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FACULDADE DE ODONTOLOGIA DE PIRACICABA

LOYSE MARTORANO FERNANDES

MECANISMOS DE INTERAÇÃO EM BIOFILMES ORAIS FÚNGICO-BACTERIANOS

MECHANISMS OF INTERACTION IN FUNGAL-BACTERIAL ORAL BIOFILMS

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

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Clínica Odontológica, in Prótese Dental area.

Orientadora: Profª Drª Altair A. Del Bel Cury

Este exemplar corresponde à versão final da tese defendida pela aluna Loyse Martorano Fernandes e orientada pela Profª Drª Altair A. Del Bel Cury.

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RESUMO

Candida albicans é um fungo oportunista mais prevalente da cavidade oral e que causa infecções biofilme-dependentes, a exemplo da Candidíase oral. Durante a formação do biofilme, os microrganismos interagem entre si por meio de adesão célula a célula ou a liberação extracelular de subprodutos (*quorum-sensing*). Sabe-se que *C. albicans* estabelece relações sinérgicas com bactérias orais, como *Streptococcus oralis* e *Streptococcus mutans*, o que aumenta a virulência e patogenicidade de *C. albicans*. Diante disso, estudos têm buscado elucidar os mecanismos de interação dos microrganismos do biofilme oral a fim de revelar potenciais alvos terapêuticos. Isto posto, esta Tese de Doutorado objetivou elucidar os mecanismos que medeiam as interações entre *C. albicans* e *S. oralis*, e *C. albicans* e *S. mutans*, já que são bactérias reconhecidas por aumentarem a virulência e patogenicidade de biofilmes de *C. albicans*. Para isso, dois estudos foram desenvolvidos. O primeiro estudo avaliou a influência de subprodutos metabólicos presentes no sobrenadante de *S. oralis* no desenvolvimento e virulência de biofilmes de *C. albicans*. Para tanto, biofilmes de *C. albicans*, *C. albicans* e *S. oralis* ou *C. albicans* e subprodutos de *S. oralis* foram desenvolvidos sobre superfícies acrílicas e avaliados quanto a atividade metabólica (redução do sal de XTT), biomassa, contagem de unidades formadoras de colônia (UFC) e formação de hifas. Como resultados, os biofilmes de *C. albicans* formados na presença dos subprodutos de *S. oralis* apresentaram maior metabolismo celular do que os demais biofilmes ($p < 0,05$), mas não foram capazes de modificar a biomassa e o número de UFC ($p > 0,05$). Adicionalmente os subprodutos de *S. oralis* aumentaram a formação de hifas de *C. albicans* quando comparado à biofilmes de *C. albicans*.. O segundo estudo, avaliou o papel das adesinas fúngicas Als1, Als3 e Hwp1 na formação de biofilmes de *C. albicans* e *S. mutans*. Para tanto, testou-se a capacidade de cepas mutantes (*als1*Δ/Δ, *als3*Δ/Δ, *als1*Δ/Δ / *als3*Δ/Δ, *hwp1*Δ/Δ) e selvagem de *C. albicans* formarem biofilmes uni e duo espécie. Os biofilmes desenvolvidos foram analisados quanto densidade óptica, metabolismo, biomassa, enumeração celular, espessura e arquitetura. Coletivamente, os resultados dessas análises apontaram que a ausência da adesina Als1 impactou negativamente a formação de biofilmes duais ($p < 0,05$). Além disso, a ausência de Hwp1 em biofilmes de *C. albicans* e *S. mutans* afetou a interação de sinergismo com a bactéria ($p < 0,05$). Por fim, a ausência simultânea de Als1 e Als3 comprometeu substancialmente à formação de biofilmes duais ($p < 0,05$). Diante dos achados dos dois estudos, conclui-se que os subprodutos de *S.*

oralis (*quorum-sensing*) contribuem para o desenvolvimento e virulência de biofilmes de *C. albicans*, e que a formação de biofilmes de *C. albicans* e *S. mutans* é mediada por Als1, Als3 e Hwp1.

Palavras-chave: Biofilmes. Candidíase Bucal. Candida albicans. Streptococcus oralis. Streptococcus mutans.

ABSTRACT

Candida albicans is an opportunistic fungus that is most prevalent in the oral cavity and causes biofilm-dependent infections, such as oral Candidiasis. During biofilm formation, microorganisms interact with each other through cell-to-cell adhesion or the extracellular release of subproducts (*quorum-sensing*). This fungus is known to establish synergistic relationships with oral bacteria, such as *Streptococcus oralis* and *Streptococcus mutans*, which increases the virulence and pathogenicity of *C. albicans*. Thus, studies have sought to elucidate the interaction mechanisms of oral biofilm microorganisms to reveal potential therapeutic targets. Therefore, this Ph.D. Dissertation aimed to elucidate the mechanisms that mediate the interactions between *C. albicans* and *S. oralis*, and *C. albicans* and *S. mutans*. In order to solve this research gap, two studies were perfomed. The first one evaluated the influence of *S. oralis* metabolic subproducts presente in the supernatant on the development and virulence of *C. albicans* biofilms. For that, biofilms of *C. albicans*, *C. albicans* and *S. oralis* or *C. albicans* and subproducts of *S. oralis* were developed on acrylic surfaces. Then, the biofilms were evaluated for metabolic activity, biomass, colony forming units (CFU) counting, and hyphae formation. As a result, *C. albicans* biofilms formed in the presence of *S. oralis* subproducts showed higher cellular metabolism than the other biofilms ($p < 0.05$). However, they could not modify the biomass and CFU ennumeration ($p > 0.05$). Additionally, *S. oralis* subproducts increased fungal hyphae formation in *C. albicans* biofilms compared to *C. albicans*. The second study evaluated the role of fungal adhesins Als1, Als3, and Hwp1 in the formation of biofilms of *C. albicans* and *S. mutans*. Therefore, the ability of mutant strains (*als1*Δ/Δ, *als3*Δ/Δ, *als1*Δ/Δ / *als3*Δ/Δ, *hwp1*Δ/Δ) and wild type of *C. albicans* to form single and dual-species biofilms was tested. Biofilms were analyzed for optical density, metabolism, biomass, cell enumeration, thickness, and architecture. Collectively, the results of these analyzes showed that the absence of Als1 adhesin negatively impacted the formation of dual biofilms ($p < 0.05$). Furthermore, the lack of Hwp1 in *C. albicans* and *S. mutans* biofilms impairs the synergistic interaction with the bacteria ($p < 0.05$). Lastly, the redundant absence of Als1 and Als3 significantly compromised the biofilm formation in dual biofilms ($p < 0.05$). In summary, *S. oralis* subproducts (*quorum-sensing*) contribute to the development and virulence of *C. albicans* biofilms, and the formation of *C. albicans* and *S. mutans* biofilms is mediated by Als1, Als3, and Hwp1.

Keywords: Biofilms. Candidiasis, Oral. Candida albicans. Streptococcus oralis. Streptococcus mutans.

SUMÁRIO

1 INTRODUÇÃO

O microbioma oral compreende uma população de, aproximadamente, 700 espécies de microrganismos organizados em uma estrutura dinâmica denominada de biofilme (Larsen & Fiehn, 2010). Os biofilmes, por sua vez, são comunidades de bactérias e fungos agregados entre si, formando um arcabouço estrutural que pode se aderir a superfícies bióticas, a exemplo do epitélio oral, ou a superfícies abióticas, como próteses dentárias (Nobile & Johnson, 2015). Essa comunidade de microrganismos está imersa em uma matriz de substâncias poliméricas, formando um microambiente que protege o biofilme contra a ação de agentes externos que possam rompê-lo, como antimicrobianos ou o sistema imune (Gulati & Nobile, 2016).

Tipicamente, a formação do biofilme é um processo dinâmico, que compreende quatro fases: i) adesão; ii) proliferação; iii) maturação; iv) dispersão (Gulati & Nobile, 2016). Durante a fase de adesão, células livres planctônicas se aderem a uma superfície (como, por exemplo, dentes ou mucosa) revestida por uma película de saliva (Hall-Stoodley et al., 2014). Algumas bactérias conhecidas como colonizadores iniciais (*Streptococcus gordonii*; *Streptococcus mitis*; *Streptococcus oralis*; *Streptococcus sanguinis*) se aderem, inicialmente, a receptores presentes na saliva, como a mucina, proteína rica em prolina e α – amilase (O'Sullivan et al., 2010; Kolenbrander et al., 2010). Esse processo de adesão inicial é mediado por adesinas da parede celular dos microrganismos constituindo, assim, uma camada basal de células que irão ancorar outras espécies e estabelecer interações poli microbianas (Kolenbrander & London, 1993; Kolenbrander et al., 2010).

Posteriormente, as células iniciam a fase de proliferação, cujas interações serão mais frequentes, fazendo com que as células se multipliquem e iniciem a produção de matriz extracelular (Gulati & Nobile, 2016). Em seguida, na terceira fase, o biofilme inicia a sua maturação e atinge uma estrutura tridimensional robusta capaz de o proteger, com diferentes gradientes de pH e disponibilidade de nutrientes (Bjarnsholt et al., 2013; Gulati & Nobile, 2016). Nesse microambiente, os microrganismos encontram condições ideais para interagirem para cooperarem (sinergismo) ou competirem (antagonismo) por nutrientes (Berne et al., 2018). Além disso, essas condições também possibilitam ao biofilme maduro ser patogênico e, assim, causar infecções. Por fim, no último estágio, os microrganismos se dispersam do biofilme como células planctônicas para colonizarem novos nichos, ou avançarem com a infecção (Peleg et al., 2010).

Dentre os microrganismos presentes no biofilme oral, *Candida albicans* é um fungo oportunista prevalente. Esse fungo é considerado um microrganismo comensal da microbiota oral e, normalmente, não causa danos ao hospedeiro (Mayer et al., 2013). Contudo, quando o hospedeiro passa por situações de desequilíbrio, a exemplo da imunossupressão, *C. albicans* invade o epitélio que reveste a mucosa oral para causar infecções (Mayer et al., 2013; Nobile & Johnson, 2015). Esse processo de invasão tecidual pode ocorrer por penetração ativa, por meio do rompimento das junções epiteliais, ou por internalização (endocitose) (Dalle et al., 2010). No processo de internalização, adesinas presentes na parede do fungo podem induzir a endocitose de *C. albicans* (Dalle et al., 2010; Naglik et al., 2011). Dessa maneira, as adesinas são relevantes não apenas para mediar as interações entre microrganismos, como citado anteriormente, mas para promover a patogenicidade do fungo.

Por se tratar de um fungo comensal oportunista, uma importante característica de *C. albicans* é a habilidade de possuir diferentes morfologia, compreendendo as formas de levedura, pseudohifa e hifas (Legrand et al., 2019). Em geral, leveduras estão envolvidas na fase de adesão do biofilme e não provocam infecções. Já as morfologias de pseudohifa e hifa estão presentes na proliferação e em biofilmes maduros. Nesses estágios, inúmeras hifas estão interconectadas, envoltas por matriz extracelular, formando uma rede tridimensional robusta (Calderone & Fonzi, 2001; Legrand et al., 2019). Além disso, a forma de hifa é a morfologia capaz de provocar doenças no hospedeiro uma vez que, apenas nesse estágio, *C. albicans* pode penetrar o epitélio oral, ou induzir endocitose (Mayer et al., 2013).

A respeito das infecções causadas por biofilme de *C. albicans*, a Candidíase oral é uma doença caracterizada clinicamente por lesões esbranquiçadas ou avermelhadas na mucosa oral. Essa doença é prevalente em idosos e crianças, indivíduos imunocomprometidos, e usuários de próteses dentárias (Millsop & Fazel, 2016). Embora os sintomas da Candidíase oral normalmente sejam amenos, essa doença é considerada um problema grave de saúde devido a infecções recorrentes ao longo da vida, podendo levar o indivíduo a comprometimentos sistêmicos e, até mesmo, à morte (Millsop & Fazel, 2016).

Apesar de *C. albicans* ser o agente etiológico primário da Candidíase oral (Mayer et al., 2013), nos últimos anos foi reconhecido que algumas bactérias orais do gênero *Streptococcus* spp. podem interagir com *C. albicans* para estabelecerem relações de sinergismo, contribuindo para a patogenicidade da doença (Xu et al., 2014a). Sabe-se

que essa parceria entre *C. albicans* e bactérias estreptocócicas aumenta a severidade e a frequência de lesões orais de Candidíase (Xu et al., 2014b). Portanto, o sinergismo fúngico-bacteriano pode desafiar terapias para o tratamento dessa doença, uma vez que o alvo desses antimicrobianos é apenas o fungo.

Adicionalmente, ao longo das décadas, as terapias para Candidíase oral têm sido pouco eficazes já que, além de não considerarem o perfil poli microbiano da infecção, não possuem um alvo terapêutico (Xu et al., 2014a; Bernard et al., 2020; Perlin et al., 2020). Agentes antimicrobianos como a Nistatina, o Fluconazol, e o Miconazol atuam promovendo a morte celular do fungo (Milsop et al., 2016), sendo, portanto, de ação inespecífica. Fundamentalmente, um alvo terapêutico possibilita a atuação das terapias sobre um componente celular do microrganismo que participa de processos chaves de seu metabolismo, assim, a ausência/inativação desse componente celular pode interromper mecanismos de virulência e patogenicidade do microrganismo (Gozalbo et al., 2014). Diante do exposto, um possível alvo terapêutico fúngico são as adesinas, pois constituem proteínas presentes na parede celular de *C. albicans* que medeiam as relações de sinergismo com bactérias estreptocócicas, e contribuem no processo de invasão tecidual (Gozalbo et al., 2014).

Além das adesinas, outro mecanismo que microrganismos utilizam para interagirem e, consequentemente, aumentarem sua virulência e patogenicidade, é a liberação extracelular de moléculas e subprodutos (Salvatori et al., 2020). Essas moléculas promovem uma espécie de comunicação celular entre microrganismos denominada de *quorum-sensing*, que sinalizará quimicamente a regulação de atividades como interação com outros microrganismos, virulência, patogenicidade e competência, (Mukherjee & Bassler, 2019).

Isto posto, pesquisas atuais estão concentradas na compreensão acerca dos mecanismos de interação fúngico-bacteriano, uma vez que esse conhecimento poderá revelar possíveis alvos terapêuticos para o tratamento da Candidíase oral. Sabe-se que *C. albicans* estabelece relações de sinergismo com algumas bactérias estreptocócicas (Falsetta et al., 2014; Xu et al., 2014b; Cavalcanti et al., 2015; Montelongo-Jauregui et al., 2018; Souza et al., 2020). Dentre essas bactérias, *Streptococcus oralis* e *Streptococcus mutans* têm um papel de destaque por serem reconhecidas por aumentarem a virulência e patogenicidade de *C. albicans* (Xu et al., 2014b; Cavalcanti et al., 2015).

Nas interações entre *C. albicans* e *S. oralis*, foi evidenciado que biofilmes maduros desses microrganismos ativam uma importante proteína de *C. albicans*,

denominada de Efg1, a qual pode ter sido mediada por moléculas *quorum-sensing* de *S. oralis* (Xu et al., 2014b). Em contrapartida, não há evidências na literatura de que o quorum-sensing de *S. oralis* possa mediar algum mecanismo de *C. albicans*.. Em relação à interação entre *C. albicans* e *S. mutans*, sabe-se que as adesinas Als1, Als3 e Hwp1 de *C. albicans* são expressas em biofilmes. No entanto, ainda não está claro o papel dessas adesinas durante a adesão entre *C. albicans* e *S. mutans*. Diante do exposto, essa Tese de Doutorado objetivou elucidar os mecanismos que medeiam as interações entre *C. albicans* e *S. oralis*, e *C. albicans* e *S. mutans*.

2 ARTIGOS

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O artigo 1, intitulado “Does *Streptococcus oralis* supernatant influence the development and virulence of *Candida albicans*?”, submetido no periódico *Archives of Oral Biology*.

O artigo 2, intitulado “*Candida albicans* adhesins modulate interactions with *Streptococcus mutans*”, a ser submetido no periódico *Microbiology*.

2.1 ARTIGO 1

Title: Does *Streptococcus oralis* supernatant influence the development and virulence of *Candida* albicans?

Running title: *S. oralis* supernatant on *C. albicans*

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Conflicts of interest

The authors declare to have no conflict of interest.

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Abbreviations:

OC: Oral candidiasis (OC)

CFU: Colony-forming units

UTYEB: Ultrafiltered tryptone-yeast extract broth

OD: optical density

PMMA: Poly(methyl methacrylate)

CLSM: Confocal laser scanning microscopy

Abstract

Objective: evaluate the influence of *Streptococcus. oralis* supernatant on the development and virulence of *Candida albicans.*

Methods: *S. oralis* subproducts was obtained by filtration of supernatant of overnight cultures. In addition, single or dual-species cultures of *C. albicans*, and *S. oralis* were cultivated. Two independent experiments were carried out: planktonic and biofilm assays. We first evaluated the growth curves of planktonic cultures. Second, mature biofilms formed on resin discs were collected to assess the biofilm's metabolic activity, total biomass, and cell enumeration (CFUs counting). Lastly, the hyphae formation (virulence factor) and biofilm thickness were analyzed by confocal laser scanning microscopy (CLSM). Data were analyzed by a one-way ANOVA test followed by Tukey posthoc ($\alpha = 0.05$).

Results: Our findings reveal that *S. oralis* subproducts did not influence the *C. albicans* development as free-floating cells. In the biofilm analysis, biofilms containing *S. oralis* supernatant showed higher cell metabolism than single *C. albicans* biofilm and *C. albicans* + *S. oralis* biofilm ($p < 0.05$). Although *S. oralis* metabolic subproducts increases the biofilm's metabolic activity, the total biomass and cell enumeration of *C. albicans* were not influenced by it ($p > 0.05$). However, representative biofilm images reveal an enhanced hyphae formation of *C. albicans* in biofilms containing *S. oralis* supernatant compared to single biofilms of *C. albicans*. Conclusions: *S. oralis* supernatant could solely contribute to *C. albicans* development and virulence.

Keywords: Biofilm; Candida albicans; Steptococcus oralis; Oral candidiasis.

1. Introduction

Due to a lack of effective antimicrobial support and fungi resistance, oral candidiasis (OC) is a life-treating infection that has been addressed worldwide. This disease is caused by biofilm and is characterized by recurrent white or red lesions in the oral mucosa (Wilson et al., 2002; Millsop & Fazel, 2016; Suleyman & Alangaden, 2016; Tsay et al., 2020). In this scenario, antimicrobial support is the first choice for treating local or systemic infections (Millsop et al., 2016). Conversely, the challenge in the OC treatment is the unspecificity of the current therapies because they do not have therapeutic targets. (Koo, Allan, Howlin, Stoodley & Hall-Stoodley, 2017). One way to detect a target is to understand how the microorganisms establish their interactions, coaggregate, and communicate to form the biofilm and provoke the disease (Rabin et al., 2015; Jiang, Chen, Yang, Yin & Yao, 2019).

In OC biofilm, *Candida albicans* is the primary microorganism (Millsop et al., 2016). Nonetheless, it is now recognized that oral bacteria, such as *Streptococcus oralis*, establish synergistic relationships with *C. albicans*, increasing the fungi's virulence and pathogenicity (Diaz et al., 2012; Xu et al., 2014b). The interaction among these microorganisms may occur via physical cell contact or quorum-sensing signals and other subproducts released extracellularly by the microorganisms (Xu, Jenkinson & Dongari-Bagtzoglou, 2014a). For example, through cellular contact, the frequency and severity of OC lesions were more than twofold in murine models co-infected with *C. albicans* and *S. oralis* compared with the fungi alone (Xu et al., 2014b). These events could be explained because the cell-cell contact between these microorganisms activates host enzymes to cleave epithelial junctions; thus, *C. albicans* actively penetrates the tissues (Xu, Sobue, Bertolini, Thompson & Dongari-Bagtzoglou, 2016).

On the other hand, little is known about *S. oralis* subproducts interacting with *C. albicans*. One investigation found that in a mature biofilm, co-cultures of these

microorganisms trigger an important growth regulator (Efg1) of *C. albicans*. As Efg1 activation does not happen in the initial cell-cell interactions, it was hypothesized that an *S. oralis* quorum-sensing molecule might trigger this response, contributing to the fungi's virulence and development (Xu et al., 2017). However, there is no evidence whether *S. oralis* supernatant, which contains quorum-sensing molecules as autoinducer 2 – (AI-2), solely impacts *C. albicans* course. Therefore, this *in vitro* study investigates the influence of *S. oralis* supernatant in the development and virulence of *C. albicans*.

2. Materials & methods

2.1 Experimental design

An *in vitro* study was conducted using *C. albicans* and *S. oralis* to develop single or dual-species cultures. Additionally, *S. oralis* supernatant was collected and filtered to obtain the bacteria's subproducts. Thus, cultures of *C. albicans*; *C. albicans* + *S. oralis*; *C. albicans* + supernatant; and *S. oralis* were submitted to two independent experimental phases: a planktonic interaction assay and biofilm model using acrylic resin discs pre-conditioned with clarified saliva (n = 10 *per* group) (Cavalcanti, Nobbs, Ricomini-Filho, Jenkinson & Del Bel Cury, 2016). Initially, planktonic cultures' growth curves were evaluated. Then, in the biofilm model, biofilms were developed until maturation (24 hours) and collected to assess the metabolic activity by XTT assay, cell enumeration by colony-forming units (CFUs) counting, biofilm's dry weight (biomass), and architecture and thickness by confocal laser scanning microscopy (CLSM). Three independent experiments were carried out. The data were statistically analyzed as described further.

2.2 Microbial strains and growth conditions

C. albicans SC5314 and *S. oralis* ATCC 35037 strains were reactivated from the original cultures and cultivated, respectively, on Agar Saboraud Dextrose (SDA) (Difco,

Detroit, MI, USA) at 37º C and Brain Heart Infusion (BHI) (Difco, Detroit, MI, USA) at 37º C, 10% CO_2. Then, *C. albicans* and *S. oralis* colonies were transferred to ultrafiltered tryptone-yeast extract broth (UTYEB) supplemented with 1% glucose and incubated overnight at 37°C, 10% CO_2. Subsequently, the cells were centrifuged (5000 *g*, 4º C, 10 min), washed twice with NaCl 0.9% solution, and resuspended in fresh UTYEB (Costa Oliveira, Cury & Ricomini Filho, 2017). Inocula were adjusted to the concentration corresponded to OD_{600} 1 × 10^5 CFU/ mL of *C. albicans* and OD_{600} 1 × 10^7 CFU/ mL of *S. oralis* (DU 800 UV/Visible Spectrophotometer, Beckman Coulter, Inc., Brea, CA, USA) (Souza et al., 2020).

Immediately prior to each experiment, *S. oralis* subproducts were obtained from *S. oralis* late exponential phase culture (Rickard et al., 2006). This culture was centrifuged (5000 *g*, 4º C, 10 min), and the supernatant was collected and filtered (0.22µm) (Corning Inc., Corning, NY, USA) (Rickard et al., 2006; Dos Santos et al., 2020).

2.3 Planktonic interaction assay

Cultures' growth curves determined the rate of cell proliferation per unit of time estimated in optical density (OD). Following *C. albicans* and *S. oralis* concentration adjustment, the cell's suspensions in UTYEB media were supplemented with 1% sucrose. During all experiments, the cultures were maintained at 37º C, 10% CO_2. At intervals of one hour, the cultures were homogenized, a sample of each was removed, and the OD was measured in a spectrophotometer. Records were carried out until the cultures reached the stationary phase.

2.4 Ethical aspects

This study was approved by the Research and Ethics Committee of the Piracicaba Dental School - University of Campinas (protocol 35443220.0.0000.5418). Three volunteers were selected according to the following criteria: ≥18 years of age, good systemic and oral health, and normal stimulated salivary flow rate (>0.7 mL/min). Participants who used

antimicrobials and antibiotic therapy in the previous two months before the experiments were excluded (Cavalcanti et al., 2016).

2.5 Specimen preparation

Poly(methyl methacrylate) (PMMA) discs (10 mm diameter x 2mm thick) were used as a substrate to form the biofilm (Cavalcanti et al., 2016). Following the manufacturer's instructions, the discs were prepared using heat-polymerized acrylic resin (VIPI Produtos Odontológicos, Pirassununga, SP, Brazil). This material was inserted and molded into a disc-shaped metallic device at the doughy stage. Subsequently, the resin was polymerized for 1 hour at 100 ºC. The discs were trimmed and progressively polished with abrasive paper (320, 400, and 600 grit) in a horizontal polisher (model APL-4; Arotec), then standardized the surface roughness at 0.30 ± 0.02 μm. The discs were immersed in distilled water at 37°C for 48 hours to allow residual monomer release. After that, the specimens were vertically positioned in 24-well plates by holders of orthodontic wire (Cavalcanti, Silva, Lucena, Pousa & Del Bel Cury, 2013; Amaechi, Tenuta, Ricomini Filho & Cury, 2019). Prior to each experiment, the samples were sterilized with ethylene oxide (Martorano-Fernandes et al., 2021).

2.6 Salivary pellicle formation and biofilm development

Each volunteer chewed a flexible film to stimulate salivation and collected the whole saliva in sterile tubes (50 mL) (Parafilm M; American Can Co, Neenah, WI). An adsorption buffer (1:1 ratio) and PMSF 0.1M buffer (1:100 ratio) were added to the saliva. Thus, the saliva was clarified (3800 *g*, 4º C, 10 mint) and sterilized by filtration (0.22μm) (Cavalcanti et al., 2016). After that, saliva was added to the 24-well plates containing the specimens to form the salivary pellicle on the discs, following incubation at 37°C, 30 min, and 75 rpm (Cavalcanti et al., 2016). Subsequently, the saliva was removed from the wells, and the inocula (supplemented with 1% sucrose) was added to promote biofilm adherence for 8 hours

at 37º C, 10% CO_2. Then, biofilm was washed twice to remove non-adherent cells, and fresh UTYEB media supplemented with 0.1 mM glucose was added. Then, the plates were incubated for 16 hours at 37º C, 10% CO_2 to allow biofilm maturation (Costa Oliveira et al., 2017). pH measurement was performed at the time points of 0, 2, 8, and 24 hours.

2.7 Metabolic activity of biofilm measurement by XTT assay

Following biofilm maturation, discs were transferred to sterile 24-well plates containing PBS enriched with 200 mM glucose, XTT solution (1 mg/mL, in ultra-purified water) (Sigma-Aldrich, San Luis, MO, USA), and menadione (0.4 mM in acetone) (Sigma-Aldrich, San Luis, MO, USA). The plates were incubated for 3 hours (35 ºC, 75rpm, darkness). After that, the absorbance of supernatants was recorded at 490nm (da Silva et al., 2008).

2.7 Biofilm biomass measurement (dry weight)

Discs were transferred to tubes with NaCl 0.9% solution and sonicated (7 W for 30 sec) to release the biofilm. Subsequently, aliquots were added to preweighed microtubes containing cold ethanol, centrifuged (10000 g, 4ºC, 10 min), and the supernatant discarded. After that, the cell pellet was dry for 3 hours. The biomass was determined by the difference between the final and initial weight of the microtubes (Costa Oliveira et al., 2017).

2.7 Cell enumeration

Biofilm was disrupted by sonication, as described above. Then, serial dilution of aliquots (10^{-1} to 10^{-6}) was used to estimate the number of cells. Aliquots of each dilution were seeded on SDA media plates for *C. albicans* and Mitis Salivarius Agar (MSA) media plates for *S. oralis* (37º C for 48 hours). The values of CFUs counting were multiplied by the serial dilution and converted to a logarithmic scale, expressed in log CFU/ mL (Sampaio et al., 2019).

2.8 Confocal laser scanning microscopy (CLSM)

To access the architecture (*C. albicans* morphology) and thickness of biofilm, CLSM was performed as reported elsewhere (Gulati et al., 2018). In brief, biofilms (n = 2 *per* group) were formed on the bottom of a sterile 6-well plate. Following biofilm maturation, *C. albicans* biofilm (single or dual-species) was stained with Concavalin A-Alexa Fluor 594 (red fluorophore - 50 μg/ml final concentration) (Thermo Fisher, Waltham, MA, USA). Thus, biofilm was visualized using a confocal microscope (555 nm diode laser) with a water-dipping 40x objective, obtaining Z-Stacks at 652 x 652 pixels (Leica Microsystems CMS, Mannheim, Germany). The ZEN software measured biofilm thickness, and ImageJ created top-views and side-views of each stack.

2.9 Statistical analyzes

Statistical Package for the Social Sciences (SPSS) software (IBM, Chicago, IL, USA) and GraphPrism (Graphpad, La Jolla, CA, USA) was used for statistical analyzes and generating graphics, respectively. Data were analyzed related to normality and homoscedasticity. Then, one-way ANOVA followed by Tukey's test was performed with a significance level fixed at 5%. Cultures' growth curves data were analyzed descriptively.

Results

First, we evaluated planktonic cultures' growth curves (Figure 1). All cultures started the log phase between 1 - 2 hours of growth. Cultures containing *C. albicans* and *S. oralis* and single *S. oralis* had a pronounced proliferation until the 5^{th} hour, reaching the stationary phase at this timepoint. On the other hand, single *C. albicans* and *C. albicans* + *S. oralis* supernatant had similar behaviors, in which the stationary phase occurred at the 9^{th} hour. Therefore, *S. oralis* supernatant did not influence the cultures' growth curve of *C. albicans*; conversely, the bacteria's physical contact favors the *C. albicans* growth.

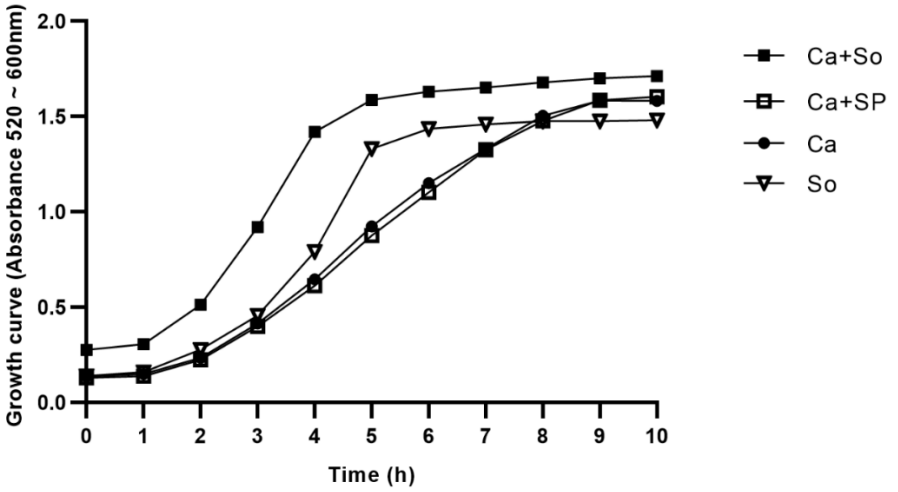

Figure 1: Planktonic cultures' growth curve per unit of time estimated in optical density. Ca + So: dual *C. albicans* and *S. oralis*. Ca + SP: *C. albicans* and *S. oralis* supernatant. Ca: single *C. albicans.* So: single *S. oralis.*

Despite this result, we hypothesized that *S. oralis* supernatant could mediate the *C. albicans* development in a complex environment as a biofilm. Interestingly, in the biofilm model, *C. albicans* biofilms containing *S. oralis* supernatant increase the cell metabolism compared to single *C. albicans* and *C. albicans* + *S. oralis* ($p < 0.05$; Figure 2).

Although the metabolism of *C. albicans* + *S. oralis* supernatant was high, our data showed that *S. oralis* supernatant did not contribute to increasing the fungi biomass and cell enumeration ($p < 0.05$; Figure 3 and Figure 4). There is no difference regarding biofilm biomass of *C. albicans* biofilms containing *S. oralis* supernatant and single *C. albicans* ($p > 0.05$; Figure 3). However, dual-species biofilm showed significantly higher biomass than single *C. albicans, C. albicans* + supernatant, and *S. oralis* ($p < 0.05$; Figure 3). Concerning cell enumeration, no differences were found for the *C. albicans* CFUs counting among single *C. albicans, C. albicans* + *S. oralis,* and *C. albicans* + supernatant ($p > 0.05$; Figure 4). In addition, *S. oralis* enumeration was no different within the single or dual-species biofilm ($p > 0.05$; Figure 4).

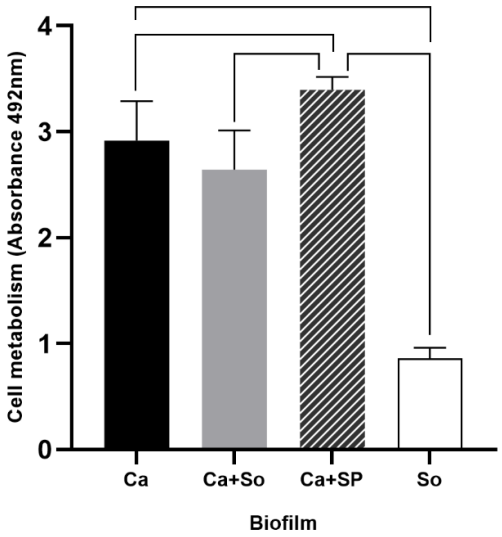

Figure 2: Cell metabolism of single or dual biofilms (n = 10 per group). A line connecting two bars means $p < 0.05$, using one-way ANOVA followed by Tukey's test. Error bars indicate standard deviations. Ca + So: dual *C. albicans* and *S. oralis*. Ca + SP: *C. albicans* and *S. oralis* supernatant. Ca: single *C. albicans.* So: single *S. oralis*.

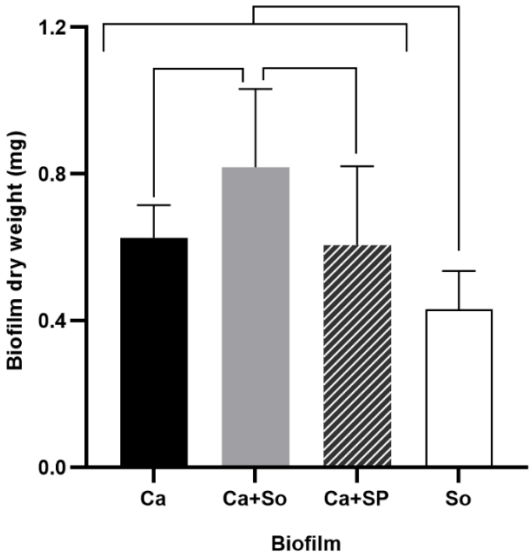

Figure 3: Biofilm biomass (n = 10 per group). A line connecting two bars means $p < 0.05$, using one-way ANOVA followed by Tukey's test. Error bars indicate standard deviations. Ca + So: dual *C. albicans* and *S. oralis*. Ca + SP: *C. albicans* and *S. oralis* supernatant. Ca: single *C. albicans.* So: single *S. oralis*.

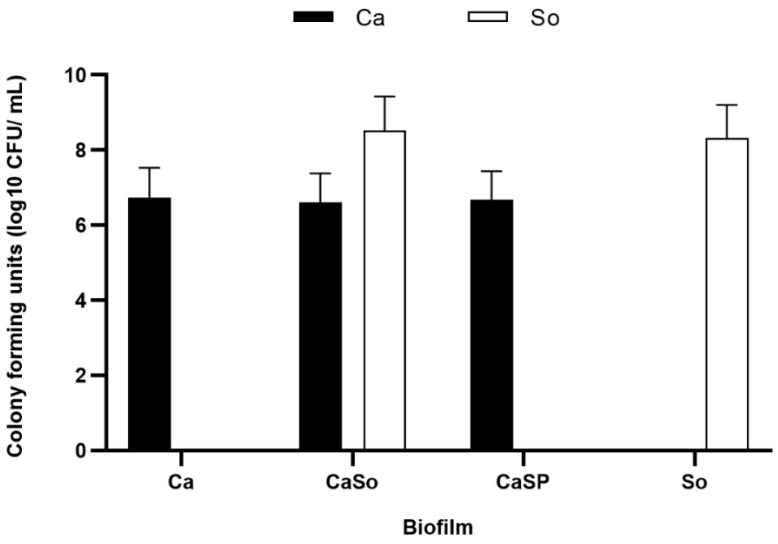

Figure 4: Cell enumeration by counting colony-forming units (CFUs) (n = 10 per group). A line connecting two bars means $p < 0.05$, using one-way ANOVA followed by Tukey's test. Error bars indicate standard deviations. Ca + So: dual *C. albicans* and *S. oralis*. Ca + SP: *C. albicans* and *S. oralis* supernatant. Ca: single *C. albicans.* So: single *S. oralis*.

The pH of biofilms was measured and shown in Supplementary Figure 1. pH of *S. oralis* and *C. albicans* + *S. oralis* ranged from 6.90 to 4.50 in 24 hours. pH of *C. albicans* and *C. albicans* + *S. oralis* supernatant in 24 hours was 6.10 and 5.98, respectively.

Based on our results that *S. oralis* supernatant increases biofilm cell metabolism, we tested the hypothesis that these bacteria's subproducts could modulate *C. albicans* virulence. By accessing the biofilm architecture and thickness, we could estimate the hyphae formation, one of the *C. albicans* virulence factors. Representative CLSM images showed that all biofilms of *C. albicans* were majority composed of hyphae in the outmost layer (Figure 5). Interestingly, images reveal that in the presence of *S. oralis* or its supernatant, *C. albicans* hyphae were more elongated than single *C. albicans* biofilms (Figure 5). These findings were confirmed by biofilm thickness measurement, in which single *C. albicans* had ± 240 µm in-depth, while *C. albicans* + *S. oralis* or *C. albicans* + *S. oralis* supernantant had ± 300 µm

each. Thus, the presence of *S. oralis* or its supernatant contributes to the hyphae formation process and potentially aids biofilm virulence.

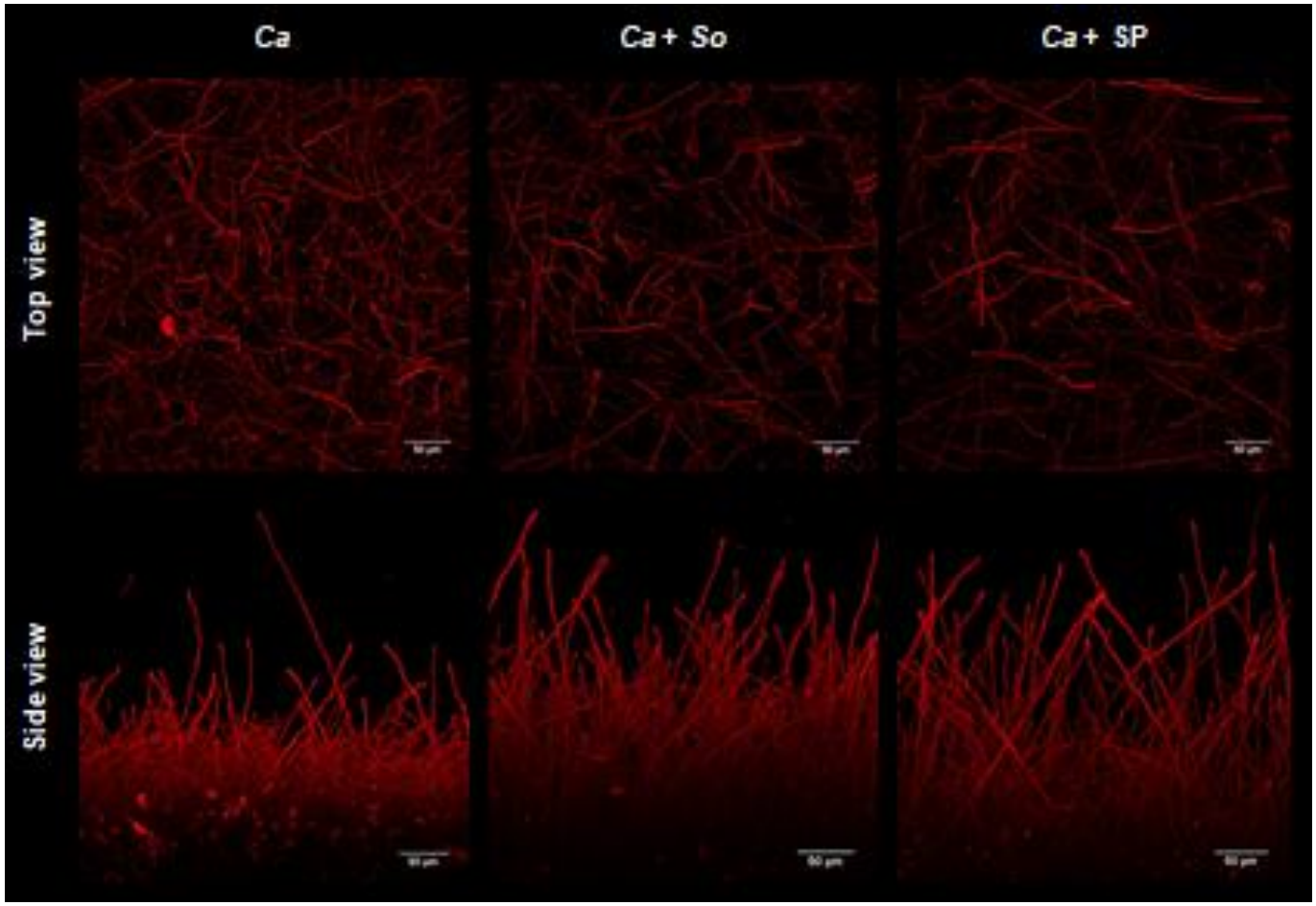

Figure 5: Representative images of dual or single biofilms by CLSM. *C. albicans* was stained with Concavalin A-Alexa Fluor 594 (red fluorophore). Biofilm was visualized using a confocal microscope (555 nm diode laser) with a water-dipping 40x objective, obtaining Z-Stacks at 652 x 652 pixels. Ca: single *C. albicans*; Ca + So: dual *C. albicans* and *S. oralis*. Ca + SP: *C. albicans* and *S. oralis* supernatant.

Supplementary Material

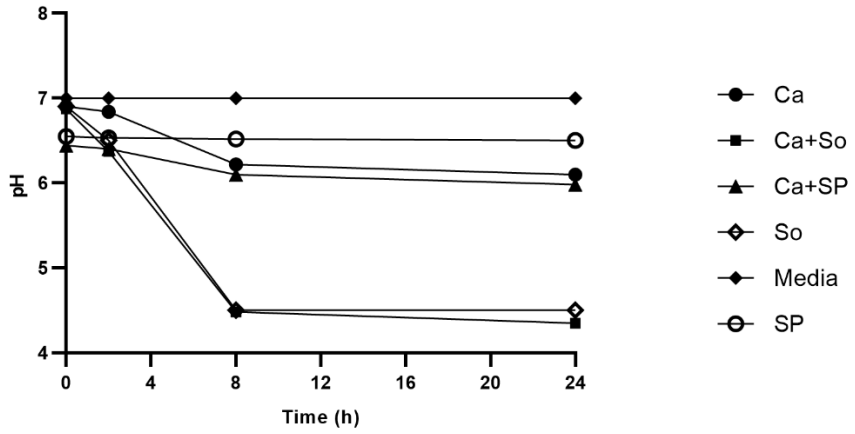

Figure S1: pH measurement per unit of time. Ca: single C. albicans. Ca + So: dual *C. albicans* and *S. oralis*. Ca + SP: *C. albicans* and *S. oralis* supernatant.. So: single *S. oralis*. SP: *S. oralis* supernantant.

Discussion

In mature biofilms, it was predicted that a *S. oralis* quorum-sensing molecule could contribute to fungal development and virulence (Xu et al., 2017). So far, no investigation evaluated *S. oralis* supernatant apart from the bacteria itself. According to our findings, *S. oralis* subproducts can play a role in the *C. albicans* virulence, albeit they do not increase the fungi biomass and population of biofilm. Thus, considering that the symbiotic relationship between *C. albicans* and *S. oralis* increases the frequency and severity of oral Candida-related diseases (Xu et al., 2014b), therapeutic targets for these infections should focus not only on the microorganisms themselves but also on the subproducts released extracellularly.

In fungi-bacteria interactions, physical contact during the planktonic stage is relevant to microorganisms coaggregate, increasing their population and forming the biofilm (Diaz et al., 2012). Besides, within this microbial community, the microorganisms can also sense and

respond to each other by a cell-cell signaling system consisting of subproducts released extracellularly, such as small chemical molecules (Miller & Lamont, 2019; Mukherjee & Bassler, 2019). In this study, in the planktonic stage, *S. oralis* supernatant did not present any effect on *C. albicans* proliferation. By contrast, the bacteria's physical contact benefits the rapid *C. albicans* development, perhaps by favoring an early-stage filamentation. These last findings are in line with a previous investigation pointing out that in the first two hours of culture, *C. albicans* and *S. oralis* present intimate proximity. Furthermore, in this cell-cell contact, the bacteria adhere to hyphal filaments (Cavalcanti et al., 2016). It is well-defined that hyphae contribute to biofilm proliferation by anchoring other cells, resulting in a cell network (Gulati & Nobile, 2016). Therefore, the physical contact between *C. albicans* and *S. oralis* not only contributes to fungi proliferation but also develops a virulent morphology, which could explain the disease's severity.

Although these results in planktonic cultures highlighted the relevance of the cell-cell contact, we hypothesized that a mature biofilm, which has a complex of several layers of polymorphic cells encased in an extracellular matrix (Chandra et al., 2001), could create ideal conditions in a local microenvironment to support *C. albicans* development by bacteria' supernatant. In these conditions, our data demonstrate that *S. oralis* supernatant significantly increases the *C. albicans* metabolism but does not affect the fungi biomass and cell enumeration. These results mean that the *S. oralis* quorum-sensing molecules could modulate the *C. albicans* mitochondrial enzymatic activity, albeit insufficient to increase the biofilm structure and population. A well-structured biofilm is relevant because it confers a robust network of cells and thickness, protecting the biofilm from chemical and physical injury (Nobile & Johnson, 2015). Meanwhile, a higher microbial population favors the biofilm's drug tolerance and resistance (Perumal, Mekala & Chaffin, 2007).

The higher metabolic activity could be associated with unsuitable pH for *C. albicans* and *S. oralis* supernatant biofilms. However, our results indicated that the pH of this biofilm remained close to neutral, which would not be sufficient to modulate metabolic activity. So, the increased metabolism of *C. albicans* and supernatant of *S. oralis* occurred by an unknown mechanism, perhaps attributed to an enhanced morphological transition of *C. albicans* in the presence of *S. oralis* quorum-sensing.

As opposed to that, physical interactions among *C. albicans* and *S. oralis* lead to a robust biofilm by enhancing its biomass, possibly attributed to an improved extracellular matrix. These results follow a previous investigation showing enhanced biomass of *C. albicans* and *S. oralis* due to a higher synthesis of the α-glucan-rich matrix (Souza et al., 2020). Of note, the major fraction of the biofilm is the extracellular matrix, which acts as a physical barrier to drug penetration as well as to biofilm drug resistance (Piece et al., 2017). Thereby, *C. albicans* and *S. oralis* physical interactions can lead to a robust biofilm, which may challenge biofilm disruption by a therapy.

Besides biofilm structure, another feature of OC biofilms is virulence (Calderone & Fonzi, 2001). Typically, the ability of *C. albicans* to cause the infection is related to filamentation because, in this morphology, the fungi can induce endocytosis or active penetrate the tissues (Calderone & Fonzi, 2001). Remarkably, one of the mechanisms that control the switch from yeast to hyphae is the *C. albicans* master regulator Efg1 (Glazier, 2022). Previous investigations showed that this regulator is activated in mature biofilms of *C. albicans* and *S. oralis*, perhaps by an *S. oralis* quorum-sensing mechanism. Our findings can support this hypothesis since *S. oralis* supernatant contributes to increasing hyphae formation on biofilms of *C. albicans* and *S. oralis*. Therefore, subproducts released extracellularly also can be attributed as contributors to increase biofilm virulence.

As a limitation, the present study does not identify the components present in *S. oralis* supernatant by gas chromatography (Dos Santos et al., 2020). However, several bacteria, including *S. oralis*, have autoinducer 2 (AI-2) as a major quorum-sensing molecule (Hardie & Heurlier, 2008), which can mediate structurally and functionally a microorganisms relationship within the biofilm (Rickard et al., 2006). Thus, to ensure that *S. oralis* quorum-sensing molecules were present in the bacteria's supernatant, our investigation used the *S. oralis* culture in the late exponential phase because it exhibits the higher activity of AI-2 in the cell-free supernatant from *S. oralis* (Rickard et al., 2006). This occurs because quorum-sensing signaling is often activated when a population density is high enough to trigger a chemical response in a coordinated manner (Solano, Echeverz & Lasa, 2014). Thereby, these previous findings endorse the presence of quorum-sensing molecules in the *S. oralis* supernatant used in this study.

Although our results bring news to the current knowledge, there is still lacking evidence regarding the role of *S. oralis* supernatant on *C. albicans* pathogenicity and host response. It is well-known that physical interactions between *C. albicans* and *S. oralis* increase in *C. albicans'* ability to invade the oral mucosa (Diaz et al., 2012) (Xu et al., 2016). Hence, the enhanced invasion process leads to severe lesions and a significant oral inflammatory response to the infection (Xu et al., 2014b). Given this evidence, future investigations should explore whether *S. oralis* supernatant contributes to the *C. albicans* tissue invasion and inflammatory host response.

In summary, our results suggest that *S. oralis* supernatant *per si* could contribute to *C. albicans* development and virulence. Therefore, alternative and novel therapies should also explore components of bacteria supernatant as targets for treating oral candidiasis. Furthermore, from a clinical viewpoint, those therapeutical targets could shed light on antifungal resistance and its implications for higher healthcare expenditures.

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2.2 ARTIGO 2

Candida albicans adhesins modulate interactions with _Streptococcus mutans_

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Abstract

Candida albicans and *Streptococcus mutans* are known to synergistically interact with each other in the oral cavity. For example, glucosyltransferase B (GtfB), secreted by *S. mutans*, can bind to the *C. albicans* cell surface, promoting dual-species biofilm formation. However, the fungal factors mediating interactions with *S. mutans* are currently unknown. The *C. albicans* adhesins Als1, Als3, and Hwp1 are key players in *C. albicans* single-species biofilm formation, but their roles, if any, in interacting with *S. mutans* are unknown. Here, we investigated the roles of the *C. albicans* cell wall adhesins Als1, Als3, and Hwp1 on forming dual-species biofilms with *S. mutans*. We assessed the abilities of the *C. albicans* wildtype, *als1*Δ/Δ, *als3*Δ/Δ, *als1*Δ/Δ/*als3*Δ/Δ, and *hwp1*Δ/Δ mutant strains to form dual-species biofilms with *S. mutans* by measuring biofilm optical densities, metabolism, cell enumeration, biomass, thickness, and architecture. We observed that the *C. albicans* wildtype strain formed enhanced dual-species biofilms in the presence of *S. mutans* in these different biofilm assays, confirming that *C. albicans* and *S. mutans* synergistically interact in the context of biofilms. Our results reveal that *C. albicans* Als1 is a major player in interacting with *S. mutans* since dual-species biofilm formation was not enhanced when the *als1*Δ/Δ strain was cultured with *S. mutans* in dual-species biofilms. In addition, our results suggest that Als3 may play a lesser role to Als1 in interacting with *S. mutans* in dual-species biofilm formation, but its role appears to be Als1-dependent. Lastly, our findings show that Hwp1 may also play a role in dual-species biofilm formation since, similar to the *als1*Δ/Δ strain, dual-species biofilm formation was not enhanced in the *hwp1*Δ/Δ strain. Overall, our data suggest that *C. albicans* adhesins Als1, Hwp1, and to a lesser extent, Als3, function to modulate interactions with *S. mutans*, and could be potential targets for future therapeutics.

Keywords: Biofilms; *Candida albicans*; *Streptococcus mutans*.

Introduction

The opportunistic fungal pathogen *C. albicans* is a normal colonizer of the oral cavity, gastrointestinal tract, and genitourinary tract of healthy humans. However, due to host environmental changes or immunocompromisation, *C. albicans* can become pathogenic and cause superficial and disseminated infections (Nobile & Johnson, 2015; Millsop & Fazel, 2016). Biofilm formation is a common virulence factor of *C. albicans* that enhances its persistence and pathogenicity in the host (Nobile & Johnson, 2015). In the oral cavity, *C. albicans* alone can form biofilms and cause infections (Millsop et al. 2016), but *C. albicans* can also form synergistic biofilms with certain bacteria, such as those in the *Streptococcus* genus (Xu, Jenkinson & Dongari-Bagtzoglou, 2014). These interspecies interactions have been shown to enhance microbial colonization and persistence, particularly in the oral cavity (Falsetta et al. 2014). The presence of *Streptococcus mutans*, for example, is known to lead to the upregulation of common *C. albicans* virulence genes, such as *HWP1*, *SAP4*, and *SAP6*, when *S. mutans* and *C. albicans* are cultured together in biofilms (Ellepola et al. 2019; Cavalcanti et al. 2015). In addition, these dual-species biofilms have been shown to cause enhanced tissue invasion and damage to the oral epithelium compared to single-species biofilms of *S. mutans* and *C. albicans* alone (Cavalcanti et al. 2015).

Cell wall proteins are important for mediating cell-cell interactions between *C. albicans* cells. For example, the *C. albicans* cell wall adhesins Als1 and Als3 in the agglutinin-like sequence (Als) family are important for *C. albicans* cell-cell interactions during single-species biofilm formation (Hoyer & Cota, 2016; Zhao et al. 2006; Nobile et al. 2008). In addition, Hwp1, a protein expressed on the *C. albicans* hyphal cell surface, is also important for promoting cell-cell interactions and single-species biofilm formation (Nobile et al. 2006a; Nobile et al. 2006b).

On the bacterial side, *S. mutans* secretes a glucosyltransferase exoenzyme, GtfB, that has been shown to promote coaggregation between *S. mutans* and other microorganisms, including *C. albicans*, enhancing dual-species biofilm formation (Gregoire et al. 2011; Falsetta et al. 2014). GtfB has been shown to bind to differnt sites along the *C. albicans* cell well (Gregoire et al. 2011; Hwang et al. 2015), suggesting that specific fungal cell wall components are required for its binding.

Here, we investigate the roles of the *C. albicans* cell wall proteins Als1, Als3, and Hwp1 on dual-species biofilms formation between *S. mutans* and *C. albicans.* Our results reveal that *C. albicans* Als1, Hwp1, and to a lesser extent, Als3, function to modulate interactions with *S. mutans*.

Material & Methods

Experimental design

This study was performed following the protocols designed to evaluate aspects of biofilm formation of *C. albicans* mutant strains (Gulati et al. 2018). Single and dual-species biofilms were compared to test whether Als1, Als3, and Hwp1 had a role in interacting with *S. mutans*. Single *C. albicans* wildtype, single *S. mutans,* and dual-species wildtype biofilms were used as control. Biofilms were formed on the bottom of 6-well or 96 well-plates until maturation (24 hours). Following that, biofilms were collected to analyze their formation by cell density, metabolism, enumeration, and biomass. Lastly, biofilms were visualized using Confocal Scanning Laser Microscopy (CLSM) to assess the biofilm architecture and thickness, and bindings between the microorganisms were visualized by optical microscopy. Experiments were performed in triplicate.

Strains and media

All strains used in this study have been previously published and are listed in Table 1. Deletion strains of *C. albicans* lacking both alleles of *ALS1*, *ALS3*, and *HWP1* as well as a double deletion strain lacking both alleles of *ALS1* and *ALS3* were used. In addition, an *S. mutans* GFP-tagged strain was used for CSLM imaging.

Table 1: strains used in this study

Strain	Source
C. albicans wildtype SC 5314	Gillum et al. 1984
S. mutans UA 159	Ajdic et al. 2002
S. mutans pDL278_P23-sfgfp	Shields et al. 2019
*C. albicans als1*Δ/Δ	Nobile et al. 2006
*C. albicans als3*Δ/Δ	Nobile et al. 2006
C. albicans als1/*als3*Δ/Δ	Nobile et al. 2008
*C. albicans hwp1*Δ/Δ	Nobile et al. 2006

C. albicans strains were grown from -80°C glycerol stocks at 30°C on Yeast Extract Peptone Dextrose (YPD) (Thermo Fisher Scientific) agar plates. Overnight cultures were grown at 30°C, 225 rpm in YPD liquid media. *S. mutans* strains were rown from -80°C glycerol stocks at 37ºC with 10% CO_2 on Brain Heart Infusion (BHI) (Thermo Fisher Scientific) agar plates. Overnight cultures were grown on BHI supplemented with 1% glucose (37ºC with 10% CO_2). RPMI 1640 medium (Sigma Aldrich) was used for the biofilm assays because it supports biofilm formation of both microorganisms (Heersema & Smyth, 2019).

Biofilm development

Cell enumeration relative to optical density readings were performed for each microorganism to establish a 1:1 ratio of each species in culture, which was equivalent to an

OD_{600} of 1 x 10^6 CFU/mL for *C. albicans* and an OD_{600} of 0.15 x 10^6 CFU/mL for *S. mutans*. This CFU/mL was added to the 6-well or 96-well plates for single-species biofilm formation and the same CFU/mL of each species was added to the 6-well or 96-well plates for dual-species biofilm formation. Plates were sealed with Breathe-Easy® sealing membranes and incubated at 37°C for 90 min at 250 rpm with 10% CO_2. Cells were washed with 1XPBS to remove non-adhered cells and fresh RPMI 1640 medium was added to each well. Biofilms were grown to maturity for 24 hours.

Biofilm formation assay (standard optical density)

Following maturation on 96 well-plates, the media was aspirated from the wells, and the biofilm formed on the bottom of each well was measured by OD_{600} reasings using a plate reader. An average of 24 reads per well were obtained and normalized by subtracting the OD_{600} reading of a blank well containing RPMI 1640 only.

Cell metabolism

The 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) reduction assay was performed to measure metabolic activity within the biofilm. A mixture of 0.5 mg/ml of XTT (Sigma Aldrich) in 1XPBS and 0.32 mg/ml of phenazine methosulfate (Sigma Aldrich) in water were added to each well and incubated in the dark for 30 minutes. After incubation, OD_{492} readings were taken in a plate reader.

Biofilm cell enumeration

After the 24-hour period of biofilm formation, media was aspirated from the 96-well plates, and the biofilms were washed to remove non-adhered cells. The biofilm formed on the bottom of each well was vigorously scraped using a pipette tip and resuspended in 1XPBS. Cell

suspensions were homogenized and serially diluted in 1XPBS. Aliquots were plated onto YPD plates for *C. albicans* and onto Mitis Salivarius Agar (MSA) (Kasvi) for *S. mutans* to enumerate colony forming units (CFUs).

Biofilm biomass determination

Biofilms were grown on the bottoms of 6-well plates. Media was aspirated from the wells and 1XPBS was added to each well. Biofilms were vigorously scraped and resuspended from the bottoms of each well using a pipette tip. Biofilm cell suspensions were aspirated and filtered onto mixed cellulose esters membranes (Millipore) using a Millipore filtration device. Subsequently, membranes were dried for 24 hours at 37ºC, and the weights of the membranes (in mg) were measured to obtain the dry weights of the biofilms. Data were normalized by subtracting the average weight of the control (media only) well.

Confocal scanning laser microscopy (CSLM) biofilm assay

Representative images of biofilms (n = 3 per group) were obtained by confocal scanning laser microscopy (CSLM). Biofilms were grown on the bottoms of silicone squares for 24 hours using the same conditions described above. Biofilms were fixed with a formaldehyde solution (36.5–38% in water). *C. albicans* biofilms were stained with Concanavalin A-Alexa Fluor 594 (Sigma Aldrich) and visualized using a 555 nm diode (red) laser. The *S. mutans* GFP-tagged strain was visualized by excitation at 488 nm (green). Z-Stacks were obtained at 652 x 652 pixels, imaging every 0.5 μm intervals using a water-dipping 40X objective lens. The .czi files were analyzed using the project stacks function in ImageJ to generate side views of each stack. Biofilm thickness was measured in μm.

Optical microscopy

Planktonic cultures of *C. albicans* and *S. mutans* were grown separately overnight in YPD (for *C. albicans*), and BHI supplemented with 1% glucose (for *S. mutans*). Cultures were diluted to an OD_{600} of 0.5 in RPMI 1640 medium and co-cultured at 37 °C with 10% CO_2 for 4 hours, with shaking at 250 rpm. An aliquot of the co-cultures was visualized using an EVOS FL microscope with a 60X oil immersion objective. The images displayed are representative of three independent experiments.

Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS) software (version 22.0). Means and standard deviations were calculated, and Student's unpaired two-tailed t-tests assuming unequal variance or one-way ANOVAs were performed for each dataset. GraphPad Prism software (version 9.4) was used to generate the graphs.

Results

Results

To test our hypothesis that Als1, Als3, and Hwp1 play roles in dual-species *C. albicans*-*S. mutans* biofilm formation, we first compared the biofilms formed by the single-species and dual-species biofilms of each strain using the standard optical density biofilm assay (Gulati et al. 2018), which correlates with biofilm thickness. Using this assay, we found a synergistic interaction between *C. albicans* and *S. mutans*, where an increase in optical density was observed for the dual-species wildtype biofilms, compared to the single-species *C. albicans* wildtype biofilms and the single-species *S. mutans* wildtype biofilms (Figure 1). We found that the presence of *S. mutans* had no effect on the biofilm formation capacity of the *C. albicans* *als1*Δ/Δ or *hwp1*Δ/Δ strains compared to the single-species *als1*Δ/Δ or *hwp1*Δ/Δ strains, respectively; however, the presence of *S. mutans* strikingly increased the biofilm formation

capacity of the *C. albicans als3*Δ/Δ strain compared to the single-species *als3*Δ/Δ strain (Figure 1). Interestingly, the presence of *S. mutans* impaired the biofilm formation capacity of the *C. albicans als1*Δ/Δ*/als3*Δ/Δ double deletion strain compared to the single-species *als1*Δ/Δ*/als3*Δ/Δ strain (Figure 1).

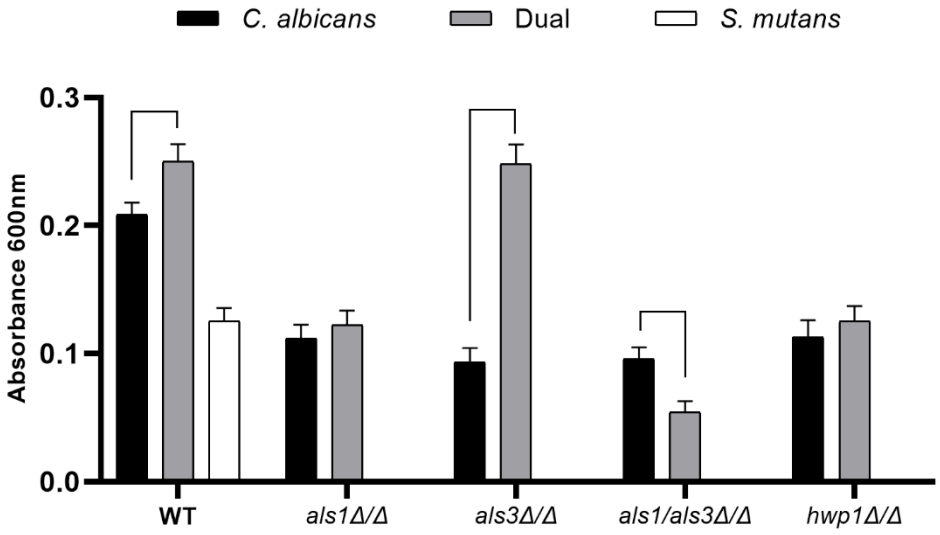

Figure 1: Biofilm density of single and dual-species biofilm measured at the OD_{600} (n = 6 per group). A line connecting two bars means $p < 0.05$, using Student's unpaired two-tailed t-test assuming unequal variance. The error bars indicate standard deviations.

Similar results to the optical density biofilm assay were obtained for the XTT cell metabolism biofilm assay and the dry weight biofilm biomass assay, where an increase in metabolic activity (Figure 2) and biomass (Table 2) was observed for the dual-species wildtype biofilms, compared to the single-species *C. albicans* wildtype biofilms and the single-species *S. mutans* wildtype biofilms. In addition, the presence of *S. mutans* increased the metabolic activity (Figure 2) and biomass (Table 2) of the *C. albicans als3*Δ/Δ strain compared to the single-species *als3*Δ/Δ strain, but had no effect on the metabolic activity (Figure 2) or biomass (Table 2) of the *C. albicans als1*Δ/Δ or *hwp1*Δ/Δ strains compared to the single-species *als1*Δ/Δ or *hwp1*Δ/Δ strains, respectively. Likewise, the presence of *S. mutans* reduced the metabolic

activity (Figure 2) and biomass (Table 2) of the *C. albicans als1*Δ/Δ*/als3*Δ/Δ double deletion strain compared to the single-species *als1*Δ/Δ*/als3*Δ/Δ strain.

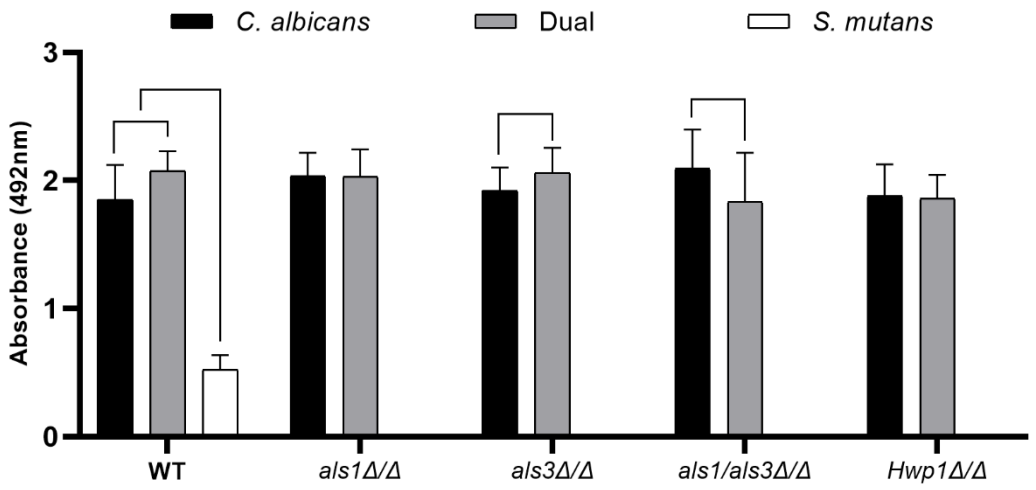

Figure 2: Metabolic activity of single and dual-species biofilm measured by XTT salt reduction (n = 6 per group). A line connecting two bars means $p < 0.05$, using Student's unpaired two-tailed t-test assuming unequal variance. The error bars indicate standard deviations.

Table 2: Biofilm dry weight in milligrams

Strain	**Dry weight (mg) (mean ± SD)**	
	Single	**Dual**
C. albicans wildtype	10.09 ± 0.26	12.03 ± 0.54
S. mutans UA159	1.88 ± 0.28	-
*C. albicans als1*Δ/Δ	4.75 ± 0.22	5.16 ± 0.27
*C. albicans als3*Δ/Δ	**4.56 ± 0.23**	**8.06 ± 0.27 ***
*C. albicans als1/als3*Δ/Δ	**4.43 ± 0.32**	**3.31 ± 0.27 ***
*C. albicans hwp1*Δ/Δ	3.38 ± 0.20	3.74 ± 0.24

* Statistical difference between single and dual biofilms according to the Student's unpaired two-tailed t-test assuming unequal variance.

To determine the number of cells of each species present in the biofilms, we next measured CFUs for each sample. Overall, *S. mutans* CFUs were higher in all dual-species biofilms compared to *S. mutans* CFUs in all single-species biofilms (Figure 3), indicating that *S. mutans* benefits from *C. albicans* by increasing its cell population. Furthermore, CFUs were lower in dual-species biofilms of the *C. albicans als1*Δ/Δ*/als3*Δ/Δ double deletion strain compared to the single-species *als1*Δ/Δ*/als3*Δ/Δ strain (Figure 3). Lastly, single-species *als3*Δ/Δ strain showed lower CFUs compared to dual-species biofilms.

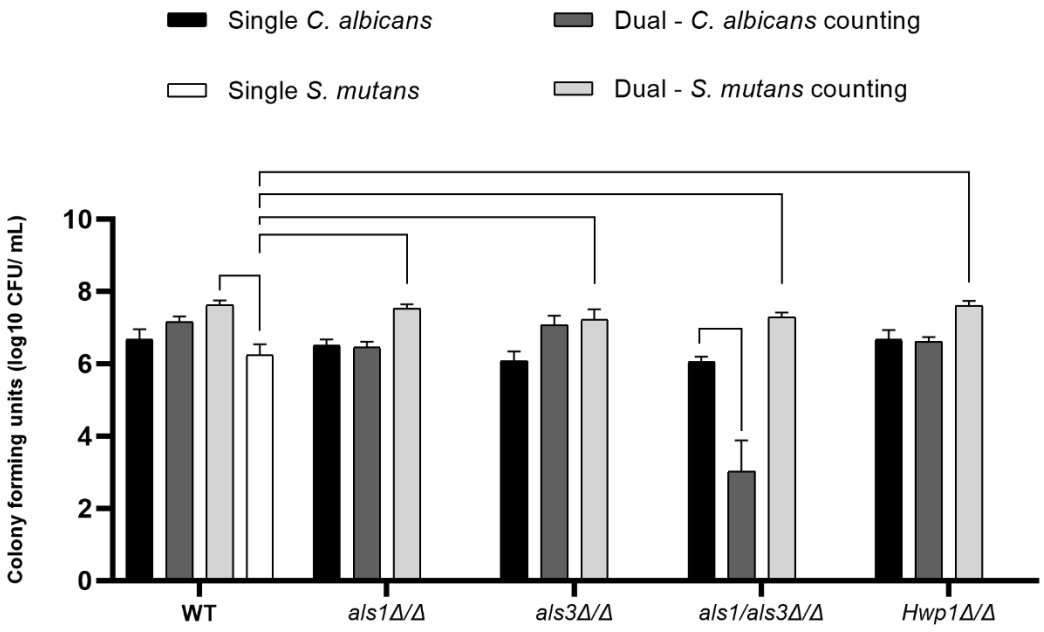

Figure 3: Cell enumeration of biofilm (n = 4 per group). Data expressed in Colony-Forming Units (CFU) on a logarithmic scale. According to the ANOVA one-way test, a line connecting two bars means $p < 0.05$. The error bars indicate standard deviations.

To assess biofilm architecture and thickness, we next performed confocal scanning laster microscopy (CSLM) assays on the biofilms. We observed that wildtype *C. albicans* dual-species biofilms were overall thicker than *C. albicans* single-species as measured in the CSLM side-view images (thickness measurements of single-species biofilms were ~230 μm on average

and those of dual-species biofilms were ~270 μm on average) (Figure 4; Table 3). In addition, as expected, all single-species *C. albicans* mutant strain biofilms produced defective biofilms ranging from 5-40 μm in thickness (Figure 4; Table 3). Similar to our results reported above for the other biofilms assays, the thickness of the biofilms of the *als1*Δ/Δ and *hwp1*Δ/Δ strains was the same between the single-species and dual-species biofilms (Table 3). In addition, the presence of *S. mutans* in the dual-species biofilms restored biofilm thickness of the *C. albicans als3*Δ/Δ strain to wildtype levels compared to the single-species *als3*Δ/Δ strain (Table 3). Overall, the CSLM images indicate that *S. mutans* benefits from the presence of *C. albicans* in all dual-species biofilms. We note that single-species *S. mutans* biofilms formed thin cell aggregates restricted to the bottom of the substrate, while in dual-species, the *S. mutans* cells were observed throughout the biofilm structure, often appearing along *C. albicans* hyphal cells.

Table 3: Range of biofilm thickness

Strain	**Thickness (μm)**	
	Single	**Dual**
C. albicans wildtype	220 – 250	250 – 280
*C. albicans als1*Δ/Δ	20 – 40	20 – 40
*C. albicans als3*Δ/Δ	20 – 40	150 – 180
*C. albicans als1/als3*Δ/Δ	20 – 40	10 – 30
*C. albicans hwp1*Δ/Δ	5 – 20	5 – 20

Lastly, we performed optical microscopy of planktonic cultures to visualize microbial interactions occurring between *C. albicans* and *S. mutans* under non-biofilm co-culture conditions. Co-cultures of the wildtype *C. albicans* and *S. mutans* strains showed binding of *S.*

mutans cells equally well to both *C. albicans* yeast-form and hyphal cells (Figure 5). Interestingly, co-cultures of the *C. albicans als1*Δ/Δ, *hwp1*Δ/Δ, and *als1*Δ/Δ/*als3*Δ/Δ strains with *S. mutans* showed strikingly fewer binding events compared to co-cultures of the *C. albicans* wildtype strain with *S. mutans* (Figure 5). Surprisingly, co-culture of the *C. albicans als1*Δ/Δ strain with *S. mutans* showed increased binding of *S. mutans* specifically to *C. albicans* hyphal cells compared to co-cultures of the *C. albicans* wildtype strain with *S. mutans* (Figure 5).

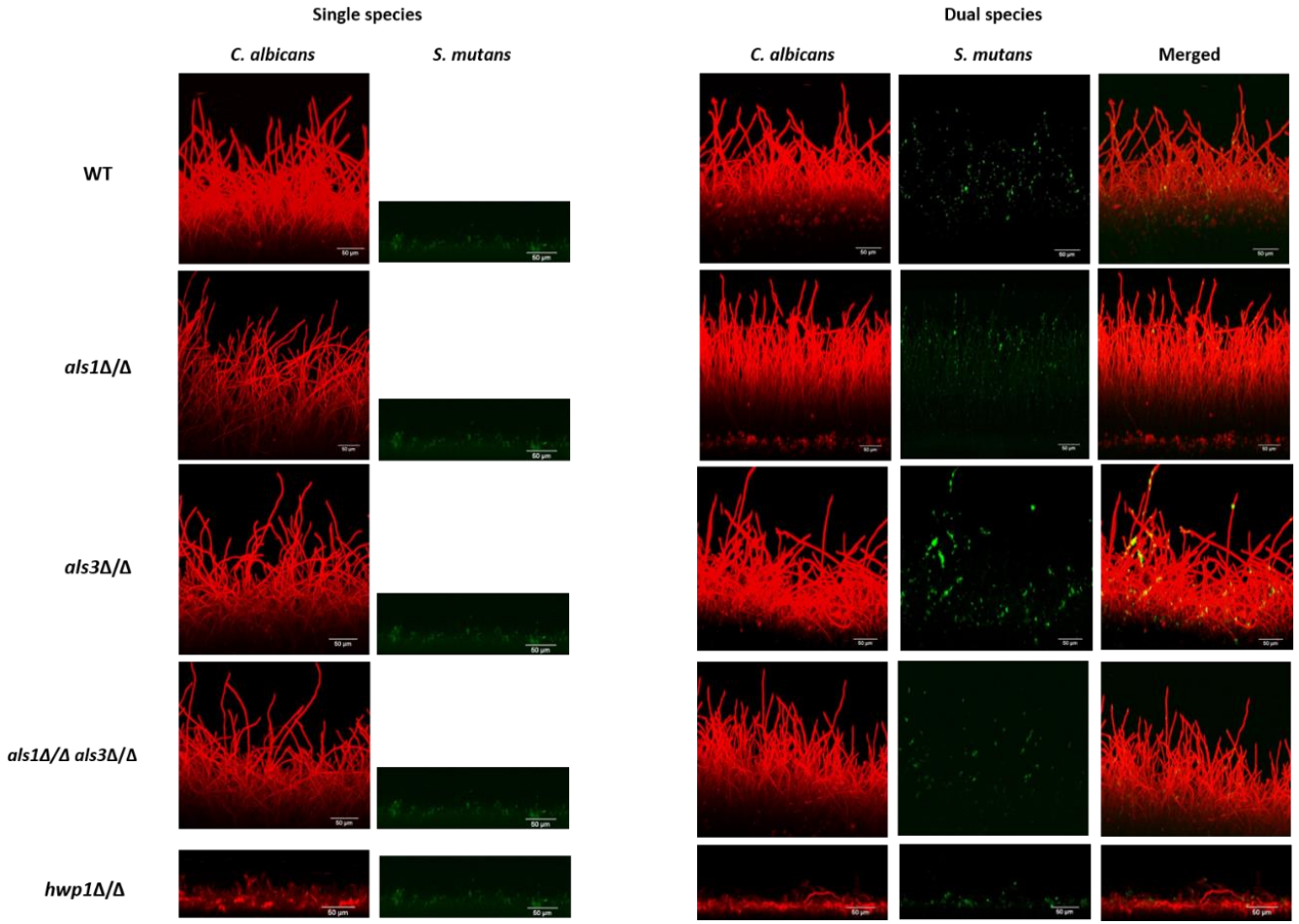

Figure 4: Representative biofilm images by Confocal Scanning Laser Microscopy (CSLM). *C. albicans* biofilm was stained with Concavalin A-Alexa Fluor 594 (red) and visualized using a 555 nm diode laser. *S. mutans* pDL278_P23-sfgfp strain was used for GFP fluorescence (green) and was detected by excitation at 488 nm. All Z-Stacks were obtained at 652 x 652 pixels, imaging every 0.5 µm intervals using a water-dipping 40x objective lens. The .czi files are analyzed using the project stacks function of ImageJ to generate side-views of each stack. Scales bars = 50 µm.

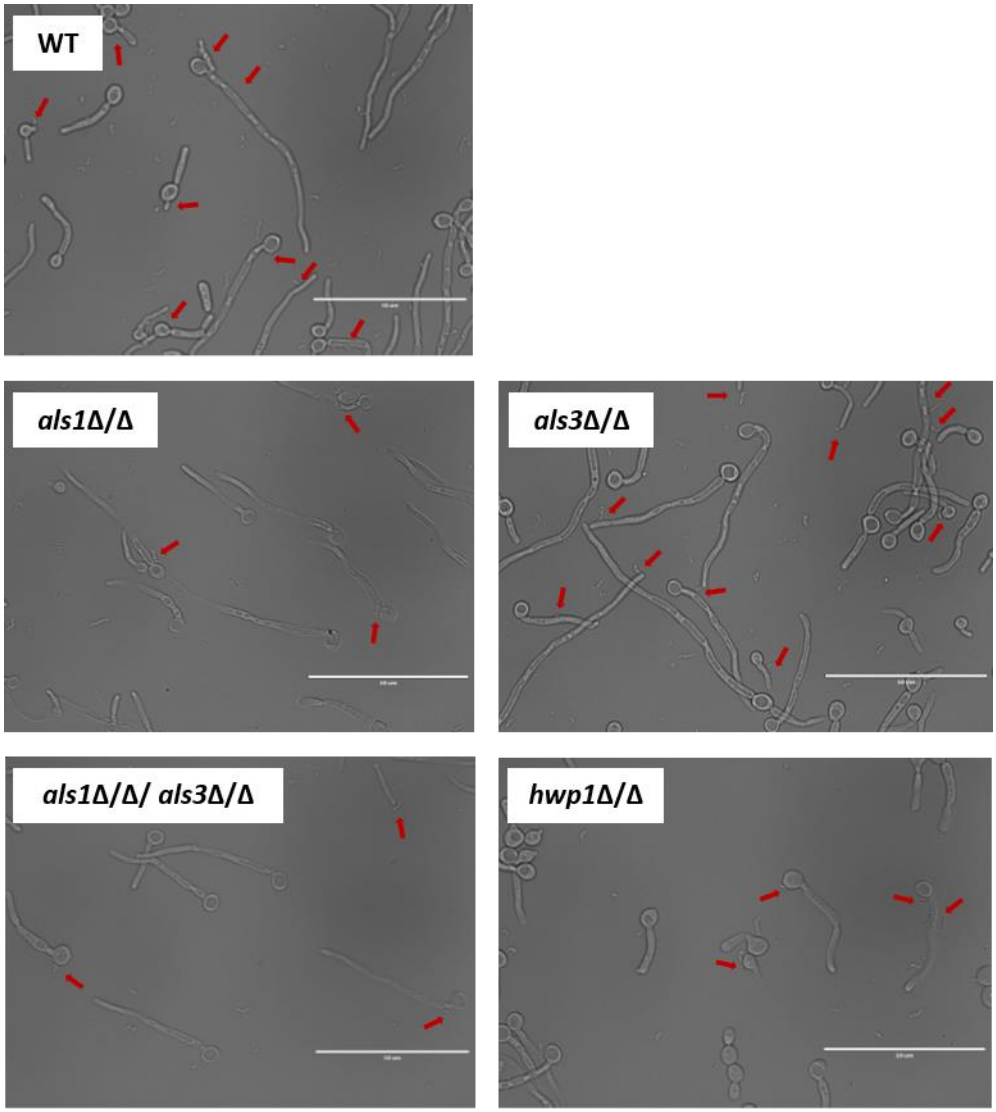

Figure 5: Representative images of co-cultures of *C. albicans* and *S. mutans* by EVOS FL microscope with a 60× oil immersion objective. *C. albicans* comprehend yeast, oval pseudohyphal, and hyphae cells. *S. mutans* presented as aggregates of colonies. Red arrows represent bindings between *C. albicans* and *S. mutans*. Scales bars = 50 μm.

D**iscussion**

In the oral cavity, biofilms establish interkingdom relationships, which can raise the potential to cause infections in case of a host imbalance (Lohse et al. 2018). *C. albicans* and *S. mutans* were among the most prevalent microorganisms in the mouth (Keijser et al. 2008; Milsop et al. 2016), synergizing during their interactions (Falsetta et al. 2014; Kim et al. 2021). On the *C. albicans* side, interacting with biofilms containing *S. mutans* enhances the fungi virulence by intensifying the switching from yeast to hyphae (Cavalcanti et al. 2015; Kim et al. 2021). Conversely, *C. albicans* produces an acidic environment, benefiting *S. mutans* metabolism (Klinke et al. 2009). In cooperation, Ca-Sm interactions lead to virulent and pathogenic biofilms (Cavalcanti et al. 2015) that could provoke prevalent oral diseases, such as oral candidiasis. Thus, comprehending how these microorganisms physically interact is relevant to create target therapies.

Binding among *C. albicans* and *S. mutans* occurs through cell-cell interactions by adhesins and proteins on the surface of both microorganisms (Hwang et al. 2015; Wan et al. 2021). It is well known that *S. mutans* GtfB binds to *C. albicans* cell wall (Gregoire et al. 2011), yet the fungi counterparts of this interaction are still a question mark. This investigation explores the roles of Als1, Als3, and Hwp1 in the interactions with *S. mutans*, because they are relevant adhesins to form *C. albicans* biofilms (Nobile et al. 2006a; Nobile et al. 2006b; Nobile et al. 2008). Thus, summarizing the findings of this study, Als1 is the major player interacting with *S. mutans*, and Als3 may play a lesser role in conjunction with Als1. In addition, the protein Hwp1 may play a secondary function since do not provide structure to form a robust biofilm.

In the absence of Als1, the presence of *S. mutans* does not modulate the metabolism, biomass, and viability when compared with single biofilms of *C. albicans* Als1 defective strain. This finding shed light on the absence of Als1 challenges the synergistic interactions between the fungi and bacteria, dropping the possibility of establishing a pathway between those

microorganisms. Clearly, microscopy images reveal that physical interactions among Ca-Sm lacking Als1 are still happening, yet substantially lesser when compared to wildtype biofilms. This interaction could be associated with other *C. albicans* ligand-binding, such as the *O*- and *N*-terminal of the mannans (Hwang et al. 2017) or other fungi cell wall components. However, in the absence of Als1, dual-biofilms were similar to single ones, highlighting the idea that Als1 is a crucial binding between *C. albicans* and *S. mutans*, perhaps more critical than other fungi proteins.

C. albicans Als1 is a cell-surface glycoprotein, which allows *C. albicans* to bind to itself, other microorganisms, abiotic surfaces (Zhao et al., 2022), and host epithelium (Kamai et al., 2002). In terms of binding other organisms, *C. albicans* strains defective in Als1 significantly impair the co-aggregation with another Streptococcus bacteria, the so-called *Streptococcus gordonii* (Klotz et al., 2007). This finding suggests that Als1 is a key player mediating interactions not only with *S. mutans* but also among other oral bacteria species.

Within the Als-family, Als1 and Als3 have 88% similarity in amino acid sequence (Sheppard et al. 2004), being adhesins with overlapping functions and required for *C. albicans* biofilm formation (Nobile et al. 2006). For that reason, we also investigate the roles of Als3 and Als1/Als3 during interactions with *S. mutans*. Interestingly, we observed that Ca-Sm biofilms formed with Als3 defective strain restored biofilm formation at wildtype levels. Furthermore, microscope images show several bindings among hyphae and *S. mutans* colonies. Previous investigations could explain this phenomenon because single *C. albicans* biofilms of *als3*Δ/Δ strain cause gene overexpression of *ALS1* (Nobile et al. 2008). Thus, we hypothesized that the absence of Als3 in dual-species biofilms also promotes *ALS1* overexpression, restoring the pathway to synergistic interactions between *C. albicans* and *S. mutans*. Therefore, this prediction argues that Als1 is required to establish the partnership aforementioned between Ca-Sm.

In addition, our results indicate that the absence of Als1/Als3 negatively impacts dual-species biofilm formation. As demonstrated in CFUs analysis, *C. albicans als1Δ/Δ /als3Δ/Δ* dual-species biofilms decrease colonies' formation compared with a single one. However, *C. albicans als1Δ/Δ* or *als3Δ/Δ* does not change its viability comparing single and dual. As these adhesins have complementary functions in single *C. albicans* biofilms and act in conjunction (Nobile et al. 2008), lacking them simultaneously decreased *C. albicans* ability to bind to *S. mutans*. Taken together, these findings suggest that Als3 has a lesser function in Ca-Sm interactions acting in conjunction with Als1. Therefore, the activity of Als3 in promoting fungi-bacteria adhesion is Als1-dependent. Although investigations about *C. albicans* and *S. mutans* biofilms are highlighted in the literature (Metwalli et al. 2013; Falsetta et al. 2014; Eidt et al. 2019; Sampaio et al. 2019; Kim et al. 2021), fewer studies explored the mechanisms behind the adhesive binding among these microorganisms (Hwang et al. 2015; Yang et al. 2018). One of them also investigates the role of Als1 and Als3, showing that these adhesins are not required for Ca-Sm interaction (Yang et al. 2018). These findings conflict with our results and could be explained due to differences between biofilm models. Yang and coworkers' formed a biofilm predominantly composed of yeasts and pseudohyphae in THB media. It is well-known that Als1 and Als3 are hypha-specific and are not present in the yeast form. Thus, only the presence of pseudo-hyphae could not provide the adhesins required for *C. albicans* to interact with *S. mutans*. Besides, THB media advantage *S. mutans* proliferation. On the other hand, in our study, dual-species biofilms were prevalent in hyphae (please see microscopy images), which allow *C. albicans* to express those adhesins. In addition, our study used RPMI-1640 as media, which is known for inducing hyphae formation (Kucharíková et al. 2011) and supports both microorganisms' proliferation (Heersema & Smyth, 2019). Therefore, we predict that a biofilm model mainly composed of hyphae better indicates the role of Als1 and Als3 in dual-biofilms.

Regarding the role of Hwp1, a protein expressed on *C. albicans* hyphae (Sundstrom, 2002), Ca-Sm biofilms formed with Hwp1 defective strain showed similar trends to single ones. CLSM imaging reveals that the architecture and thickness of single and dual-biofilms of *hwp1*Δ/Δ do not demonstrate differences. Either single or dual-biofilms formed a rudimentary layer (~ 5 - 20 μm in depth) without elongated hyphae compared to wild-strain. These findings suggest that *S. mutans* may have some difficulties with the fungi physically interacting with *C. albicans* lacking Hwp1. Since *hwp1*Δ/Δ is hyphae-deficient (Nobile et al. 2008), our first hypothesis was that *S. mutans* could not bind to *C. albicans* because it has more affinity for hyphae form. However, optical microscopy reveals that *S. mutans* bind to the *hwp1*Δ/Δ mutant strain, even though lesser than the wildtype. Thus, we predict that the challenge for *hwp1*Δ/Δ-Ca and *S. mutans* interaction is not due to a lack of protein but because this biofilm can not provide enough structure for *C. albicans* and *S. mutans* to interact and form a robust biofilm. Given these findings, we conclude that Hwp1 has a secondary role in *C. albicans* and *S. mutans* interactions.

In parallel to demonstrating the role of Als1, Als3, and Hwp1 in dual-species biofilms, this study also pointed out that *S. mutans* benefits from *C. albicans* relationship independent of the strain. Even without relevant *C. albicans* biofilm promoters, *S. mutans* is still proliferating, as demonstrated by CFU counting results. One explanation is that *C. albicans* is still creating a lower pH and oxygen microenvironment to assist *S. mutans* metabolism, even without Als1, Als3, and Hwp1. From the clinical viewpoint, this is relevant data since current therapies for oral candidiasis consider only fungi as a target. Hence, oral bacteria are unharmed to co-aggregate and metabolize in the biofilm. Thereby, future approaches to treating those infections should also consider targets for the bacteria in the oral microbiome.

Concerning the ability of *C. albicans* promotes infections, Als1, Als3, and Hwp1 are proteins required for the adhesion and invasion of epithelial cells (Ponniah et al. 2007; Phan et

al. 2007; Zhu et al. 2009). Mainly, Als3 is an invasin-like molecule that binds to host cell receptors and induces *C. albicans* endocytosis (Phan et al. 2007). As a limitation of this study, we did not explore the role of Als1, Als3, and Hwp1 in dual-biofilms during tissue invasion. This analysis could predict whether lacking those proteins in biofilms with *S. mutans* impairs the ability of *C. albicans* to invade epithelial tissues. Considering that *S. mutans* heightens *C. albicans* pathogenicity, we do not exclude the possibility of somehow *S. mutans* bypassing the lacking of *C. albicans* proteins to enable the fungi to invade the epithelium. Thus, we encourage future investigations to explore the role of Als1, Als3, and Hwp1 in dual-species biofilms.

Finally, research about therapeutic targets is highlighted due to the antimicrobial resistance problem worldwide (Lee et al. 2021). To date, novel therapy strategies targeting Als3 are under investigation (Kioshima et al. 2019; Singh et al. 2022). In fact, Als3 is relevant for *C. albicans* adhesion, co-aggregation, and tissue invasion (Liu & Filler, 2011). However, our results indicated that Als1 is the key player in forming dual-species biofilms with *S. mutans* in conjunction with Als3. In addition, Hwp1 develops a secondary function in this interaction. Therefore, our findings provide not only new insights into the mechanism of interaction between *C. albicans* and Streptococcus bacteria but further targets for experimental therapies.

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Data availability statement

Raw data were generated at University of Campinas. Derived data supporting the findings of this study are available from the corresponding author A.A.D.C. on request.

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3 DISCUSSÃO

Embora os antimicrobianos atuais utilizados para o tratamento da Candidíase oral sejam eficientes, há uma demanda global por alternativas a essas terapias, uma vez que os tratamentos atuais apresentam problemas como resistência antimicrobiana, ausência de alvo terapêutico, e seletividade, apenas para o fungo (Xu et al., 2014a; Bernard et al., 2020; Perlin et al., 2020; Lee et al., 2021). Portanto, pesquisas atuais têm focado em elucidar os mecanismos que medeiam a formação e as interações fúngico-bacterianas do biofilme, na tentativa de estabelecerem potenciais alvos terapêuticos. Dessa forma, será possível a formulação de novas terapias antimicrobianas que posam atuar sobre componentes específicos dos microrganismos que são relevantes para o estabelecimento do biofilme (Gozalbo et al., 2014). Isto posto, a Tese em pauta objetivou investigar os mecanismos que medeiam as interações entre *C. albicans* e *S. oralis*, e *C. albicans* e *S. mutans*, já que se trata de patógenos reconhecidos por estabelecerem relações de sinergismo e aumentarem a virulência do biofilme causador da Candidíase oral.

No primeiro estudo, evidências mostraram que os subprodutos de *S. oralis*, liberados extracelularmente, podem aumentar a virulência de biofilmes de *C. albicans*. Dessa maneira, considerando-se que a frequência e a severidade das lesões de Candidíase oral são maiores em biofilmes mistos de *C. albicans* e *S. oralis* (Xu et al., 2014b), novas terapias devem explorar como alvos terapêuticos moléculas *quorum-sensing* bacterianas. Esses achados, apesar de serem preliminares e apresentarem algumas limitações, sugerem um componente modulador da virulência do biofilme.

No segundo estudo explorou-se o papel das adesinas Als1, Als3 e Hwp1 de *C. albicans* na formação de biofilmes com *S. mutans*. Os achados desse estudo indicaram que Als1 é adesina fúngica que medeia as interações entre esses microrganismos. Além disso, evidenciou-se que as adesinas Als3 e Hwp1 têm um papel coadjuvante nas interações entre *C. albicans* e *S. mutans*. Embora a literatura seja rica em evidências sobre a relação de sinergismo entre esses microrganismos e sobre a maneira como esse biofilme fúngico-bacteriano contribui para a severidade de lesões cariosas (Falsetta et al., 2014), o ponto de vista da Candidíase oral e dos componentes da parede celular de *C. albicans* que interagem com *S. mutans* ainda é pouco explorado. Dessa forma, o presente estudo apresenta novidades relevantes ao atual estado da arte, uma vez que evidencia três componentes da parede celular de *C. albicans* que medeiam as interações com a bactéria.

Como limitações, não foi avaliada a patogenicidade dos biofilmes. Nesse estudo evidenciou-se que moléculas *quorum-sensing* de *S. oralis* aumentam a virulência de *C. albicans*

e sabe-se que, na forma de hifa, o fungo é capaz de penetrar na mucosa oral (Mayer et al., 2013). Adicionalmente, é bem estabelecido que as adesinas Als1, Als3 e Hwp1 desempenham um papel importante na adesão de *C. albicans* às células epiteliais do hospedeiro (Gulatti & Nobile, 2016). Entretanto, o papel dos subprodutos de *S. oralis* e das adesinas de *C. albicans* na patogenicidade do biofilme ainda não foi elucidado. Por meio dessa avaliação será possível elucidar a defesa inicial dada pela barreira epitelial à infecção por um biofilme fúngico-bacteriano. Assim, para avaliação do dano tecidual inicial provocado por um biofilme, podem ser utilizados modelos *in vitro* de epitélio oral humano (Dongari-Bagtzoglou et al., 2016; Klausner et al., 2021). Embora não seja possível introduzir células do sistema imune, estes modelos têm sido amplamente empregados, uma vez que permitem visualizar a invasão tecidual inicial e verificar o dano causado pelos microrganismos aos tecidos (Dongari-Bagtzoglou et al., 2016; Klausner et al., 2021). Portanto, sugere-se que investigações futuras explorem o papel dos subprodutos de *S. oralis* e das adesinas Als1, Als3 e Hwp1 de *C. albicans* na patogenicidade do biofilme.

Coletivamente, os achados indicam componentes fúngico-bacterianos que medeiam interações no biofilme oral e podem ser utilizados como potenciais alvos terapêuticos. Atualmente, as Equinocandinas são uma classe de drogas baseadas na estratégia de alvo terapêutico (Perlin et al., 2010). Esses fármacos são representados pela Caspofungina, Micafungina e Anidulafungina, tendo sido aprovados pela *Food and Drug Administration* (FDA) dos Estados Unidos, desde 2001. Essas drogas têm como alvo a síntese de β-1,3-glucano, um importante constituinte da parede celular de *C. albicans*. Nos últimos vinte anos, estudos clínicos evidenciaram que essas drogas são altamente eficazes e possuem pouca resistência antimicrobiana (Pappas et al., 2016). Apesar do considerável sucesso clínico com o uso de Equinocandinas, a parede celular de *C. albicans* é uma estrutura dinâmica e complexa em sua composição (Gozalbo et al., 2004). Portanto, outros alvos na parede celular ou subprodutos liberados extracelularmente, como os apontados no presente estudo, devem ser explorados.

4 CONCLUSÃO

Com base nos resultados obtidos no presente estudo, conclui-se que em biofilmes mistos de *Candida albicans* e *Steptotoccus oralis*, os subprodutos liberados extracelularmente pela bactéria medeiam as relações de sinergismo entre esses patógenos e a virulência do fungo. Ademais, as adesinas Als1, Als3 e Hwp1 de *Candida albicans* são os componentes fúngicos responsáveis por interagir com *Streptococcus mutans*. Portanto, do ponto de vista clínico, moléculas *quorum-sensing* de *S. oralis* e adesinas Als1, Als3 e Hwp1 de *C. albicans* são promissores alvos terapêuticos para o tratamento da Candidíase oral.

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ANEXOS

Anexo 1: Parecer consubstanciado do Comitê de Ética em Pesquisa da Faculdade de Odontologia de Piracicaba/Unicamp

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DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Virulência e patogenicidade de biofilmes duo-espécies de Candida albicans e Streptococcus spp

Pesquisador: ALTAIR ANTONINHA DEL BEL CURY

Área Temática:

Versão: 2

CAAE: 35443220.0.0000.5418

Instituição Proponente: Faculdade de Odontologia de Piracicaba - Unicamp

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.234.284

Apresentação do Projeto:

Transcrição editada do conteúdo do registro do protocolo e dos arquivos anexados à Plataforma Brasil

A EQUIPE DE PESQUISA citada na capa do projeto de pesquisa inclui ALTAIR ANTONINHA DEL BEL CURY (Cirurgiã-Dentista, Docente da Área de Prótese Parcial Removível da FOP-UNICAMP, Pesquisadora responsável, Orientadora) e LOYSE MARTORANO FERNANDES (Cirurgiã-Dentista, Doutoranda no PPG em Clínica Odontológica da FOP-UNICAMP, Orientanda), o que é confirmado na declaração dos pesquisadores e na PB.

Delineamento e resumo da pesquisa: Trata-se de estudo experimental, laboratorial, comparativo, transversal, in vitro, cego e aleatorizado (em relação às análises) que envolverá amostras de saliva total estimulada de três indivíduos adultos, sem distinção de sexo, alunos de Pós-Graduação da FOP-UNICAMP. Objetivo: o objetivo deste estudo será avaliar a interação e patogenicidade de biofilmes duo-espécie de Candida albicans e Streptococcus spp, e analisar a virulência destes biofilmes em contato com o modelo in vitro de epitélio oral. Metodologia: Os biofilmes de Candida albicans (SC5314) e de Streptococcus spp serão desenvolvidos sobre superfícies de poli(metil)metacrilato. Anteriormente ao desenvolvimento do biofilme será formada a película salivar, a qual a saliva humana será coletados de três voluntários saudáveis, após concordar com o termo de

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PIRACICABA, 25 de Agosto de 2020

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jacks jorge junior
(Coordenador(a))

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