crescimento bacteriano não terem sido inibidos (Willems *et al.*, 2016).

Em suma, existe evidência experimental de que fungos como *C. albicans* podem apresentar potencial cariogênico (Klinke *et al.*, 2011; Charone *et al.*, 2013; Brighenti *et al.*, 2014; Falsetta *et al.*, 2014; Szabó *et al.*, 2014; Caroline De Abreu Brandi *et al.*, 2016; Charone *et al.*, 2017). Entretanto, a placa dentária não se torna necessariamente mais cariogênica pela sua simples presença. Interações entre fungos e bactérias, de acordo com os resultados de nosso estudo e de outros estudos, podem até mesmo levar a uma redução no potencial cariogênico de biofilmes (Klinke *et al.*, 2011; Willems *et al.*, 2016).

Em conclusão, a relação das doenças orais mediadas por biofilme com os fungos e outros “coabitantes” do ecossistema bucal, como vírus, protozoários e *archae*, merece ser mais profundamente estudada (Rosier *et al.*, 2014). Porém, não podemos nos esquecer de estudar as relações desses componentes “esquecidos” do microbioma também com a manutenção da saúde oral (Buchen, 2010; Krom *et al.*, 2014). Afinal, apesar de invisível a olho nu, o microbioma está diretamente relacionado com o processo de saúde e doença. Sendo a integridade do ecossistema bucal vital na manutenção tanto da saúde oral quanto da saúde geral do organismo humano (Zarco *et al.*, 2012).

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