UNESP - Universidade Estadual Paulista

"Júlio de Mesquita Filho"

Faculdade de Odontologia de Araraquara

KÁSSIA DE CARVALHO DIAS

EFEITO DAS TOXINAS MICROBIANAS PROVENIENTES DE BIOFILME SIMPLES OU MISTO DE *Staphylococcus aureus* E *Candida albicans* SOBRE MONOCULTURAS OU CULTURAS 3D DE CÉLULAS DA MUCOSA ORAL

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Orientador: Prof. Dr. Carlos Eduardo Vergani
Co-orientadora: Dra. Paula Aboud Barbugli

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DADOS CURRICULARES

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Onde houver erro, que eu leve a verdade

Onde houver desespero, que eu leve a esperança

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Dias KC. Efeito das toxinas microbianas provenientes de biofilme simples ou misto de *Staphylococcus aureus* e *Candida albicans* sobre monoculturas ou culturas 3D de células da mucosa oral [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2016.

RESUMO

Esta presente tese foi dividida em quatro estudos que tiveram como objetivos. 1. Validar um protocolo e comparar o efeito dos tampões RPMI/MOPS e RPMI/HEPES no desenvolvimento de biofilmes e na viabilidade celular de queratinócitos (NOK-si e HaCat); 2. Comparar o dano celular e a resposta inflamatória induzidos pelos metabólitos de biofilmes simples e misto de *Staphylococcus aureus* e *Candida albicans;* 3. Avaliar o tipo de morte celular (apoptose *vs*. necrose) e a ativação de caspases relacionadas aos metabólitos desses biofilmes e 4. Caracterizar um tecido oral reconstituído e analisar o dano tecidual causado pelo sobrenadante e biofilme propriamente dito desses microrganismos. No estudo 1, a viabilidade celular foi avaliada pelo método colorimétrico do MTT e por imagens da cultura após 12 horas em contato com os meios de cultura. Ambos os tampões permitiram similar crescimento do biofilme. Efeito citotóxico do MOPS foi verificado após 6 horas de crescimento de NOK-si e HaCat. Houve preservação da viabilidade e morfologia quando as células foram expostas a RPMI/HEPES. Conclui-se que RPMI/HEPES pode ser utilizado como um meio tamponamente viável para estudos que avaliam o efeito do biofilme em cultura de queratinócitos ao longo do tempo. No estudo 2, o sobrenadante dos biofilmes de 36 h de *C. albicans* e *S. aureus*, isolados ou em associação, foi colocado em contato com NOK-si, HaCat e macrófagos (J774A.1). O dano celular foi avaliado por meio de ensaios de viabilidade celular (MTT) e liberação da enzima LDH. A produção de citocinas foi analisada pelo método de ELISA e a avaliação do tipo de morte celular pela marcação das células apoptóticas com anexina V e necróticas com iodeto de propídio. Biofilme misto e de *C. albicans* foram mais citotóxicos e o biofilme misto causou maior dano celular pela liberação da enzima LDH. Metabólitos do biofilme de *S. aureus* estimularam maior produção de NO, IL-6 e TNF-α. Maior quantidade de células marcadas com anexina V (apoptose) foram detectadas após exposição ao sobrenadante de *C. albicans*, e maior marcação para o iodeto de propídio (necrose) para as células expostas ao sobrenadante do biofilme misto. Os metabólitos de biofilmes mistos foram capazes de induzir maior dano celular e os metabólitos de *S. aureus*, maior resposta inflamatória. No estudo 3, a indução de morte celular em cultura de NOK-si foi avaliada após o contato com sobrenadante de biofilmes simples ou mistos, com diferentes tempos de formação (08, 16 e 24 horas). As células

microbianas foram quantificadas em UFC/mL e os danos aos queratinócitos foram avaliados pelos ensaios de MTT e de liberação da enzima LDH. Houve marcação de apoptose e necrose como no estudo anterior e ensaio colorimétrico para detecção dos marcadores de apoptose celular (caspases). A morfologia celular dos queratinócitos foi avaliada por meio de microscopia eletrônica de varredura (MEV). Houve um aumento progressivo nos valores de UFC/mL obtidos dos biofilmes de 08 horas para 16 horas, tanto para os biofilmes simples, quanto para os mistos. Houve uma redução progressiva na viabilidade celular após exposição ao biofilme de 16 horas quando comparado ao biofilme de 08 e 24 horas. As imagens de MEV mostraram uma diminuição das microvilosidades e a formação de vesículas na membrana das células expostas ao sobrenadante do biofilme de 16 horas. Houve também maior marcação de anexina V quando as células foram expostas ao sobrenadante de biofilmes simples de *C. albicans* e *S. aureus*; e com iodeto de propídio para o biofilme misto. A apoptose induzida pelos sobrenadantes do biofilme de *S. aureus* e misto foi associada à ativação das caspases -2, -3, -6 e -8; e o aumento da atividade da caspase -3 para sobrenadante de *C. albicans*. Assim, maior dano celular pode ser observado quando queratinócitos foram expostos a metabólitos dos microrganismos em sinergismo no biofilme. No estudo 4, tecido oral reconstituído foi obtido pela formação in vitro de um equivalente de tecido conjuntivo (fibroblastos e colágeno) e uma camada epitelial (queratinócitos). A caracterização histológica foi obtida pelas colorações de Hematoxilina-Eosina, Ácido Periódico de Schiff - P.A.S. e Picrosirius e a caracterização imunohistoquímica pela evidenciação de citoqueratina 13 e 14, Ki-67 e colágeno IV. A análise do dano causado ao tecido pelo sobrenadante e biofilmes, com 08 e 16 horas, foi observada por meio da liberação da enzima LDH e avaliação histológica (Hematoxilina-Eosina). O biofilme misto foi responsável pela maior destruição tecidual. O tecido epitelial reconstituído apresentou características histológicas e imunohistoquímicas semelhantes ao epitélio oral e pode ser utilizado para investigar padrões de infecção causada por biofilmes em culturas simples e mista de *C. albicans* e *S. aureus*.

Palavras-chave: Biofilmes. Queratinócitos. *Candida albicans*. *Staphylococcus aureus*. Necrose. Apoptose.

Dias KC. Effect of microbial toxins from single or mixed biofilms of *Staphylococcus aureus* and *Candida albicans* on monocultures and 3D cultures of oral mucosal cells [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2016.

ABSTRACT

The present thesis was divided into four studies with the following objectives. 1. Validate a protocol and compare the effect of RPMI/MOPS and RPMI/HEPES buffers on the development of biofilms and keratinocyte cell viability (NOK-si and HaCat); 2. Compare the cellular damage and the inflammatory response induced by the metabolites of simple and mixed biofilms of *Staphylococcus aureus* and *Candida albicans*; 3. Evaluate the type of cell death (apoptosis vs. necrosis) and the activation of caspases related to the metabolites of these biofilms and 4. Characterize the reconstituted oral tissue and analyze the tissue damage caused by the supernatant and biofilm of these microorganisms. In study 1, cell viability was evaluated by the MTT colorimetric method and by culture images after 12 hours in contact with the culture media. Both buffers permitted similar biofilm growth. The cytotoxic effect of MOPS was observed after six hours of NOK-si and HaCat growth. There was preservation of viability and morphology when cells were exposed to RPMI/HEPES. It was concluded that RPMI/HEPES can be used as a buffering medium for studies evaluating the effect of biofilm on keratinocyte culture over time. In study 2, the supernatant of the 36-hour biofilms of *C. albicans* and *S. aureus*, isolated or in combination, was placed in contact with NOK-si, HaCat and macrophages (J774A.1). Cell damage was assessed by cell viability assays (MTT) and LDH enzyme release. Cytokine production was analyzed by the ELISA method and evaluation of the type of cell death by the staining of the apoptotic cells with annexin V and the necrotic cells with propidium iodide. The mixed biofilm and biofilm of *C. albicans* were more cytotoxic, and the mixed biofilm caused greater cellular damage through the release of the LDH enzyme. *S. aureus* biofilm metabolites stimulated greater production of NO, IL-6 and TNF-α. Greater numbers of cells labeled with annexin V (apoptosis) were detected after exposure to the supernatant of *C. albicans*, and there was greater propidium iodide staining (necrosis) for cells exposed to the supernatant of the mixed biofilm. The mixed biofilm metabolites were able to induce greater cellular damage and the metabolites of *S. aureus* produced a greater inflammatory response. In study 3, cell death induction in NOK-si culture was evaluated after contact with the supernatant of single or mixed biofilms, with different formation times (08, 16 and 24 hours). Microbial cells were quantified in terms of CFU/mL and damage to keratinocytes was assessed by MTT

assay and LDH enzyme release. Apoptosis and necrosis staining was evaluated as in the previous study and colorimetric assay was performed for the detection of cellular apoptosis markers (caspases). The cellular morphology of the keratinocytes was evaluated by scanning electron microscopy (SEM). There was a progressive increase in the CFU/mL values obtained from biofilms from eight to 16 hours for both single and mixed biofilms. There was a progressive reduction in cell viability after exposure to the 16-hour biofilm in comparison with the 08 and 24-hour biofilm. SEM images exhibited a decrease in microvilli and the formation of vesicles on the membrane of the cells exposed to the supernatant of the 16-hour biofilm. There was also greater annexin V staining when the cells were exposed to the supernatant of the single biofilms of *C. albicans* and *S. aureus*; and greater propidium iodide staining with the mixed biofilm. Apoptosis induced by supernatants of *S. aureus* biofilm and mixed biofilm was associated with the activation of caspases -2, -3, -6 and -8, while increased caspase -3 activity was associated with the *C. albicans* supernatant. Thus, greater cellular damage can be observed when keratinocytes were exposed to the metabolites of the microorganisms in synergism in biofilm. In study 4, reconstituted oral tissue was obtained by the *in vitro* formation of an equivalent of connective tissue (fibroblasts and collagen) and an epithelial layer (keratinocytes). Histological characterization was performed by Hematoxylin-Eosin, Periodic Acid Schiff - P.A.S. and Picrosirius staining, and immunohistochemical characterization by cytokeratin 13 and 14, Ki-67 and collagen IV. The analysis of the tissue damage caused by the supernatant and biofilms at eight and 16 hours was observed through the release of the LDH enzyme and histological evaluation (Hematoxylin-Eosin). The mixed biofilm was responsible for the greatest tissue destruction. The reconstituted epithelial tissue presented histological and immunohistochemical characteristics similar to the oral epithelium and can be used to investigate infection patterns caused by biofilms of *C. albicans* and *S. aureus* in single and mixed cultures.

Keywords: Biofilms. Keratinocytes. *Candida albicans*. *Staphylococcus aureus*. Necrosis. Apoptosis.

LISTA DE SIGLAS E ABREVIATURAS

3D	Three-dimensional
C. albicans	*Candida albicans*
CK	Cytokeratin
ELISA	Enzyme-Linked Immunosorbent Assay
HaCat	Human adult skin keratinocytes propagated under low Ca2+ conditions and elevated temperature
HE	Haematoxylin and eosin
HEPES	N-(2-hidroxietil)piperazina-N'-(2-ácido etanosulfônico)
IL-6	Interleukin-6
LDH	Lactate dehydrogenase
MOPS	3-[N-morfolino]propano-ácido sulfônico
MSSA	Methicillin-susceptible *Staphylococcus aureus*
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NO	Nitric oxide
NOK-si	Spontaneously immortalized normal oral keratinocytes
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
PI	Propidium iodide
RPMI	Roswell Park Memorial Institute
S. aureus	*Staphylococcus aureus*
SEM	Scanning electron microscope
TEM	Transmission electron microscopy
TNF-α	Tumour Necrosis Factor alpha
UFC/mL	Colony-forming unit per millilitre

SUMÁRIO

1 INTRODUÇÃO

Os microrganismos presentes na cavidade bucal apresentam a capacidade de se aglutinar e interagir, promovendo o desenvolvimento de biofilmes polimicrobianos (Chandra et al.[5], 2001; Douglas[11], 2003), que correspondem a uma comunidade de células sésseis aderidas a um substrato, embebidas em uma matriz de polímeros extracelulares (Finkel, Mitchel[12], 2011). A presença de *Staphylococcus aureus*, uma bactéria gram-positiva; e *Candida albicans*, um fungo pleomórfico, nesses biofilmes tem sido relacionada à maior frequência e severidade de doenças, como, por exemplo, no desenvolvimento de infecções sistêmicas (Morales et al.[23], 2010). Co-infecções, especialmente na presença de biofilmes de espécies mistas, são de difícil tratamento (Li et al.[19], 2015). A relevância clínica da presença dos biofilmes nas infecções humanas relaciona-se ao fato dessa estrutura complexa apresentar características fenotípicas distintas das células microbianas no estado planctônico, pois são mais tolerantes aos agentes antimicrobianos e menos vulneráveis às defesas do hospedeiro (Costerton et al.[6], 1995; Gilbert et al.[13], 1997; Habash et al.[14], 1999).

Embora *C. albicans* seja um organismo comensal, esse microrganismo é capaz de produzir infecções oportunistas quando o equilíbrio entre o patógeno e o hospedeiro é comprometido (Lynch[21], 1994). Como resultado de complexas interações, diversas manifestações clínicas da infecção com *C. albicans* têm sido relatadas, desde envolvimentos mucosos superficiais até óbitos de indivíduos por infecções disseminadas, em decorrência da falência de múltiplos órgãos (Lynch[21], 1994; Dalle et al.[7], 2010). Esses microrganismos apresentam a capacidade de colonizar a mucosa oral e superfícies duras, como os dentes e as próteses dentárias, formando assim, estruturas complexas de biofilmes polimicrobianos (Zijnge et al.[40], 2010). A candidose é a infecção fúngica mais comum encontrada na boca, sendo a espécie *C. albicans* a mais prevalente (Akpan et al.[1], 2002). *C. albicans* é capaz de se aderir à superfície das próteses, de modo que as mesmas podem atuar não só como um fator de predisposição, mas também como reservatório para esse microrganismo, sendo este fator considerado relevante para que as reinfecções ocorram (Von Fraunhofer et al.[39], 2009). A patogenicidade dos fungos do gênero *Candida* tem sido atribuída a sua capacidade de aderir e formar biofilmes, bem como de liberar enzimas hidrolíticas (Sánchez-Vargas et al.[30], 2013).

O microrganismo *S. aureus*, apesar de também ser considerado um colonizador normal da cavidade oral (Sánchez-Vargas et al.[31], 2002), tem sido reconhecido como um dos principais patógenos humanos, devido à sua elevada virulência e por apresentar capacidade de

desenvolver resistência às terapias medicamentosas (Chadwick[4], 1999). *S. aureus*, quando presente em infecções polimicrobianas, apresenta relação de cooperação com *C. albicans*, exibindo um aumento da severidade de doenças como no caso da estomatite protética (Nair et al.[25], 2014), manifestação de uma forma de candidose que afeta normalmente o palato de 42,4% dos pacientes usuários de próteses dentais removíveis (Marchini et al.[22], 2004).

As interações entre fungos e bactérias estão presentes na natureza e têm grande relevância clínica. Assim, estudos sobre biofilmes mistos de espécies de *Candida* associados com bactérias têm despertado interesse sobre o papel das interações entre espécies de reinos diferentes, em sua fisiologia e virulência (Thein et al.[35], 2009; Sztajer et al.[34]; 2014). No biofilme, os microrganismos desenvolvem várias relações de cooperação, de forma sinérgica ou inibitória, que influenciam na patogenia e na saúde do paciente (Peleg et al.[27], 2010). Além da elevada prevalência e capacidade de causar infecções severas, tanto locais quanto sistêmicas, a capacidade que os microrganismos *S. aureus* e *C. albicans* cooperarem entre si, promove um sinergismo entre as espécies (Shirtliff et al.[32], 2009).

Harriot e Noverr[15] (2009) demonstraram sinergismo na relação entre *C. albicans* e *S. aureus* em modelo de biofilme heterotípico propiciando maior resistência à vancomicina, ao estudar a interação in vitro entre essas espécies. Peters et al.[28] (2010) também demonstraram que as bactérias possuem capacidade de aderência às células de *C. albicans*, sendo *S. aureus* a espécie bacteriana com maior capacidade de aderência às hifas de *Candida*.

A interação entre *S. aureus* e *C. albicans* resulta em forte resposta inflamatória em infecções polimicrobianas. A capacidade dos microrganismos de induzir danos às células do hospedeiro pode desempenhar um papel na promoção de respostas pró-inflamatórias que resultam no recrutamento e ativação de células imunes, como neutrófilos e macrófagos (Villar, Zhao[38], 2010). Citocinas pró-inflamatórias locais e sistêmicas (Interleucina-6, Fator de Necrose Tumoral-Alfa, Interleucina-1β) apresentaram níveis significativamente elevados durante a co-infecção em momentos iniciais, quando comparados à infecção simples, independente da morfogênese de *C. albicans* (Nash et al.[26], 2014). Tem sido relatado na literatura que Als3 é uma proteína de superfície de *C. albicans* responsável por regular a sua aderência ao *S. aureus*, sendo um receptor importante na ligação de bactérias com hifas de *C. albicans* (Demuyser et al.[9], 2014). Por sua vez, *S. aureus* produz diferentes proteases que aumentam a adesão de *C. albicans* à mucosa oral (Nair et al.[25], 2014).

A mucosa oral é constituída pela associação do epitélio, rico em queratinócitos, e tecido conjuntivo, rico em fibroblastos, responsáveis por sintetizar colágeno e substâncias de matriz tecidual. A presença de *C. albicans* no epitélio oral é capaz de estimular vias de sinalização que

promovem a morte celular por apoptose, mediante a ativação de caspases celulares, seguida de necrose tardia (Villar, Zhao[38], 2010). A síntese de enzimas degradativas específicas com atividade proteolítica, como as proteases e fosfolipases, são características desses patógenos, promovendo a destruição de células e tecidos colonizados por esses microrganismos (Pinto et al.[29], 2008; Motta-Silva et al.[24], 2010).

Os fatores de virulência utilizados por *S. aureus* podem ser constituintes da parede celular, que contribuem para indução do dano, como o ácido teicóico, ácido lipoteicóico e proteína A; podem ser enzimas produzidas por esse microrganismo, que participam do mecanismo de patogenicidade, como, por exemplo, a coagulase, hialuronidade e catalase; ou ainda podem ser toxinas microbianas, como a alfa, beta, delta e gama-hemolisina. Essas toxinas desempenham um importante papel na agressão tecidual, à medida que formam poros na membrana e causam a destruição da célula do hospedeiro. (Dinges et al.[10], 2000; Archer et al.[3], 2011). A perda da viabilidade e a indução de apoptose em queratinócitos humanos foi observada quando as células foram expostas aos biofilmes de *S. aureus.* (Kirker et al.,[17] 2009).

A apoptose e a necrose são mecanismos pelos quais as células eucarióticas podem morrer. A apoptose está envolvida em vários processos fisiológicos, incluindo a homeostase celular e morfogênese, como também ocorre em resposta às condições de estresse, tais como infecções intracelulares (Thompson[36], 1995; Zong et al.[41], 2006; Alberts et al.[2], 2008). A ativação das caspases resulta em traços característicos de apoptose, os quais incluem condensação citoplasmática e nuclear, ruptura da membrana mitocondrial, fragmentação do DNA e manutenção da membrana plasmática intacta (Thompson[36], 1995; Zong et al.[41], 2006; Alberts et al.[2], 2008). O conteúdo das células apoptóticas é removido por fagocitose, resultando na ausência de inflamação (Thompson[36], 1995; Zong et al.[41], 2006; Alberts et al.[2], 2008). Por outro lado, a necrose é considerada como sendo uma reação patológica às principais perturbações no ambiente celular, conduzindo ao aumento de volume do citoplasma, a lise osmótica e a liberação de conteúdos intracelulares para o meio extracelular, desencadeando resposta inflamatória (Thompson[36], 1995; Zong et al.[41], 2006; Alberts et al.[2], 2008).

As vias apoptóticas dependentes das caspases podem ser ativadas durante infecções causadas por *C. albicans* e *S. aureus* e isso pode contribuir para a morte celular induzida por esses microrganismos (Villar, Zhao[38], 2010; Hu et al.[16], 2009). As vias intrínseca e extrínseca foram identificadas nos casos de apoptose dependente das caspases (Sun et al.[33], 1999; De Martino et al.[8], 2010). A apoptose induzida pela ativação da via intrínseca é iniciada pela interrupção da integridade mitocondrial e a translocação do citocromo c para o citosol que catalisa a formação do apoptossomo e conduz a ativação da caspase iniciadora 9, que por sua

vez, cliva e ativa a caspase efetora 3, responsável pela amplificação proteolítica (Lavrik et al.[18], 2005). Já a via de morte extrínseca é desencadeada pela sinalização dos receptores de morte localizados na superfície da célula, que incluem o receptor de fator de necrose tumoral ou ligante Fas, por exemplo, e isso é capaz de ativar a cascata das caspases (Locksley et al.[20], 2001). A apoptose induzida por *S. aureus* está associada à ativação das caspase-3 e caspase-8 (Hu et al.[16], 2009) e o aumento da atividade das caspases 3 e 9 foi observado durante as primeiras horas de infecção por *C. albicans* (Villar et al.[37], 2012).

Para o estabelecimento de estratégias de tratamento mais eficientes para as infecções que envolvem a presença conjunta de *C. albicans* e *S. aureus*, é fundamental compreendermos, mais detalhadamente, o impacto da interação microrganismo-hospedeiro nos processos de resposta inflamatória e indução de morte celular. Dessa forma, o presente estudo tem como objetivo responder questões ainda pouco abordadas sobre o tema, como o comportamento em comunidade desses microrganismos e o seu efeito sinérgico na indução de morte celular e no dano tecidual.

2 PROPOSIÇÃO

O objetivo do presente estudo foi caracterizar o biofilme simples e misto de *C. albicans* e *S. aureus* e comparar o efeito do sobrenadante desses biofilmes na viabilidade celular, liberação da enzima lactato desidrogenase, tipo de morte celular e indução de resposta inflamatória in vitro, em cultura de células em monocamada (NOK-si e HaCat). Além de caracterizar um tecido epitelial reconstituído e analisar o dano causado pelo sobrenadante e biofilme propriamente dito. Dessa forma, os estudos foram conduzidos com os seguintes objetivos específicos:

1. Comparar o efeito da utilização de dois diferentes tampões (MOPS e HEPES) em combinação com o RPMI no desenvolvimento de biofilmes e na viabilidade celular de queratinócitos;
2. Avaliar a citotoxicidade e o tipo de morte celular (apoptose *vs.* necrose) induzida pelo sobrenadante dos biofilmes simples e mistos de *S. aureus* e *C. albicans* em queratinócitos;
3. Analisar o efeito do sobrenadante de biofilmes simples e mistos de *S. aureus* e *C. albicans* na produção de citocinas pro-inflamatórias por macrófagos;
4. Desenvolver, validar um tecido epitelial oral reconstituído e comparar os danos teciduais produzidos por biofilmes simples e mistos de *S. aureus* e *C. albicans* e por seus respectivos sobrenadantes, utilizando o tecido desenvolvido experimentalmente.

3 PUBLICAÇÃO

3.1 Publicação 1

Influence of different buffers (HEPES/MOPS) on keratinocyte cell viability and microbial growth*

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Abstract

This study assessed the effect of the buffers *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid* (HEPES) and 3-(N-morpholino) propanesulfonic acid (MOPS) on Keratinocyte cell viability and microbial growth. It was observed that RPMI buffered with HEPES, supplemented with L-glutamine and sodium bicarbonate, can be used as a more suitable medium to promote co-culture.

Highlights:

- MOPS buffer (164 mM) is toxic for keratinocytes cells.
- HEPES buffer promotes the development of biofilm efficiently.
- HEPES buffer (25 mM) is non-toxic for keratinocytes cells.
- RPMI buffered with HEPES can be used as a viable alternative.

Keywords: Cell Survival; Keratinocytes, HEPES, *Candida albicans*, *Staphylococcus aureus*.

Short Communication

Studies of co-culture with keratinocytes cells and microorganisms have been fundamentally important in assessing the virulence and cytotoxicity of biofilms. The loss of keratinocytes cell viability when cells are exposed to biofilms of *Staphylococcus aureus* can be observed after 3 hours of exposure[1]. The co-culture of *Candida albicans* and oral epithelium cells stimulate signaling pathways that promote cell death[2].

The culture medium used for biofilm growth should not interfere with cell viability. Although RPMI (Roswell Park Memorial Institute) buffered with MOPS has been used, the concentration of MOPS[4] is greater than that permitted for cell cultures[3], and could compromise co-culture studies. As an alternative, HEPES could be used for biofilm formation of *C. albicans* and *S. aureus*[5]. The present research compared the effect of RPMI buffered with HEPES and MOPS on keratinocyte cell viability and microbial growth.

C. albicans SC5314 and *S. aureus* ATCC 25923 microorganisms were used to produce single and dual species biofilms, in accordance with the methodology described by Zago et al. (2015)[4]. To prepare the yeast and bacteria pre inoculum, a loop full of agar stock cultures was

transferred to 10 mL of Yeast Nitrogen Base broth (YNB - Difco, Becton Dickinson Sparks, MD, USA) supplemented with 100 mM glucose and Tryptic Soy Broth (TSB - Acumedia Manufactures Inc., Baltimore,MD, USA), respectively, and incubated at 37°C overnight. Thereafter, the dilution of the inoculum was performed and cultures were incubated until they reached mid-exponential grow phase. Cells of the resultant cultures were harvested and washed twice with sterile phosphate-buffered saline solution (PBS, pH 7.2) in sterile tubes at 5,000 × g for 5 min.

Microorganisms were re-suspended (about 10^7 cells per mL^{-1}) in culture medium RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA), using two different buffers: Buffer 1 (pH 7.0±0.1, 164 mM MOPS) (Sigma-Aldrich, St. Louis, MO); Buffer 2 (pH 7.0±0.1, 25 mM HEPES) (Sigma-Aldrich, St. Louis, MO) 2 mM L-glutamine (Lonza, Walkersville, USA), and 2.0 g/L sodium bicarbonate (Synth, São Paulo, Brazil). The adhesion of microorganisms was performed on 24-wells sterile plates (TPP Techno Plastic Products AG, Switzerland) at 37°C in an orbital shaker (75 rpm) for 90 min[6]. The pH was measured using a benchtop pH meter (QX 1500 Plus-Qualxtron, São Paulo, Brazil).

Cell cultures were prepared with the Normal Oral Keratinocytes (NOK-SI)[7] and Human Keratinocyte Cell Line (HaCat) (BCRJ 0341). The cells were cultivated until they reached confluency (48 hours), washed with 10 mM PBS, centrifuged and re-suspended in Dulbecco's Modified Eagle Medium (DMEM; (Gibco BRL, Grand Island, NJ, USA) and placed (about 4.5 x 10^4 cells/well) in 24 well plates. Live cells were counted in a Neubauer chamber (magnification ×10).

MTT assay [3-(4.5- dimethylthiazole-2-yl)2.5- diphenyl tetrazolium bromide] (Sigma-Aldrich, St. Louis, MO, USA) was performed at 02, 04, 06, 08 and 12 hours after incubation at 37ºC in 5% CO_2 with the RPMI/MOPS, RPMI/HEPES and DMEM (control cells). After each period of contact, the culture mediums were removed and 250 µl of MTT/PBS solution (5.0 $mg.mL^{-1}$) were added to each well and incubated for 4 hours at 37ºC. Next, MTT solution was removed and the formazan crystals were solubilized in 250 µL of 2- propanol[8]. The spectrophotometric measurements were performed at 562 nm (Reader 400 EZ – Biochrom**,** Cambridge, UK), using isopropanol as the blank.

To observe the changes in morphology, cells were analyzed and photographed under a Leica DMI 3000B inverted microscope, after 12 hours of contact with the culture media.

The colony forming units (CFU/mL) and cell viability (MTT) results were statistically evaluated using ANOVA one-way followed by Tukey's test for significance $p<0.05$. The values

were expressed as the mean and standard deviation of three independent replications, in triplicate for each experimental condition (n=9).

As RPMI supplemented with 25 mM of HEPES did not result in the growth of *S. aureus* biofilm, a further experiment was conducted where RPMI/HEPES was supplemented with L-glutamine (2.0 mM) and sodium bicarbonate (2.0 g/L). The biofilm results indicated a slight change in pH from 7.0 to a maximum of 7.8 after 24 hours. However, there was no difference between RPMI buffered with MOPS and HEPES (L-glutamine/sodium bicarbonate), for the number of CFU/mL variable, showing that both buffers promoted similar biofilm growth (Table 1).

Keratinocyte cell viability was studied for up to 24 hours of incubation (data not shown) with the media RPMI/HEPES pH 7.0 and RPMI/HEPES pH 8.2, resulting in a cell viability of 100% for NOK-SI and HaCat in both conditions (Figure 1a/1b).

The cytotoxic effect of MOPS was not observed over short periods. However, the kinetic study revealed high cytotoxicity after 6 hours, with similar results observed for the two cell lines (Figure 1a/1b). These results demonstrate that co-culture protocols using MOPS as buffer may not be valid, particularly for periods of incubation longer than 4 hours. These results agree with existing literature regarding the toxicity of this buffer in mammalian cells in concentrations greater than 20 mM[3]. The HEPES buffer has buffering properties similar to MOPS and has been used for *S. aureus* and *C. albicans* biofilm formation[5]. The medium prepared with RPMI/HEPES 25 mM, supplemented with L-glutamine (2mM) and sodium bicarbonate (2.0g/L) was capable of promoting both the growth of single and dual species *C. albicans* and *S. aureus* biofilms, while maintaining the cell viability of two keratinocytes cell lines around 100% until up to 12 hours of incubation (Table 1, Table 2 and Figure 1b). The period of 12 hours was selected to examine the morphology of the cells exposed to the different media. When cells were exposed to the RPMI/MOPS medium, a major change in viability, about 80% of cell death, were identified through MTT (Figure 1a/1b). From Figure 2, cellular degradation in RPMI/MOPS and cell proliferation in RPMI/HEPES can be observed.

The RPMI/HEPES medium supplemented with L-glutamine (2.0 mM) and sodium bicarbonate (2.0 g/L) can be used as a viable alternative for studies willing to investigate the effects of biofilms on keratinocytes over time.

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Tables

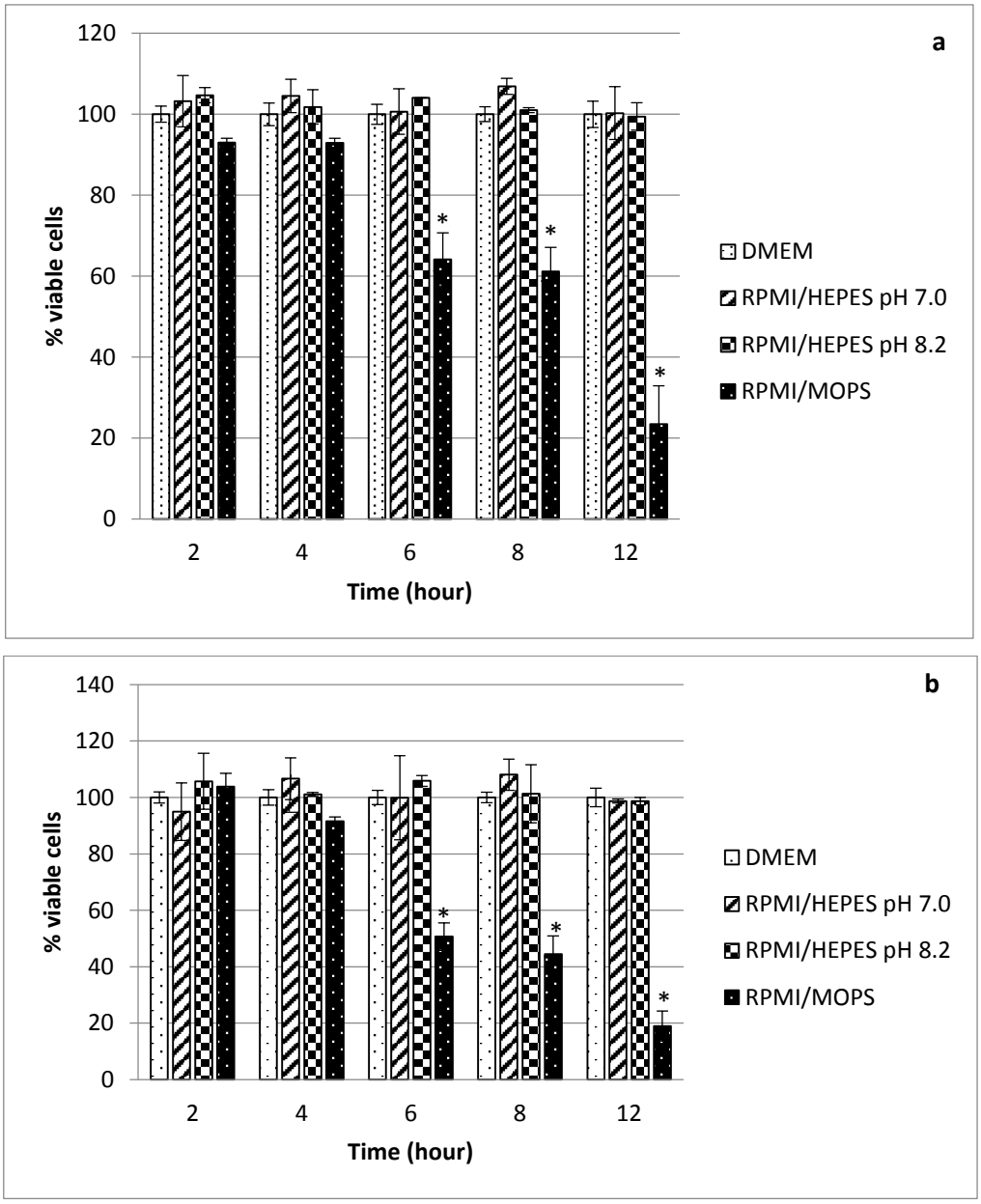
Table 1- Mean and standard deviation values of log_{10} CFU/mL for the growth of *C. albicans* and *S. aureus* in single and mixed biofilms in the **RPMI/MOPS** and **RPMI/HEPES** media, with 24 hours of biofilm formation on three occasions performed in triplicate (n= 9 samples).

Group	RPMI WITH MOPS	RPMI WITH HEPES	*p**
C. albicans	5.44 (± 0.30)[a]	5.52 (±0.20)[a]	0.01
S. aureus	7.60 (±0.07)[b]	7.68 (±0.10)[b]	
Mixed (*C. albicans*)	6.13 (±0.25)[c]	6.13 (±0.02)[c]	
Mixed (*S. aureus*)	8.22 (±0.14)[d]	8.09 (±0.02)[d]	

* ANOVA. Tukey post-test = equal letters for results without a significant difference; the same letters for results with a significant difference.

Table 2- Mean and standard deviation values (SD) of pH of supernatant of *C. albicans* and *S. aureus* single- and mixed-species biofilms in the **RPMI/MOPS** and **RPMI/HEPES** media on three occasions performed in triplicate (n=9 samples).

pH of culture media			
	C. albicans	*S. aureus*	Mixed-species
RPMI/MOPS	6.91 (±0.02)	6.88 (±0.04)	6.89 (±0.04)
RPMI/HEPES	7.88 (±0.09)	7.53 (±0.35)	7.81 (±0.08)



F**igure 1 – (a)** Viability percentage based on different culture media and pH in contact with **NOK-SI** (n=9 samples). (**b**) Viability percentage based on different culture media and pH in contact with **HaCat** (n=9 samples). ANOVA. Tukey post-test. *significant difference ($p<0.05$).

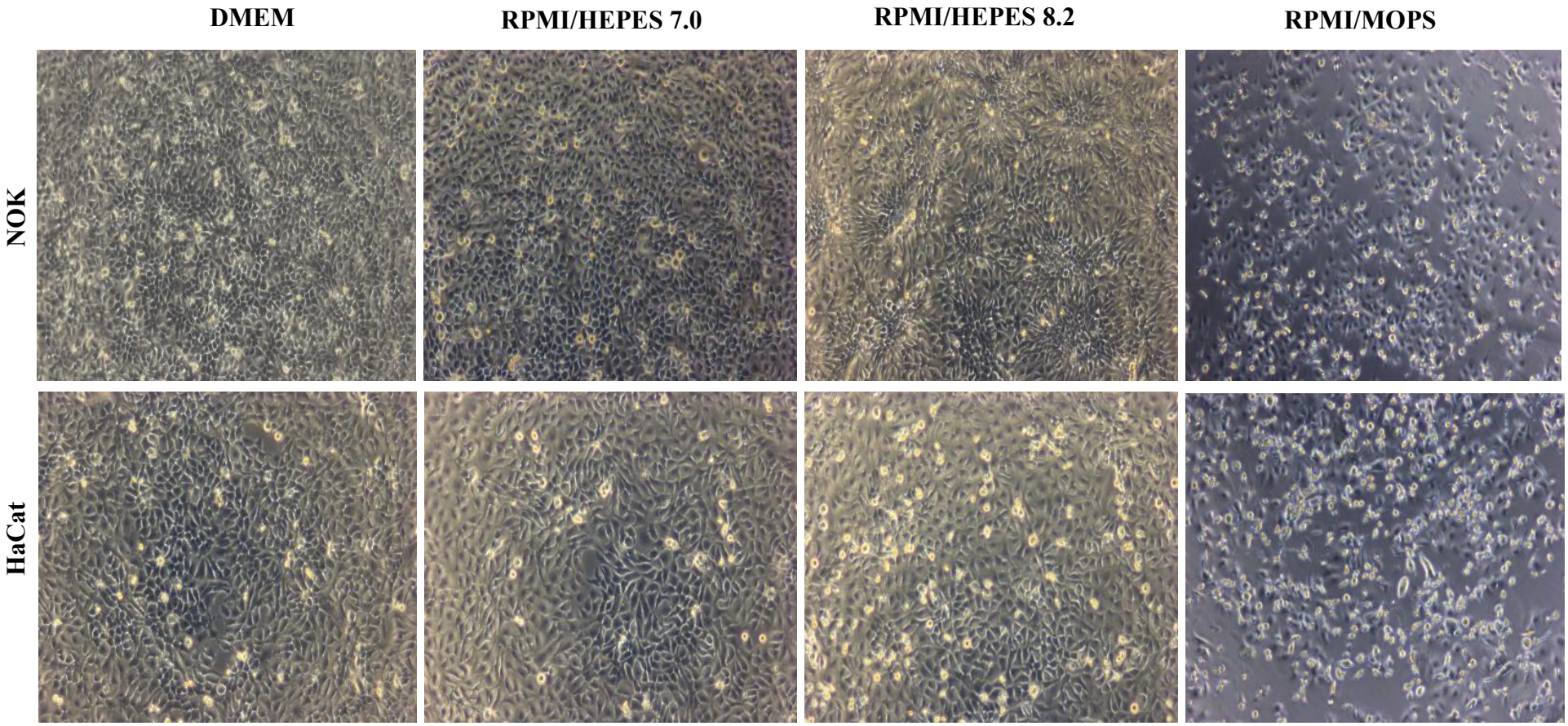


Figure 2. Images obtained by inverted microscope for the DMEM, RPMI/HEPES pH 7.0, RPMI/HEPES pH 8.2 and RPMI/MOPS with 12 hours of incubation in NOK-SI and HaCat cells groups. Magnification ×20.

3.2 Publicação 2*

Biofilms metabolites from *Candida albicans* and *Staphylococcus aureus* promote cell death and inflammatory response.

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Abstract

The aim of the present study was to further understand the effect of biofilm metabolites from single and mixed cultures of *Candida albicans (C. albicans)* and methicillin-sensitive *Staphylococcus aureus* (MSSA), after 36 h of formation, on keratinocytes (NOK-si and HaCat) and macrophages (J774A.1). Biofilm metabolites from *C. albicans* and MSSA were collected and placed in contact with keratinocytes and macrophages for cell viability assay (MTT). Lactate dehydrogenase (LDH) enzyme release was measured to assess the cell membrane damage of keratinocyte, the cells were analyzed after 02 and 24 h of contact with biofilms metabolites by brightfield microscopy. The determination of cell death was performed by labeling the apoptotic cells with annexin V and the necrotic cells with propidium iodide (PI) by fluorescence microscopy. Biofilm metabolites were also placed in contact with J774A.1 cells for 24 h to measure cytokine production (IL-6, TNF-α and NO) by ELISA assay. The results were analyzed statistically with ANOVA, followed by the Tukey test, with a significance level of 5%. For cell viability assay, metabolites from single-species *C. albicans* were as toxic as mixed biofilm metabolites, and the MSSA metabolites were less toxic than the others. The mixed biofilm metabolites were the most aggressive to the cellular membrane, which could be confirmed by the expressive marking of PI in this group, as well as the morphology of the cells. Single- MSSA and mixed-species biofilms metabolites induced the production of IL-6, NO and TNF-α by J744A.1 macrophages. A reduced production of IL-6 and NO by mixed biofilm was observed in comparison with the single MSSA, *C. albicans* biofilms metabolites were able to induce only low levels of NO. Mixed biofilms metabolites are able to induce greater cellular damage, whereas the MSSA metabolites are able to induce increased inflammatory response.

Keywords: Biofilm, Cytokines, *Candida albicans*, *Staphylococcus aureus*, Keratinocytes, Macrophages.

Introduction

Biofilm is a microbial community embedded in a self-made matrix of extracellular polymeric substances (EPS). It is a mode of microbial growth where dynamic communities of interacting sessile cells are irreversibly attached to a solid substratum, as well as to each other [1, 2]. Among the opportunistic pathogens in the oral cavity, *Candida albicans* (*C. albicans*) is the microorganism most frequently isolated in denture bases (64.4%) [3]. Methicillin-sensitive *Staphylococcus aureus* (MSSA) is recovered from 34.4% of the denture wearers, while the association between these two microorganisms is found in 8.8% of patients [3]. *C. albicans* and MSSA form a mutual alliance that promotes a positive synergism between species [4, 5], which has been related to increase frequency and severity of infectious disease [6], as the development of prosthesis stomatitis, that is the most common form of oral candidosis, with overall incidence of 11–65% in complete prosthesis wearers [7-9]. Moreover, *C. albicans* and MSSA can also be coisolated from several diseases such as cystic fibrosis, superinfection of burn wounds, urinary tract infections, diabetic foot wounds, and from the surfaces of various biomaterials, including implants, endotracheal tubes, feeding tubes, and catheters [10,11].

These opportunistic pathogens can colonize mucous membranes, invade tissues and cause infections [12]. Their pathogenicity is related to several factors such as the ability to develop biofilms, drug resistance, and to produce toxic metabolites and toxins [13]. *C. albicans* and MSSA biofilms are rich in proteases and phospholipase. When these microorganism biofilm are put together, both phospholipase C (PL-C) and proteases (SAP) can be encountered [5]. The interaction between MSSA and *C. albicans* results in strong inflammatory response in polymicrobial infections. These microorganisms can modulate one another's proteomic profile during *in vitro* coculture biofilm growth, including the expression of several defined and putative virulence proteins such as the CodY protein, which regulates nutrient acquisition and toxin production [4].

The ability of microorganisms to induce cell damage may play a role in promoting pro-inflammatory responses that result in recruitment and activation of immune cells, such as neutrophils and macrophages [14]. Local and systemic pro-inflammatory cytokines (*e.g.* IL-6, TNF-α and IL-1β) showed significantly elevated levels during co-infection in the early stages, when compared to the simple infection, regardless of the morphogenesis of *C. albicans* [15]. The concentration of IL-6 and IL-8 by HaCat keratinocytes increased with incubation by *C. albicans* filtrates [16]. The pathogenicity of MSSA is due to the repertoire of toxins, exoenzymes, adhesins, and immune-modulating proteins [17]. A unique subset of innate

proinflammatory cytokines (IL-6, granulocyte colony-stimulating factor, keratinocyte chemoattractant, monocyte chemoattractant protein-1, and macrophage inflammatory protein-1α) were significantly increased during polymicrobial versus monomicrobial peritonitis [11]. MSSA biofilm metabolites induced the IL-1β, IL-6, TNF-α, CXCL-8 and CXCL-1 cytokine production in human keratinocytes by ELISA assay [18]. The highest concentration of IL-6 and for TNF-α were detected in MSSA coincubated with human mononuclear cells [19].

Therefore, the aim of this research was to evaluate the effect of metabolites derived from *C. albicans* and MSSA biofilms on epithelial cell death and macrophage inflammatory responses. To identify synergism in pathogenicity and virulence of these microorganisms these effects were compared between mono- and mixed-species biofilms.

Materials and Methods

Microbial strains and growth conditions

C albicans SC5314 and MSSA ATCC 25923 reference strains were used in this study. *C. albicans* was maintained in Yeast Peptone Glucose medium (YEPD: 1% yeast extract, 2% Bacto peptone and 2% D-glucose, 2% agar) and MSSA in Tryptic Soy Broth medium (TSB-Acumedia Manufactures Inc., Baltimore, MD, USA), both frozen at -80°C until use. *C albicans* was subcultured onto Sabouraud Dextrose Agar plates (SDA - Acumedia Manufactures Inc., Baltimore, MD, USA) supplemented with chloramphenicol (0.1 g/L) and incubated at 37°C for 48 h for colony growth. MSSA were subcultured onto brain heart infusion (BHI) agar plates (Acumedia Manufactures Inc., Baltimore, MD, USA) supplemented with amphotericin B (0.025 g/L) and incubated at 37°C for 48 h.

Adhesion and biofilm formation

After incubation of MSSA plates, seven freshly grown colonies were transferred to 10 mL of TSB medium used for the pre-inoculum growth in an incubator at 37°C for 18 h. The dilution of the inoculum was performed in a ratio of 1:20 (0.5 mL pre-inoculum in 9.5 mL of TSB medium); and these cultures were then incubated until they reached mid-exponential phase growth: optical density (OD) 600nm= 0.597±0.019, which corresponds to 8.37 ± 8.23 $\log_{10}$(CFU/mL).

For *C. albicans*, 10 freshly grown colonies were transferred to 10 mL culture media Yeast Nitrogen Base broth (YNB - Difco, Becton Dickinson Sparks, MD, USA) supplemented with 100 mM glucose, and used for the growth of pre-inoculum at 37°C for 16 h. The dilution of the inoculum was performed in the ratio of 1:10 (1 mL pre-inoculum and 9 mL of sterile liquid culture); and these cultures were incubated until they reached mid-exponential phase growth: OD 540nm=0.557±0.148, which corresponds to 6.72 ± 5.98 $\log_{10}$(CFU/mL).

Cultures were then centrifuged at 3.220 *xg* for 10 minutes at 4°C and pellets were washed twice with sterile phosphate buffered saline solution (PBS; 100 mM NaCl, 100 mM NaH_2PO_4, pH 7.2, Products Labsynth Laboratories Ltda., Diadema, SP, Brazil) by stirring and centrifugation. After washing, cells were resuspended in RPMI-1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA) which was buffered with HEPES 5,95g/L (Sigma-Aldrich, St. Louis, MO, USA) pH 7.0 to a final concentration of 25 mM, supplemented with L-glutamine (2.0 mM) and sodium bicarbonate (2.0 g/L) [20], and the optical densities of the suspensions were standardized to the concentration of $1x10^7$ CFU/mL for both microorganisms.

For biofilm formation, adherence of microorganisms was carried out in pre-sterile 24-well culture plates (TPP Techno Plastic Products AG, Switzerland) [21]. For the tests with isolated cultures, a final solution (1.5 mL) was prepared with 750 uL of the suspensions of each of the microorganisms (about $1x10^7$ cells per mL) and 750 uL RPMI 1640, which was individually transferred to the wells of a sterile culture plate. For tests on mixed cultures, it was also prepared a final 1.5 mL solution, containing 750 uL of the solution of each of the microorganisms: *C. albicans* + MSSA. All plates were incubated at 37°C in an orbital shaker (75 rpm) for 90 min, after which the orifices were washed 2 times with 1.5 mL of sterile PBS, followed by addition of 1.5 mL of fresh RPMI 1640, to each well. The plates were incubated for 24 h, when 750 μL of the suspensions were removed and an equal volume of fresh RPMI 1640 was added. The number of adhered cells and those present in the biofilms was determined by counting colony-forming units (CFUs). The wells were briefly washed twice with PBS to remove loosely attached cells and resuspended in 1000 μL of PBS. Next, the adhered biofilm was carefully scraped of the wells with a sterile pipette tip for 1 min. The 1000 μL suspensions were then vigorously vortexed to separate a possible aggregation among the cells. Serial decimal dilutions (in PBS) were made and the number of *C. albicans* was determined by pipetting replicate specimens (25 μL) of the suspensions on SDA medium supplemented with chloramphenicol. The same procedures were performed for the MSSA cells, which were plated on BHI agar supplemented with amphotericin B. For the dual species studies, serial dilutions were plate onto both media. The plates were incubated for 24h-48 h at 37°C and the values of

CFU/mL were counted. The experiments were performed in three replicates and repeated in three independent assays. The pH was measured using a benchtop pH meter (QX 1500 Plus-Qualxtron, São Paulo, Brazil).

After 36 h of formation, the biofilm medium (supernatant) and the microorganisms attached in the wells were removed and filtered together in low protein binding filter 0.22 µm (SFCA, Corning, Germany) to obtain the metabolites for the following assays. For epithelial cells (NOK-si and HaCat), the undiluted metabolites were incubated for 02, 04, 06, 08, 12 and 24 h. For macrophages, biofilms metabolites were diluted in the ration of 1:4 (metabolites: DMEM), and stimulated for 24 h, ensuring cell viability ≥ 95%.

Keratinocytes cell cultures

NOK-si was kindly provided by Prof. Dr. Carlos Rossa Junior (Department of Periodontology, School of Dentistry of Araraquara-UNESP, Brazil) [23], HaCat (BCRJ 0341) and J774A.1 macrophages (BCRJ 0121) were purchase from Rio de Janeiro Cell Bank (BCRJ, RJ, Brazil). The cells were cultured in DMEM (Dulbecco's Modified Eagle's, Gibco BRL, Grand Island, NJ, USA) containing 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY), 100 IU/mL penicillin, 100 mg/mL streptomycin (Sigma Chemical Co., St. Louis, MO, USA) and 2 mL/L glutamine (GIBCO, Grand Island, NY). The cells were maintained at 37°C under of 5% CO_2 and 80% humidity. The cells were grown to reach confluence (90%), counted in a Neubauer chamber (magnification ×10) and plated (4.5×10^4 cells/well for NOK-si and HaCat and 1.0×10^5 cells/well for J774A.1). For the experiments, cells were used between the 3rd and 8th passage.

Protein contained in the supernatant

The total level of soluble protein contained in the biofilms metabolites was measured by Bradford protein assay [22], using bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA), as standard. The spectrophotometric measurements were performed at 595 nm (400 EZ Reader - Biochrom, Cambridge, UK).

Cell viability

For the analysis of cellular metabolism, the mitochondrial activity of the keratinocytes was measured using the MTT assay [3-(4.5-dimethylthiazole-2-yl) 2.5-diphenyl tetrazolium

bromide] (Sigma-Aldrich, St. Louis, MO, USA) [24], that was performed at 02, 04, 06, 08, 12 and 24 h after incubation at 37°C in 5% CO_2 with the RPMI (negative control), *C. albicans*, MSSA and mixed biofilm metabolites; and lysis buffer containing Triton X-100 (LB, positive control). After each period of contact, the cells were washed with PBS and 250 µL of MTT (2.5 mg/mL) was added to each well and incubated for 4 h. Next, MTT was removed and the formazan crystals were solubilized in 250 µL of 2-propanol. The spectrophotometric measurements were performed at 562 nm (Reader 400 EZ - Biochrom, Cambridge, UK).

Cell membrane damage

The release of the enzyme lactate dehydrogenase (LDH) was determined after 02, 04, 06, 08, 12 and 24 h in contact with the metabolites, using the CytoTox-96 nonradioactive cytotoxicity assay (Promega, Madison, WI) according to the manufacturer's recommendations. Initially, 100 µL of each control (RPMI, DMEM and lysis buffer containing TritonX-100) and experimental sample (biofilm metabolites) were added in triplicate in 96 well plate (Thermo Scientific; #31125). Thereafter, 100 µL of the "CytoTox-ONE TM" reagent was added and incubated for 10 minutes. After this period, 50 µL of the "stop" solution was added to each well and the fluorescence was then measured with a filter combination of 544 nm/590 nm (Fluoroskan, FL Ascent, Labsystems, Helsinki, Finlan). Culture medium and biofilm metabolites were used as blank in the absence of the cells.

Evaluation of keratinocytes morphology

Keratinocytes were analyzed and photographed in a Leica DMI 3000B microscope (Leica Microsystems, Wetzlar, Germany), after 02 and 24 h of contact with biofilms metabolites. The cells were analyzed by brightfield microscopy.

Cell death assay

The type of cell death (apoptosis/necrosis) induced by MSSA, *C. albicans* and mixed biofilms metabolites was investigated in NOK-si and HaCat cells by annexin V/Alexa Fluor 488 and PI (594nm) (Molecular Probes, Invitrogen). Annexin V binds specifically to phosphatidylserine residue on the cell membrane during apoptosis. PI intercalates into the broken DNA, typical process of cell necrosis [25, 26]. After 08 h of contact with the single and

mixed biofilms metabolites, the cells were washed twice with binding buffer (10 mM Hepes/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH = 7.4) and incubated with annexin V (10 uL/well) and PI 2 uL (100 ug/mL) for 20 min at room temperature. Cells were then washed with PBS and maintained in DMEM 10% for analysis with a fluorescence inverted microscope (Leica DMI 3000B; Leica Microsystems, Wetzlar, Germany).

Cytokines produced by macrophages

J774A.1 macrophages were keep in contact with *C. albicans*, MSSA and mixed biofilm metabolites for 24 h, in a ratio of 1:4 (biofilm metabolites: cell culture medium) to avoid cell death, determined experimentally by MTT (Fig 6A). After that, macrophages supernatants was collected and cytokines production were measured (IL-6, TNF-α and nitric oxide – NO) by enzyme-linked immunosorbent assay - ELISA (BD Biosciences, San Jose, CA).

Statistical analysis

Normality and homogeneity of the variances were checked using the Shapiro-Wilk and Levene tests. Results were statistically evaluated using one-way analysis of variance (ANOVA) followed by Tukey's test. A 5% significance level was adopted for all statistical tests performed ($p<0.05$). All studies were performed in triplicate on three independent occasions.

Results

Characterization of single- and mixed-species biofilms

MSSA biofilm in mixed cultures showed higher values of log_{10}CFU/mL, comparing the single cultures, which was not observed when comparing the *C. albicans* in single and mixed biofilms (table 1). The pH of *C. albicans* (pH=7.03), MSSA (pH=6.89), and mixed biofilms metabolites (pH=7.00) remained near physiologic pH (pH=7.0) showing that the pH values did not affect the cell viability rates.

The protein measurements by the Bradford assay showed lower amount of total protein on *C. albicans* metabolites (3.12 μg/ml±1.10), when compared to MSSA (21.44 μg/mL±0.53) and mixed biofilm (19.21 μg/mL±1.35) metabolites, with no significant differences between them (Fig 1).

Cell viability

Biofilms metabolites were able to significantly decrease the cell viability of NOK-si and HaCat cells after 06 h incubation with metabolites of *C. albicans* single- and mixed-species biofilm, and after 12 h of incubation with metabolites of single MSSA biofilms, in both cells. The maximum reduction in cell viability was reached within 24 h for all groups. *C. albicans* metabolites (about 40%), mixed group (about 40%) and MSSA group (about 78%) (Figs. 2A and 2B).

Cell membrane damage

The metabolites from mixed biofilms significantly increased LDH release compared to the single *C. albicans* and MSSA after 8 h of incubation, reaching the maximum LDH release in 24 h. The MSSA metabolites caused less LDH release (Figs. 3A and 3B).

Cell morphology of keratinocytes

No changes in cell morphology of NOK-si and HaCat were observed after only 2 h of contact with metabolites from single-species *C. albicans*, MSSA and mixed-species biofilms. The major changes in the morphology (loose and spherical cells, cellular debris, decreasing the amount of cells) were evident after 24 h of incubation with metabolites from the three types of biofilms (Figs. 4A and 4B).

Cell death by necrosis or apoptosis

After 8 h of exposure to the biofilms metabolites, the NOK-si and HaCat cells that were in contact with mixed biofilm metabolites showed more staining with annexin V and PI, suggesting that the microorganism synergism disrupt more the integrity of the epithelial cells membrane, and cell death by late apoptosis/necrosis. Cells were also stained with annexin V and lower stained with PI when exposed to the single *C. albicans* metabolites. Lower labeling of annexin V and PI was observed when the cells were exposed to the MSSA metabolites (Fig. 05).

Expression of cytokines by macrophages

ELISA assay revealed that J774A.1 macrophages stimulated with metabolites from MSSA biofilm induced the production of high levels of NO, IL-6 and TNF-α in comparison with metabolites from *C. albicans* biofilm, whose cytokine levels remained low. TNF-α production by J774A.1 stimulated with mixed biofilms remained as high as MSSA, with the exception of IL-6 and NO, which was higher in MSSA (Fig 6B).

Discussion

This *in vitro* study investigated the effects of soluble metabolites produced by single- and mixed-species biofilms of *C. albicans* and MSSA on human keratinocytes and macrophages. First, the biofilm development was characterized. Then, keratinocytes cell viability was assessed by MTT and cell membrane damage by LDH release. Changes in morphology, apoptosis/necrosis cell death were also investigated in keratinocytes, in addition to cytokines production by macrophages**.** This study showed that metabolites from mixed-species biofilm are more pathogenic to epithelial cells than those from single-species biofilm. Concerning the inflammatory response, metabolites from single-species *C. albicans* weakly poorly induce cytokine production, while metabolites from single MSSA yielded higher levels of cytokines, with a down-regulation process when this microorganism was cultured in the presence of *C. albicans* (mixed metabolites).

The biofilm characterization showed a higher value of $\log_{10}$CFU/mL of MSSA in mixed-species culture biofilms when compared to the single-species (Table 1). It is known that prostaglandin E2 produced by *C. albicans* stimulates the growth of MSSA in mixed-species biofilms formed by these two microorganisms [27]. Moreover, MSSA possesses affinity for *C. albicans* hyphae [4, 5] and therefore more MSSA biomass is retained after washing steps, which may explain the higher values of $\log_{10}$CFU/mL for MSSA in mixed–species biofilms. In addition, single-species MSSA biofilm and the mixed-species biofilm produced higher levels of total protein than *C. albicans* biofilms in their soluble factors used as "metabolites" (Fig 1). Although no difference in the amount of total proteins were observed between metabolites from MSSA and mixed biofilms, differential in-gel electrophoresis have shown that a total of 27 proteins were significantly differentially produced by MSSA and *C. albicans* during co-culture biofilm growth, such as the CodY protein, which regulates the acquisition of nutrients and the production of toxins. [4].

The metabolites from the mixed-species biofilms were the most damaging for both NOK-si and HaCat cells in comparison to single *C. albicans* and MSSA groups. It is not very evident in the MTT assay, in which the decrease rates of cellular viability were similar between single *C. albicans* and mixed biofilm metabolites. However, for LDH assay, is evident most LDH release in both cells incubated with the mixed biofilm. Hydrolytic enzymes such as phospholipases (PL-C) and proteinase are metabolites known to be present in *C. albicans* biofilms [28, 29]. Moreover, it has been reported higher production of proteinase in single-species MSSA biofilms, while single *C. albicans* biofilms presented higher levels of PL-C. When both microorganisms were co-cultured, both enzymes were produced [5]. The activation of phosphatidylinositol-specific PL-C increases the cellular calcium level and, in doing so, it may participate in the apoptosis process [30]. Thus, PL-C could be associated with the greater number of keratinocytes apoptotic cells when exposed to metabolites from *C. albicans*-single and mixed-species biofilm (Fig 5). In addition, *C. albicans* continuously secretes farnesol as a quorum sensing molecule [31]. Like other isoprenoid alcohols, farnesol induces apoptosis in a variety of mammalian cells [10, 32, 33]. Previous investigations have clearly shown that farnesol triggers an apoptotic process in eukaryotic cells via the induction of caspase, the production of reactive oxygen species (ROS) and the disruption of mitochondrial integrity, resulting in mammalian cell death [10].

The LDH results demonstrated that metabolites from 36 h-old biofilm proved to be harmful to NOK-si and HaCat, regardless the source of the metabolites. However, metabolites from mixed-species biofilm produced higher cell membrane damage, indicating increased pathogenicity of the metabolites from mixed-species biofilm (Figs 3A and 3B). This more pathogenic behavior was also demonstrated in the annexin V/PI assay. In such condition, NOK-si and HaCat had a double staining with annexin V and PI. On the other hand, when the cells were exposed to metabolites from single-species biofilm, there was little staining, especially in the MSSA group, and annexin V labelling was predominant in the *C. albicans* group (Fig 5).

Interestingly, higher levels of Interleucin-6 (IL-6) were induced by MSSA metabolites and lower levels were detected when macrophages were exposed to mixed biofilm metabolites. IL-6 is able to block apoptosis in cells during the inflammatory process, keeping them alive in very toxic environments [34]. This mechanism could be involved in the damage caused by MSSA metabolites, which promoted the smallest labeling of cells with annexin V (apoptosis), as well as minor values of LDH and cytotoxicity in NOK-si and HaCat (MTT). MSSA toxins (e.g. exotoxins, protein A and α-toxins) do not possess direct cell damaging action, but have a potent effect on cells of the immune system by inducing the overproduction of cytokines [35].

Further, lipotheichoic acid have been shown to strongly induce IL-6 production in monocyte-like cell line [36, 37]. Although in a lower extent, IL-6 was also produced by mixed-species biofilm metabolites, suggesting that somehow the decrease or absence of IL-6 could trigger cell death mechanisms. Previously, a significant increase of IL-6 levels were found during *in vivo* peritoneal co-infection with *C. albicans* and MSSA, compared to monomicrobial infection [11]. The same behavior was observed regarding NO production in J774A.1 cells after being exposed to biofilm metabolites for 24 h. There was an expressive production of NO, when macrophages were incubated with metabolites from single-species MSSA biofilm, and a repression of NO production by metabolites from mixed- biofilm comparing to the metabolites from single-species MSSA. Differently, *C. albicans* metabolites induced low levels on NO in J774A.1 cells (Fig 6b), which is in agreement with another study in which *C. albicans* was capable of blocking nitric oxide (NO) production from macrophages [38].

The induction of NO production have been related with higher levels of TNF-α [39]. This may partially explain our results, as higher levels of TNF-α induced by metabolites from single- MSSA and mixed-species biofilm metabolites on J774A.1 cells were may be related to NO production (Fig 6b). Although it is known that infections with *C. albicans* and MSSA are associated with the production of proinflammatory cytokines, notably IL-1β and TNF-α [40], the results in the present investigation showed that single *C. albicans* was not able to induced TNF-α. Accordingly, TNF-α expression in perfused rat liver was induced after intraportal candidemia *vs* MSSA bacteremia, although *Candida* spp. promoted the weakest stimulatory response [41].

Therefore, our results support further investigations to determine which mechanisms are involved in keratinocyte cell death by single and mixed *C. albicans* and MSSA biofilm metabolites as well as the inflammatory pathways induced by them.

Conclusion

Our data suggest that metabolites from 36h-old biofilms decreased cell viability on keratinocytes, especially when *C. albicans* and MSSA are co-cultured (metabolites from mixed-species biofilm), triggering apoptosis and necrosis cell death. Regarding the inflammatory response of macrophages, metabolites from single- MSSA and mixed-species biofilms induced pro-inflammatory cytokines, with a down-regulated production of IL-6 and NO by mixed biofilm in comparison to the single MSSA, which may be associated to the presence of *C. albicans*.

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Table 1- Mean value, standard deviation, confidence interval, minimum and maximum value of log_{10} CFU/mL for the growth of single and mixed *C. albicans* and MSSA biofilms with 36 h, performed three times in triplicate (n = 9 samples).

	Log_{10} CFU/mL		**IC (95%)**				
Group	Mean	SD	LL	UL	Minimum	Maximum	*p**
Ca[a]	5.75	0.18	5.61	5.89	5.46	5.99	0.01
MSSA[b]	7.85	0.03	7.82	7.88	7.78	7.91	
Mixed (Ca)[a]	5.84	0.06	5.79	5.90	5.75	5.95	
Mixed (MSSA)[c]	8.39	0.18	8.25	8.53	8.04	8.68	

ANOVA. Tukey post-test = letters equal to results with no significant difference; different letters for results with a significant difference.

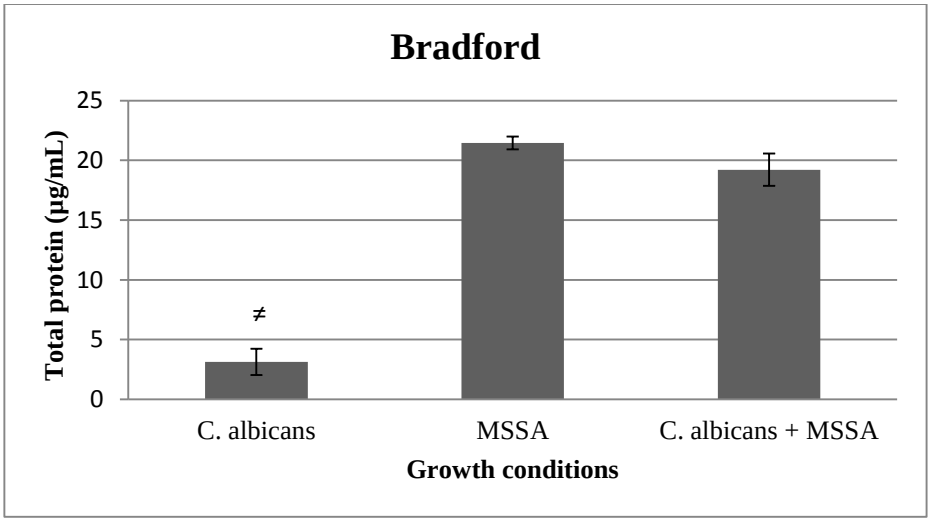

Fig 1: Total protein contained in the biofilm supernatant by the Bradford method. ≠ significant difference ($p<0.05$).

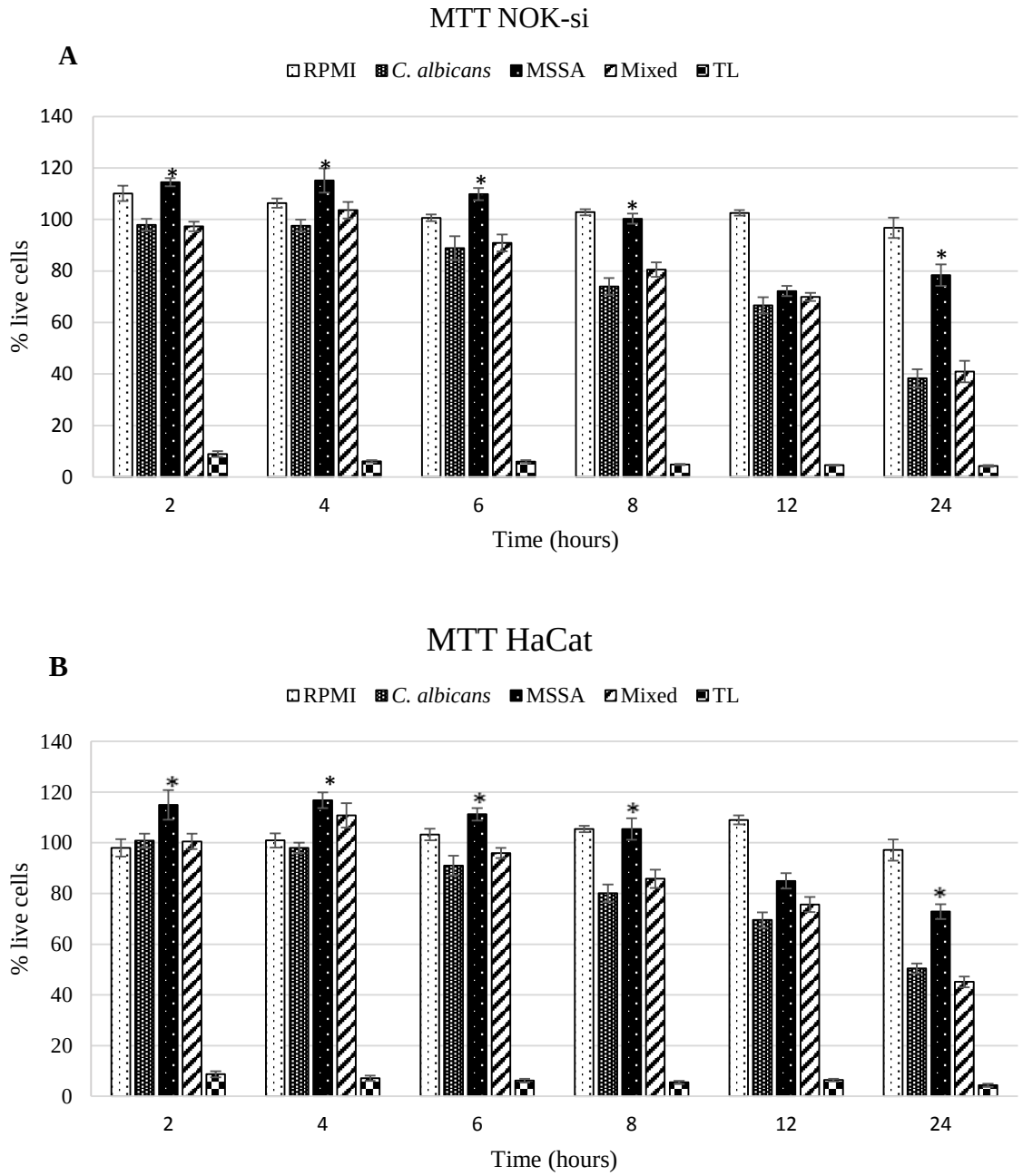

Fig 2- NOK-si (A) and HaCat (B) cells viability after being in contact with biofilm metabolites for 2, 4, 6, 8, 12 and 24 hours of three independent experiments, in triplicate for each experimental condition (n=9). RPMI= Negative control, *C. albicans*= *C. albicans* metabolites, MSSA = MSSA metabolites, Mixed = mixed metabolites, TL = lysis buffer. Error bars represent standard error. ANOVA. Tukey post-test.* Difference compared to the experimental group.

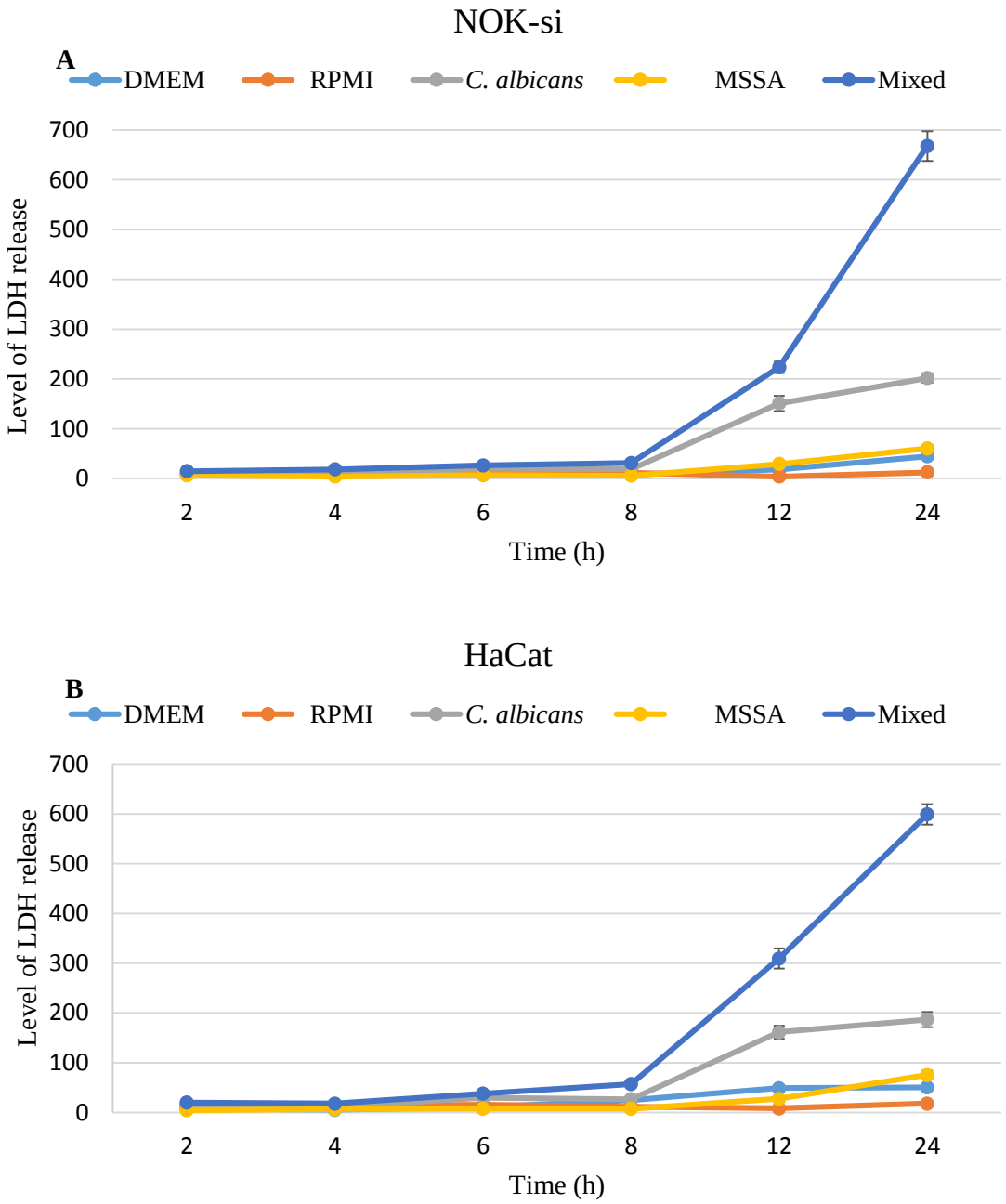

Fig 3- LDH release from NOK-si (A) and HaCat (B) cells after incubation with biofilm metabolites during 2, 4, 6, 8, 12 and 24 hours. Three independent experiments were performed in triplicate for each experimental condition (n=9). DMEM = negative control; RPMI = negative control; *C.albicans* = *C. albicans* metabolites; MSSA = MSSA metabolites; Mixed = mixed metabolites.

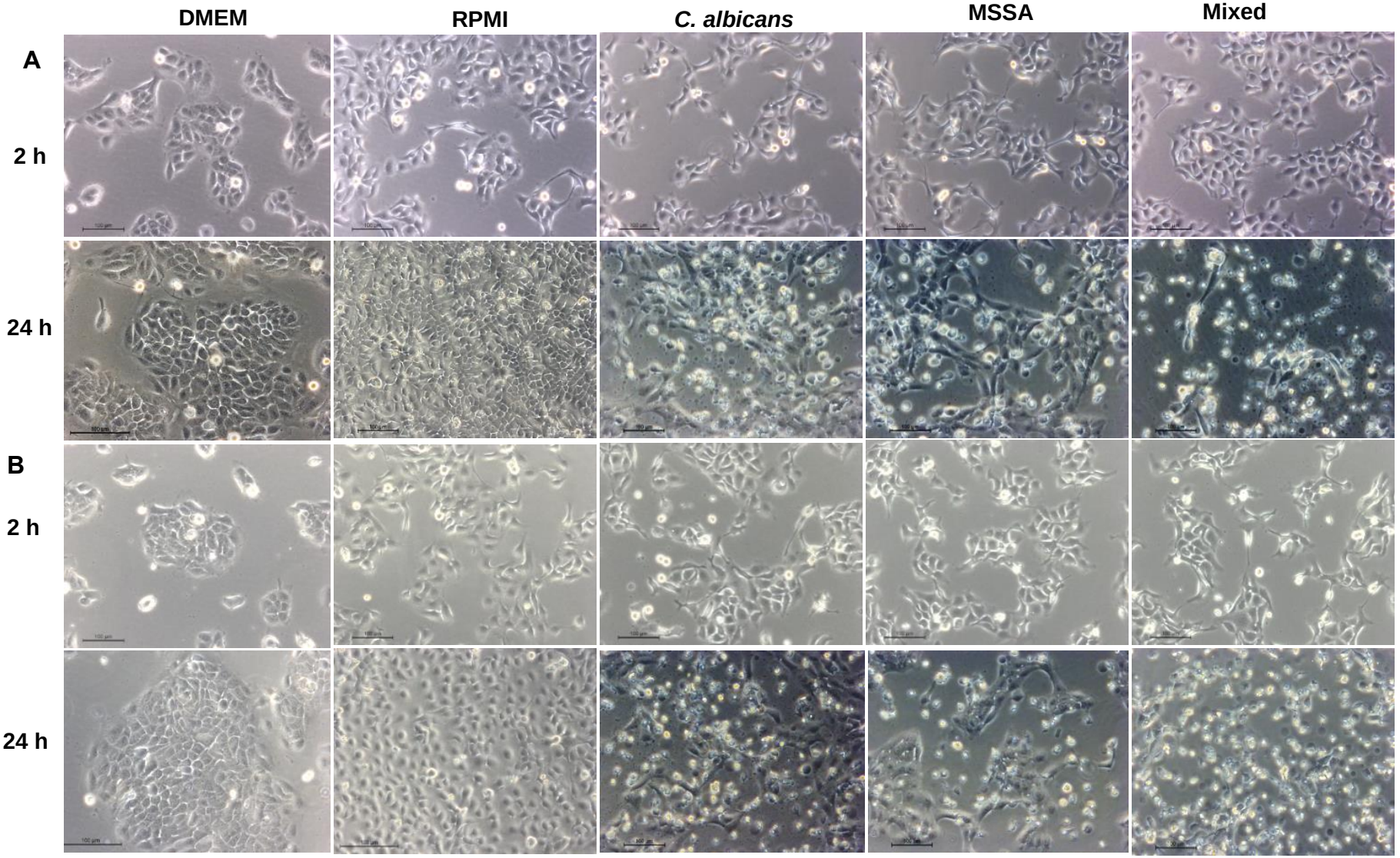

Fig 4 – A) Images obtained to inverted microscope for the control group (DMEM and RPMI) and experimental groups (*C. albicans*, MSSA and Mixed) with 2 and 24 h in contact with NOK-si. **B)** Images obtained to inverted microscope for the control group (DMEM and RPMI) and experimental groups (*C. albicans*, MSSA and Mixed) with 2 and 24 h in contact with HaCat. The bar in images corresponds to 100 µm.

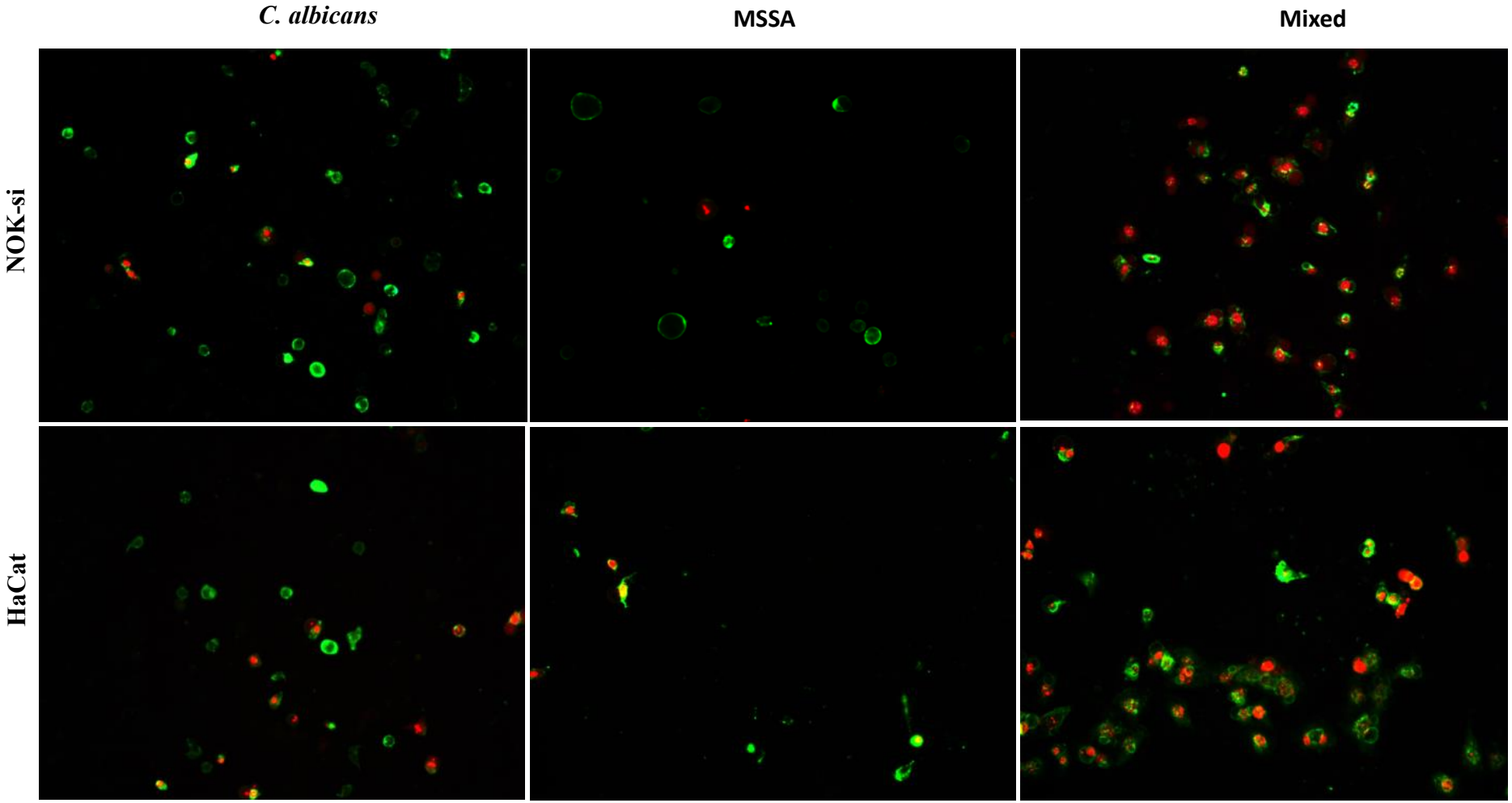

Fig 5: Detection apoptotic cells (staining positively for annexin V in green) and necrotic cells (staining positively for PI in red) after contact for 08 h with *C. albicans*, MSSA and mixed biofilm metabolites. Magnification x200.

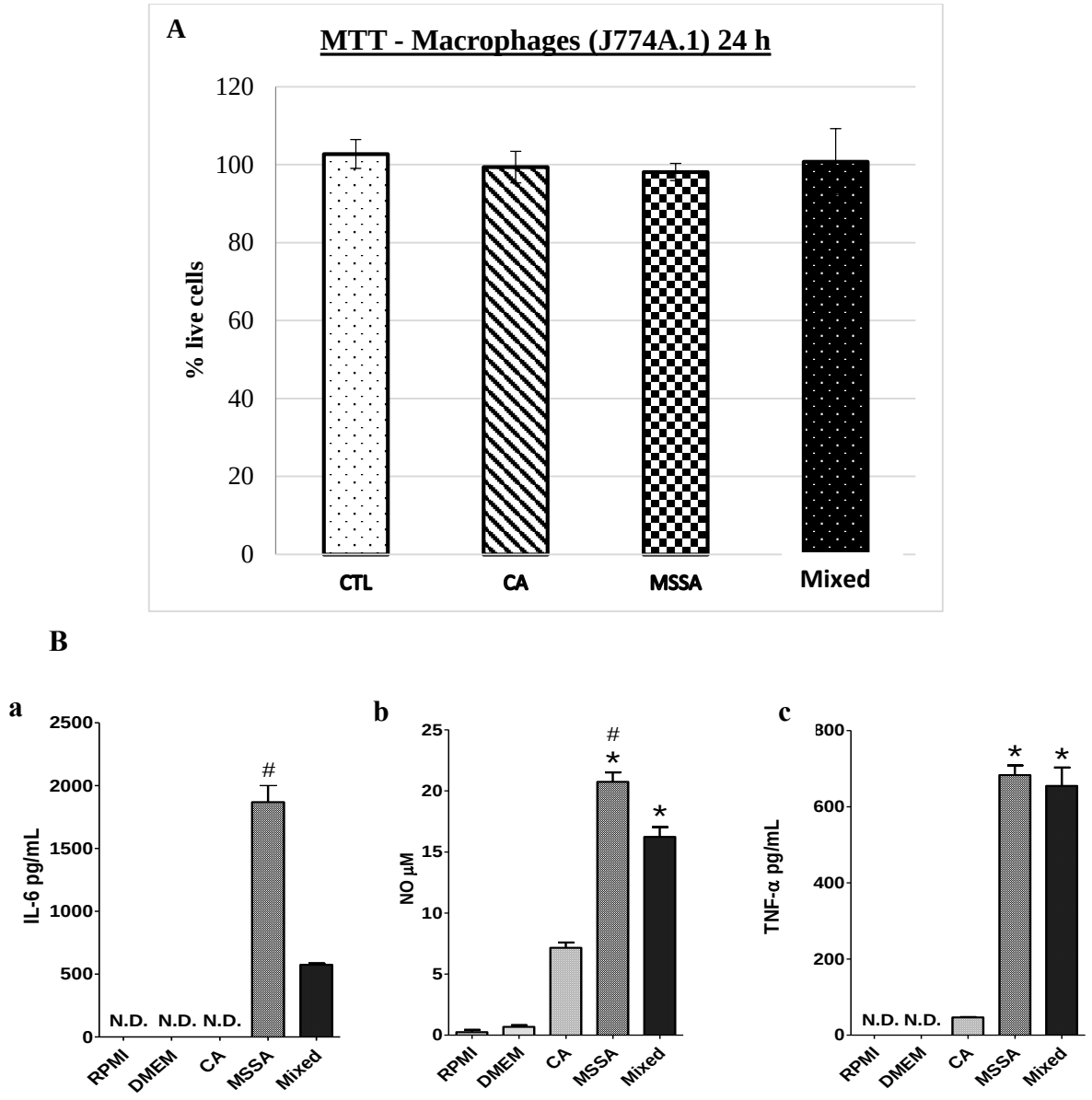

Fig 6. Macrophage response after challenge with metabolites produced by *C. albicans* and MSSA single- and mixed-species biofilms. **A)** Percentage according toxicity to biofilm metabolites in contact with the J774A.1 macrophage, three occasions performed in triplicate (n = 9 samples). **B**) IL-6 (A), NO (B) and TNF-α (C) production from J774A.1 macrophages after 24 h stimulation with MSSA, *C. albicans* and mixed metabolites. Error bars represent standard deviation. Symbols represent statistical differences. a) # *versus* Mixed; b) * *versus C. albicans*; # *versus* Mixed; c) * *versus C. albicans.* Analysis by ANOVA, Tukey post-test, $p \geq 0.05$. CLT = control (DMEM), *C. albicans* = *C. albicans* metabolites, MSSA = MSSA metabolites, Mixed = mixed metabolites. N.D.= not detectable.

3.3 Publicação 3

The effect of toxins from single- and dual-species biofilms of *Candida albicans* and *Staphylococcus aureus* on keratinocyte cell death and caspase activation*

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Running Head: The effect of biofilm toxins on keratinocyte

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ABSTRACT

Microorganisms can interact and develop polymicrobial biofilms. Biofilms comprising of *Staphylococcus aureus* and *Candida albicans* can increase the frequency and severity of oral diseases. The present *in vitro* study assessed cell death of oral keratinocytes (NOK-si) after contact (for 04, 12 and 24 h) with the toxins from single- and dual-species biofilms of *S. aureus* and *C. albicans* produced by 08, 16 and 24 h-old biofilms. The log_{10}(CFU/mL) values were calculated to quantify the total number of microbial cells. NOK-si cell viability was assessed by colorimetric MTT assay. The cell membrane damage caused by the toxins was assessed by measuring the release of the lactate dehydrogenase (LDH). Scanning Electron Microscopy (SEM) was used to assess the morphology of the NOK-si after biofilm exposure to toxins. Determination of cell death by apoptosis/necrosis was assessed using annexin V and propidium iodide (PI). The evaluation of the cell death pathway was carried out by detecting the apoptosis markers (caspase -2, -3, -6, -8 and -9). Statistical analysis was performed through ANOVA followed by Tukey test (α=5%). Higher values of log_{10}(CFU/mL) of *C. albicans* were observed when this microorganism was cultivated with *S. aureus* for 08 and 16 h of biofilm formation.The toxins of dual-species biofilms of 16 h were the most aggressive for NOK-si cells, with cell viability rates of only 31.76%, within 24 h of incubation. The toxins of *S. aureus* biofilm were the least harmful at all assessment times. The SEM images showed that the destruction of cell membranes was more pronounced when cells were incubated with 16 h dual-species biofilm toxins. A greater amount of annexin V and PI stained cells was observed when exposed to dual-species biofilm toxins. The 16 h dual-species biofilms had higher percentages of necrotic cells (24.95%). Apoptosis induced by *S. aureus* and dual-species biofilm was associated with activation of caspases -2, -3, -6 and -8; and increased activity of caspase -3 was observed during contact with *C. albicans* toxins. The toxins derived from the supernatants of biofilms with 08, 16 and 24 h of formation were harmful to the oral epithelial cells. The synergism between the species of *C. albicans* and *S. aureus* promote caspases activation of NOK-si and greater cell death, with higher rate of necrosis.

Keywords: Biofilms. Keratinocytes. Necrosis. Apoptosis. Caspase. *Staphylococcus aureus. Candida albicans.*

INTRODUCTION

Biofilms correspond to a community of sessile cells adhered to a substrate, embedded in a matrix of extracellular polymers (1). The presence of *S. aureus* and *C. albicans* in these biofilms has been related to greater frequency and severity of diseases, *e.g*., in the development of systemic infections (2). Moreover, hospital-acquired infections (HAIs) can be caused by bacterial, viral and fungal pathogens that easily spread through the body. Co-infections, especially in the presence of dual-species biofilms are difficult to treat (3). *C. albicans* and *S. aureus* form a mutual alliance that promotes an synergism between species (4, 5).

S. aureus virulence factors are constituents of the cell wall that contribute to the damage induction; these include teichoic acid, lipoteichoic acid and protein A; additionally factors may be enzymes involved in the pathogenic mechanism, *e.g*. coagulase, catalase and hyaluronidase; or they may be microbial toxins, such as alpha (pore-forming toxins), beta, delta, and gamma-haemolysin (6, 7). In contrast, *C. albicans* is capable of producing degradative enzymes with proteolytic activity, such as proteases and phospholipases, which promote the destruction of epithelial cell membranes and cause the invasion and destruction of cells and tissues (8, 9). Moreover, *C. albicans* continuously secretes farnesol as a quorum sensing molecule (10), which can also be considered as a virulence factor. Like other isoprenoid alcohols, farnesol induces apoptosis in a variety of mammalian cells (11, 12). Previous investigations have clearly shown that farnesol triggers an apoptotic process in eukaryotic cells via the induction of caspases, the production of reactive oxygen species (ROS) and the disruption of mitochondrial integrity, ultimately resulting in cell death (13).

The activation of caspases results in nuclear and cytoplasmic condensation, rupture of mitochondrial membrane, DNA fragmentation, with no effect on the plasma membrane integrity (14, 15, 16). Intrinsic and extrinsic pathways have been identified in caspase-dependent apoptosis (17, 18). Apoptosis induced by activation of the intrinsic pathway is initiated by disruption of mitochondrial integrity and translocation of cytochrome c into the cytosol that catalyses the formation of the apoptosome and leads to activation of caspase prime -9, which in turn cleaves and activates caspase effector -3, responsible for the proteolytic amplification (19). The signalling of the death receptors located on the cell surface triggers the extrinsic death pathway, including TNF receptor or Fas ligand, and it is able to activate the caspase cascade (20).

The loss of viability and induction of apoptosis in keratinocytes has been observed when cells were exposed to *S. aureus* biofilms (21). Apoptosis induced by *S. aureus* was associated

with the activation of caspase-3 and caspase-8 (22). The presence of *C. albicans* in the oral epithelial cells is also able to induce early apoptosis followed by secondary necrosis (23), and increased activities of caspase -3 and -9 have been observed during infection with *C. albicans* (24). However, the mechanisms involved in the induction of keratinocytes cell death by toxins from *C. albicans* and *S. aureus* grown as dual biofilm have not yet been investigated.

The aim of this study was to assess whether the toxins produced by *C. albicans* and *S. aureus* in dual-species biofilms would cause greater keratinocyte cell death than single-species biofilms, and determine the type of cell death (*i.e*. apoptosis or necrosis). It was also investigated which caspases were activated when cells were exposed to different biofilm toxins.

MATERIALS AND METHODS

Strains and growth conditions

Two reference strains were used in this study. *C. albicans* ATCC SC5314 and *S. aureus* ATCC 25923. *C. albicans* was maintained in Yeast Peptone Glucose medium (YEPD: 1% yeast extract, 2% Bacto peptone and 2% D-glucose, 2% agar) and *S. aureus* was maintained in Tryptic Soy Broth medium (TSB-Acumedia Manufactures Inc., Baltimore, MD, USA) and these cultures were frozen at -70°C until use. *C. albicans* was subcultured onto Sabouraud Dextrose Agar plates (SDA - Acumedia Manufactures Inc., Baltimore, MD, USA) supplemented with chloramphenicol (0.1 g/L) and incubated at 37°C for 48 h to generate the *C. albicans* yeast used for all the experiments. *S. aureus* was subcultured onto brain heart infusion (BHI) agar plates (Acumedia Manufactures Inc., Baltimore, MD, USA) supplemented with amphotericin B (0.025 g/L) and incubated at 37°C for 48 h to generate the bacteria cells used for the experiments.

Adhesion and biofilm formation

For *S. aureus*, seven freshly grown colonies were transferred to 10 ml of TSB medium used for the starter growth in an incubator at 37°C for 18 h. The dilution of the inoculum was performed at a ratio of 1:20 (0.5 ml starter in 9.5 ml of TSB medium); and these cultures were incubated until they reached mid-exponential phase growth: optical density (OD) 600nm=0.597±0.019, which corresponds to 2.35E+08±1.70E+08 CFU/ml.

For *C. albicans*, 10 freshly grown colonies were transferred to 10 ml culture media Yeast Nitrogen Base broth (YNB - Difco, Becton Dickinson Sparks, MD, USA) supplemented with

100 mM glucose, used for the growth of pre-inoculum in an incubator at 37°C for 16 h. The dilution of the inoculum was performed in the ratio of 1:10 (1 ml pre-inoculum and 9 ml of sterile liquid culture); and these cultures were incubated until they reached mid-exponential phase growth: OD 540nm=0.557±0.148, which corresponds to 5.20E+06 ± 9.47E+05 UFC/ml.

The cultures were centrifuged at 3.220 x 'g' for 10 min at 4°C and pellets were washed twice with sterile phosphate buffered saline solution (PBS; 100 mM NaCl, 100 mM NaH_2PO_4, pH 7.2, Products Labsynth Laboratories Ltda., Diadema, SP, Brazil) by stirring and centrifugation. After washing, cells were resuspended in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) which was buffered with 5,95g/L HEPES (Sigma-Aldrich, St. Louis, MO, USA) at pH 7.0 to a final concentration of 25 mM, supplemented with 2.0 mM L-glutamine and 2.0 g/L sodium bicarbonate (25), and the optical densities of the suspensions were standardized to a concentration of $1x10^7$ CFU/ml for both microorganisms.

For biofilm formation, adherence of microorganisms was carried out in pre-sterile 24-well culture plates (TPP Techno Plastic Products AG, Switzerland) (26). For the tests with single cultures, a final solution was prepared in 1.5 ml containing 750 µL of the suspensions of each of the microorganisms (about $1x10^7$ cells per ml) and 750 µL RPMI 1640, which was individually transferred to wells of a sterile culture plate. For tests on dual-species cultures, it was also prepared a final 1.5 ml solution, containing 750 µL of the solution of each of the microorganisms: *C. albicans* + *S. aureus*. All plates were incubated at 37°C in an orbital shaker (75 rpm) for 90 min. After this period, the wells were washed with 1.5 ml of sterile PBS. After 08, 16 and 24 h of culture, single- and dual-species biofilms of *C. albicans* and *S. aureus* were removed from the plates and processed to obtain toxins, which were filtered in low protein binding filter 0.22 µm (SFCA, Corning, Germany) and then placed in direct contact with the NOK-si for 04, 12 and 24 h.

Protein quantification of biofilm metabolites

The total level of soluble protein contained in the "toxin" preparation was measured by Bradford assay (27), using bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) as a standard. The spectrophotometric measurements were performed at 595 nm (400 EZ Reader - Biochrom, Cambridge, UK).

Cell culture

NOK-si cells were kindly provided by Prof. Rossa Junior (Department of Periodontology, School of Dentistry of Araraquara-UNESP, Brazil) (28). The cells were cultured in DMEM (Dulbecco's Modified Eagle's, Gibco BRL, Grand Island, NJ, USA) containing 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY), 100 IU/ml penicillin, 100 mg/ml streptomycin (Sigma Chemical Co., St. Louis, MO, USA) and 2 ml/L glutamine (GIBCO, Grand Island, NY). The cells were maintained at 37°C under of 5% CO_2 and 80% humidity. The cells were grown to reach confluence (90%), counted in a Neubauer chamber (magnification ×10) and plated at 4.5×10^4 cells/well. For the experiments, cells were used between the 3rd and 8th passage.

NOK-si cellular metabolism

For the analysis of cellular metabolism, the mitochondrial activity of the keratinocytes was measured using the MTT assay [3-(4.5-dimethylthiazole-2-yl) 2.5-diphenyl tetrazolium bromide] (Sigma-Aldrich, St. Louis, MO, USA) (29). MTT was performed at 24 h after incubation at 37°C in 5% CO_2 with RPMI (as negative control), *C. albicans*, *S. aureus* and dual-species biofilm toxins, respectively. After the period of culture, the cells were washed with PBS and 250 μL of MTT (2.5 mg/ml) was added to each well and incubated for 4 h. Next, MTT was removed and the formazan crystals were solubilized in 250 μL of 2-propanol. The spectrophotometric measurements were performed at 562 nm (Reader 400 EZ - Biochrom, Cambridge, UK).

Membrane damage of NOK-si

The release of the enzyme lactate dehydrogenase (LDH) was determined after 04, 12 and 24 h of contact with the biofilm toxins, using the CytoTox-96 nonradioactive cytotoxicity assay (Promega, Madison, WI) according to the manufacturer's recommendations. First, 100 μL of control (RPMI) and experimental sample (NOK-si cell supernatant) were added in triplicate to 96 well plate (Thermo Scientific; #31125). Subsequently, 100 μL of the "CytoTox-ONE TM" reagent was added and incubated for 10 min. After this period, 50 μL of the "stop" solution was added to each well and then the fluorescence was measured with a filter combination of excitation 544 nm/ emission 590 nm (Fluoroskan, FL Ascent, Labsystems, Helsinki, Finland). RPMI and biofilm toxins were used as blank in the absence of the NOK-si cells.

Cell morphology

For Scanning Electron Microscopy **(**SEM), NOK-si were seeded on glass slides incubated with biofilm toxins for 24 h were fixed with 2.5% glutaraldehyde for 1 h. Post-fixed cells were washed in three changes of phosphate buffer and then dehydrated in increasing ethanol concentrations (70%, 90%, and 100%) for 1 h in each solution. After dehydration, the samples were placed in a desiccator for 7 days before gold sputter coating. The images were captured using the SEM JEOL JSM-6610LV (JEOL Ltd., Akishima,Tokyo, Japan) under 12 mm working distance and 12 kV. The experiment was conducted in triplicate on three independent occasions.

Cell death assay

The induction of apoptosis by biofilm toxins was confirmed using fluorescence staining and microphotographs taken of cells stained with Hoechst 33342, annexin V/Alexa Fluor 488 and PI (Molecular Probes, Invitrogen). Annexin V binds specifically to phosphatidylserine residue on the cell membrane during apoptosis. PI intercalates into the damaged DNA, a typical consequence of cell necrosis (22, 30). After overnight contact with the single and dual-species biofilm toxins, NOK-si cells were washed twice with binding buffer (10 mM Hepes/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH = 7.4) and incubated with Hoechst (1.5 µL/ml), annexin V (10 µL/well) and PI 2 µL (100 µg/ml) for 20 min at room temperature, after which cells were washed with PBS. The analysis of apoptotic, necrotic or live cells was performed using the IN Cell Analyser 2000 (GE Healthcare, Salt Lake City, Utah, USA) and an image analysis software (GE Healthcare, Salt Lake City, Utah, USA).

Caspase activity assay

The activation of caspase -2, -3, -6, -8, and -9 was measured using the ApoTarget Caspase Colorimetric Protease Assay Kit (BioSource International, CA) according to the manufacturer's protocol, after 8 h of incubation with biofilm toxins. Briefly, control and experimental cells were collected, washed with ice-cold PBS, and resuspended in chilled cell lysis buffer (20 µl per 3.2 X 10^5 cells) for 10 min on ice. Lysates were clarified by centrifugation (10,000 x g, 1 min, 4ºC). The protein concentration was determined by Bradford method (1-4 mg/ml). For the assay, all samples were standardized to 2.6 mg/ml, and protein was incubated

with 50 µl reaction buffer and 5 µl caspase fluorometric substrate (VDVAD-pNA for caspase-2, DEVD-pNA for caspase-3, VEID-pNA for caspase-6, IETD-pNA for caspase-8 and LEHD-pNA for caspase-9) at 37ºC for 2 h. Camptothecin (CPT - 0.2 µg/ml) was used as apoptosis-inducing agent (Positive Control). Enzyme-catalysed release was monitored at 405 nm on a 96-well microtiter plate reader (Reader 400 EZ - Biochrom, Cambridge, UK).

Statistical analysis

The results were statistically evaluated using two-way analysis of variance (ANOVA), followed by a post-hoc Tukey's test. Normality and homogeneity of the variances were checked using the Shapiro-Wilk and Levene tests. A 5% significance level was adopted for all statistical tests performed. All studies were performed in triplicate on three independent experiments.

RESULTS

Biofilm characterization

Higher values of $\log_{10}$(CFU/ml) of *C. albicans* were observed when this microorganism was cultivated with *S. aureus* for 08 and 16 h of biofilm formation (p=0.01) (Table 1). There was no statistically significant difference between $\log_{10}$(CFU/ml) values of *C. albicans* and *S. aureus* biofilms at 24 h of formation, in either single- or dual-species cultures (p=0.64). There was a progressive and significant increase (p=0.01) in $\log_{10}$(CFU/ml) values of the biofilm between 08 and 16 h for both single- and dual- biofilms. The same increase was not observed at 16 h and 24 h (p=0.06).

The total amount of proteins of supernatant of biofilm suspension assessed by the Bradford assay showed that *S. aureus* biofilm produced higher values at 08 h and 16 h relative to *C. albicans* biofilm formation (p=0.01), while higher protein value was observed for the dual biofilm at 24 h (p=0.01). No protein production was detected in the dual biofilm at 8 h. *C. albicans* produced a lower amount of total protein, irrespective of biofilm growth conditions (p=0.01) (Fig. 1).

NOK-si viability via MTT assay

Reduced cell viability values (p=0.01) of NOK-si cells were produced by 16 h biofilm toxins in comparison with 08 and 24 h toxins, regardless of microorganisms and biofilm growth conditions (single or dual). The dual-species biofilm was the most damaging (p=0.03), with cell viability rates of only 31.76%, when NOK-si cells were exposed to 16 h biofilm toxins. The *S. aureus* biofilm toxins were the least harmful at all assessment times of cell viability (p=0.01) (Fig. 2).

NOK-si cell membrane damage by Lactate Dehydrogenase assay

Fig. 3 shows that, after 24 h of incubation, the toxins produced by dual-species biofilms grown for 16 h and 24 h, respectively, caused the greatest cell damage (p=0.01), with the former producing the highest LDH release values (p=0.01). In general, *S. aureus* biofilms toxins caused the least cell damage (p=0.01), regardless of the time of cell exposure to toxins.

NOK-si cell morphology by SEM

SEM findings corroborated with the MTT data. Minor NOK-si cell morphology changes were observed when cells were exposed to 08 h biofilm toxins. When cells were incubated with the *C. albicans* biofilm toxins, characteristic signs of cell death were observed with the formation of surface blebbings. Apoptotic bodies were evident on cells incubated with 16 h biofilm toxins, particularly when the cells were stimulated with the dual biofilm toxins. Further cell damage was observed when the cells were exposed to 24 h dual biofilm toxins, where pores in the membrane were evident. *S. aureus* biofilm toxins caused less cell damage, showing characteristics more similar to control (Fig. 4).

NOK-si cell death evaluation from Annexin V/PI assay

The toxins from 08 h and 16 h-old dual-species biofilms produced higher percentages of necrotic cells (24.51% and 24.95%, respectively) compared to *C. albicans* and *S. aureus* toxins (p=0.01) (Fig. 5). A higher percentage of apoptotic cells were also observed for 16 h dual biofilm (32.52%, p=0.01).

A greater amount of annexin V stained cells was observed when the cells were exposed to *S. aureus* single biofilm toxins (Fig. 6). A significant number of cells were labelled with PI

when cells were exposed to dual-species biofilm toxins, suggesting a cytotoxic effect related to membrane damage comparable with necrosis or late apoptotic cell death.

NOK-si caspase activity after exposure to biofilm toxins

S. aureus and dual-species biofilm toxins induced activation of caspase -2, -3, -6 and -8 when compared to control cells (p=0.01), as shown in Fig. 7. *C. albicans* toxins were able to activate only caspase -3 (p=0.01). The caspase -9 values in the experimental groups were similar to the negative control (p=0.53).

DISCUSSION

The data obtained here demonstrated that the association between toxins from dual-species biofilms promoted more pronounced cell death of NOK-si (68.24%). In addition, there was a greater release of LDH after contact with toxins of these biofilms. In contrast, low LDH levels were observed after NOK-si contact with *S. aureus* toxins. These findings suggest that *C. albicans* can enhance the damage caused by *S. aureus* (31). In addition, formation of apoptotic bodies and pores in the membrane were more evident after contact with dual-species biofilm toxins, and there was stronger labeling of the annexing V/PI cells and high percentage of necrotic cells (24.95%). Previous proteomic analyses have shown that a total of 27 proteins were significantly differentially produced by *S. aureus* and *C. albicans* during co-culture biofilm growth (4). In a recent study, high production of proteinase in single-species *S. aureus* biofilm was found, while *C. albicans* single-species biofilm presented high phospholipase levels, but when both microorganisms were co-cultured, both enzymes were produced (5). This synergism may have accounted for the higher toxicity of the dual-species biofilm toxins.

When the times of biofilm formation were evaluated, toxins from 16 h-old biofilm produced higher percentage of cell death than toxins from 24 h-old biofilm. This finding can be explained because at 16 h biofilm could have more metabolic active cells, so the microorganism can produce more toxins; confirmed by the $\log_{10}$(CFU/ml) results, where there is an increase in the $\log_{10}$(CFU/ml) values when comparing 08 h and 16 h (Table 1).

Here, toxins from 16 h-old dual-species biofilm were associated with late apoptosis or necrosis, with high percentage of necrotic cells (24.95%) and apoptotic cells (32.52%), suggesting that when the cells are exposed to dual-species biofilm toxins, they undergo a rapid and extensive damage of the plasma membrane. Apoptosis and necrosis are mechanism by

which eukaryotic cells may die. Necrosis is considered to be a major pathologic response to perturbations in the cellular environment, leading to increase in cytoplasmic volume, osmotic lysis and release of intracellular content to the extracellular medium (14, 15). This process promotes potentially harmful inflammatory and immune responses; necrotic cells can activate the inflammasomes, induce cytokine production, facilitate inflammatory cell recruitment to the site of infection and stimulate dendritic cell maturation (32), making the local treatments on mucosal tissue more challenging to perform and succeed.

A high rate of apoptotic cells was observed when the cells were exposed to 08 h *C. albicans* (24.29%) and 08 h *S. aureus* biofilm toxins (20.64%), indicating that single biofilm toxins are more related to apoptosis, which is less harmful as compared to dual-species biofilm toxins, once apoptosis does not involve inflammation, as the membrane remains intact. Apoptosis is involved in many physiological processes, including cell morphogenesis and homeostasis, but it also occurs in response to stress conditions, such as intracellular infections (14, 15).

This study also evaluated the effects of biofilm toxins in caspase activation. It was observed that *S. aureus* and dual-species biofilm toxins activated caspase -2, -3, -6 and -8 production, with no significant differences in caspase activation between these biofilms. *C. albicans* single biofilm toxins activated only caspase -3 production, which demonstrates that the presence of *S. aureus* in dual-species biofilm may be associated with increased caspase activation by this biofilm. Caspase-2 is an initiator caspase during apoptosis mediated by the pore-forming toxin (33). Here, it was observed that production of caspase -2 was induced by *S. aureus* and dual-species biofilm toxins. Although the toxins forming pores are related to virulence factors of *S. aureus* (7, 33), scanning electron microscopy shows that the pores on the NOK-si membranes were more detected after stimulation with dual-species biofilm toxins, which may indicate an increase in the activation of this pathway in the presence of *C. albicans*. The activation of caspase-3 and -8 was involved in cell death induced by lipoteichoic acid from *S. aureus* (34). The same mechanism may be involved in the present study.

S. aureus biofilms have been shown to induce apoptosis by activation of caspase -3 (35). Likewise, the presence of *C. albicans* in epithelial cells is capable of stimulating signaling pathways that promote cell death by apoptosis through the activation of cellular caspases, followed by late necrosis (23). Further, the activation of caspase -3 induced by *S. aureus*, *C. albicans* and dual-species biofilm toxins may be associated with the apoptotic cell death when NOK-si cells were exposed to these biofilm toxins. Furthermore, little labeling with annexin V/PI was observed when cells were stimulated by *C. albicans* biofilm toxins, when compared

to the other groups. This may have occurred because *C. albicans* biofilm toxins are responsible only for the activation of caspase -3. Annexin V labeling was significantly lower when NOK-si cells were incubated with *C. albicans* biofilm toxins. The relationship with the apoptosis inhibition has been found with the activation of caspase-3 (36), which is confirmed in the present study, once it was found lower percentages of apoptotic cells when NOK-si were exposed to *C. albicans* biofilm toxins. Moreover, there was a statistically significant difference when *C. albicans* biofilm toxins were compared to 16 h and 24 h *S. aureus* and dual biofilm toxins.

This study demonstrated that the synergistic effect of *C. albicans* and *S. aureus* biofilms toxins resulted in the loss of cell viability, cell membrane damage, late apoptosis or necrosis and activation of caspase -2, -3, - 6 and -8. To the best of our knowledge, this is the first study to characterize, in a more comprehensive manner, the oral keratinocytes (NOK-si) cell death promoted by toxins of single and dual biofilms of *S. aureus* and *C. albicans*.

CONCLUSION

The toxins derived from the supernatants of biofilms with 08, 16 and 24 h of formation were harmful to the oral epithelial cells, and the supernatant of the dual biofilms was more toxic, inducing more cell death and promoting apoptosis/necrosis related to the activation of caspases.

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Table 1 - Mean value and standard deviation (SD) value of log_{10}(CFU/ml) for the growth of single- and dual-species *C. albicans* and *S. aureus* biofilms at 08, 16 and 24 h (n = 9).

Time Period (h)	**Growth conditions**			
	Ca (±SD)	**Sa(±SD)**	**Dual Ca (±SD)**	**Dual Sa (±SD)**
08	5.46 (±0.31)^{Aa}	7.64 (±0.17)^{Ab}	6.04 (±0.31)^{Ac}	7.83 (±0.13)^{Ab}
16	5.94 (±0.18)^{Ba}	8.34 (±0.36)^{Bb}	6.50(±0.20)^{Bc}	8.43 (±0.34)^{Bb}
24	6.27 (±0.35)^{Ba}	8.43 (± 0.16)^{Bb}	6.43 (±0.22)^{Ba}	8.35 (±0.35)^{Bb}

The uppercase letters show differences among time periods and lowercase letters show differences among the growth conditions.

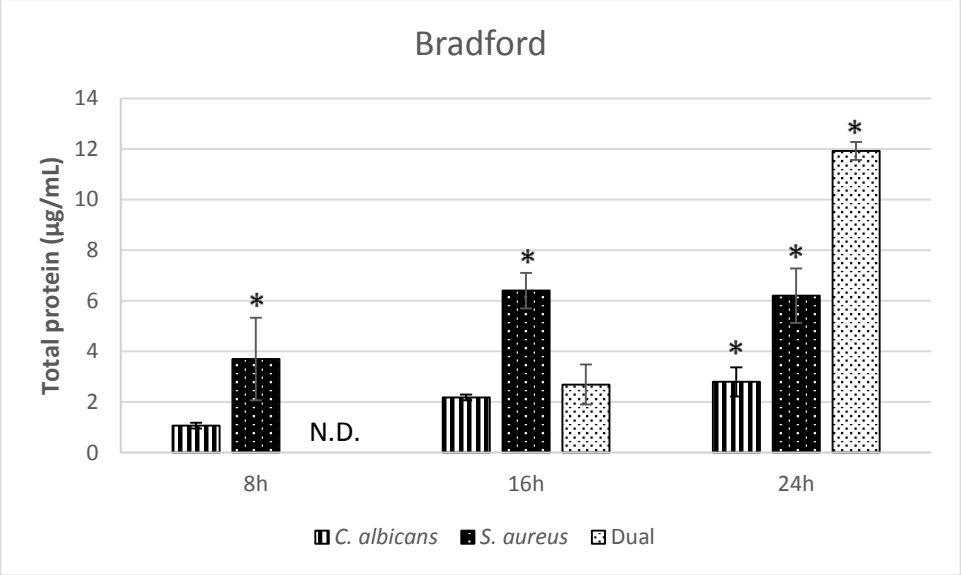

Fig. 1: Quantification of single- and dual-species biofilm total protein production by the Bradford method. Differences with $p<0.05$ (*) are considered statistically significant. N.D.= not detected. (Error bars = ± SEM; n = 9).

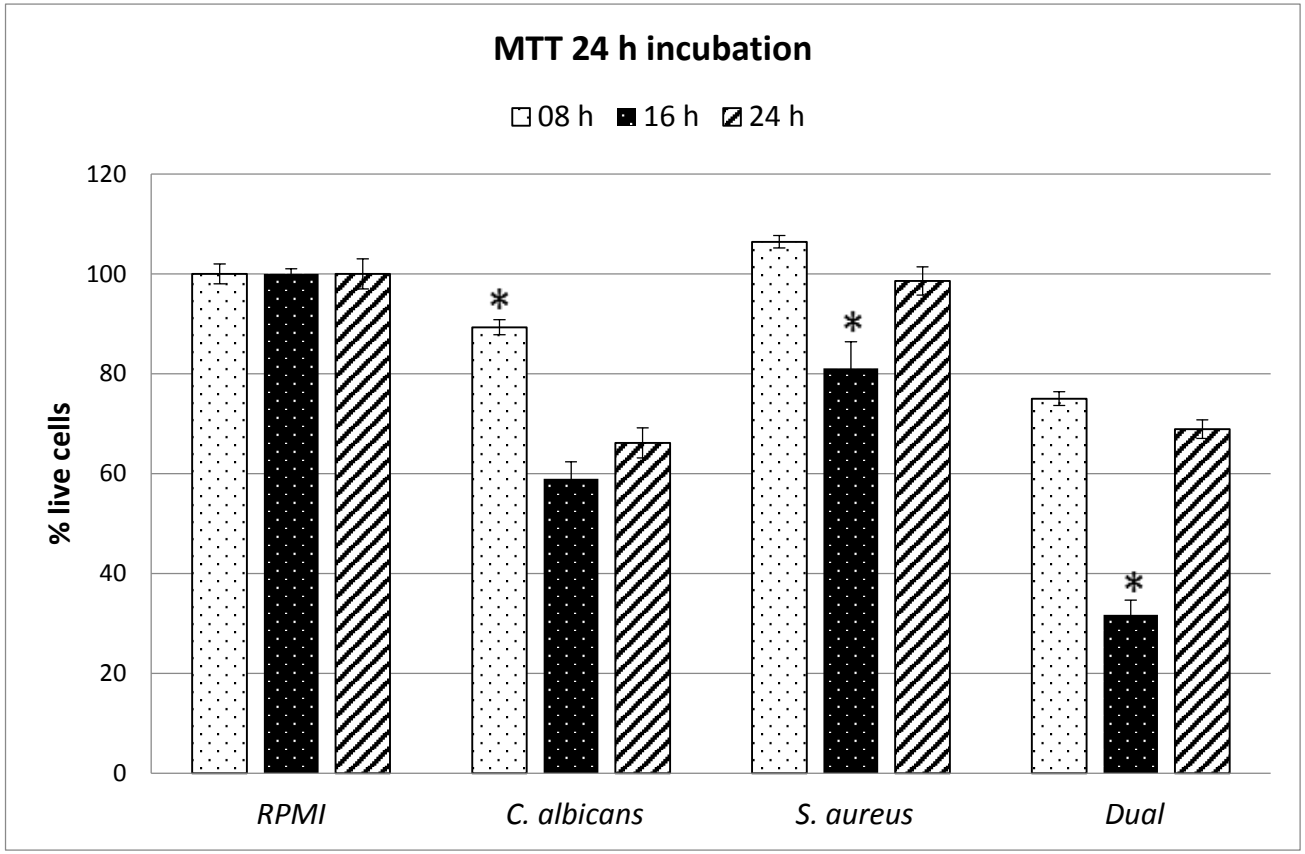

Fig. 2: NOK-si cell viability after contact with biofilm "toxins" relative to the control (RPMI) of three independent replications, in triplicate for each experimental condition (n=9). Differences with $p<0.05$ (*) are considered statistically significant. Bars represent standard error of the mean (SEM). (Error bars = ± SEM; n = 9).

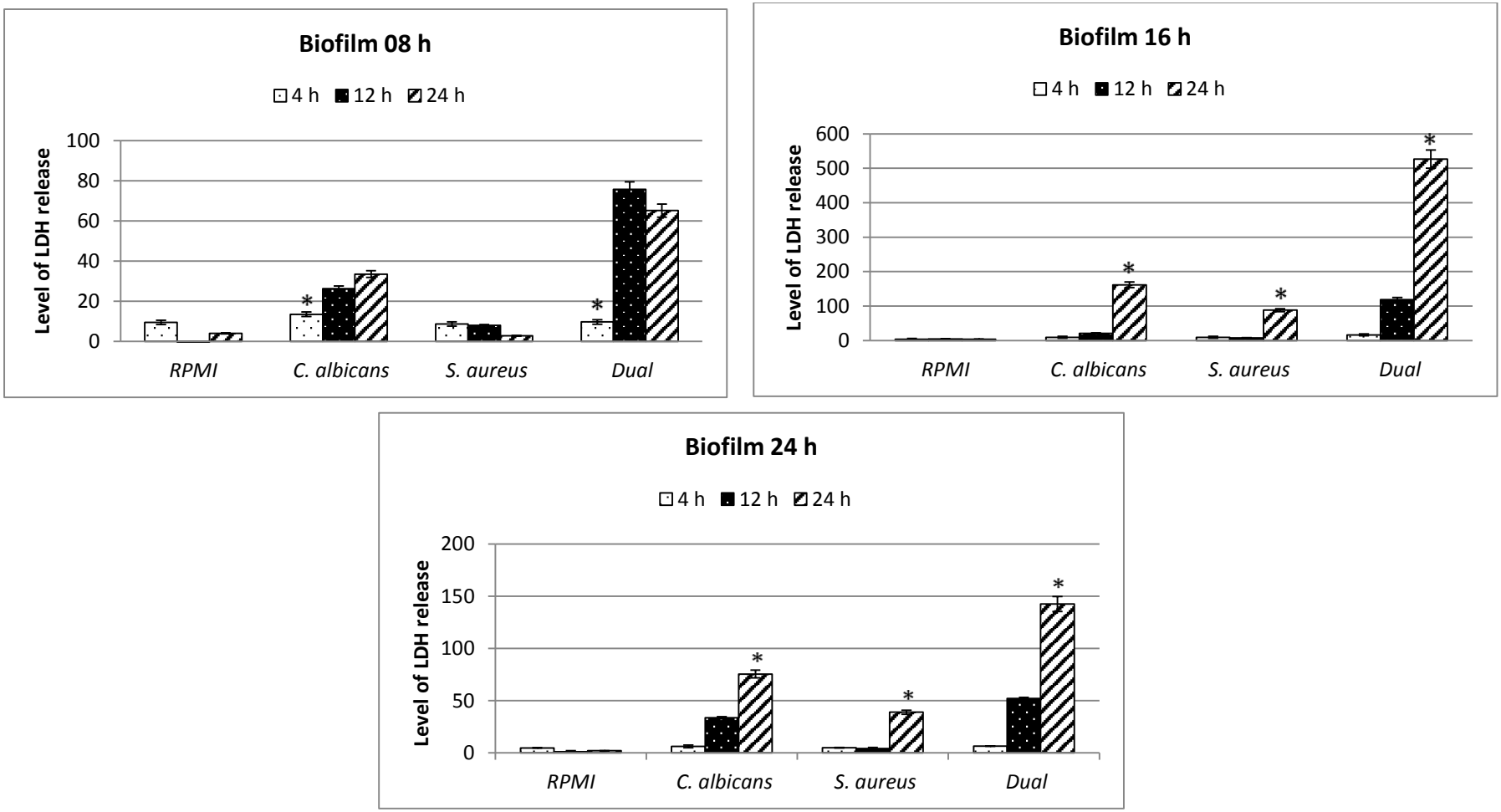

Fig. 3. Level of LDH release from NOK-si cells at different incubation times with biofilm "toxins", three times performed in triplicate (Error bars = ± SEM; n = 9). RPMI = negative control; *C. albicans* = supernatant of *C. albicans* biofilm; *S. aureus* = supernatant of *S. aureus* biofilm; Dual = supernatant of dual biofilms.

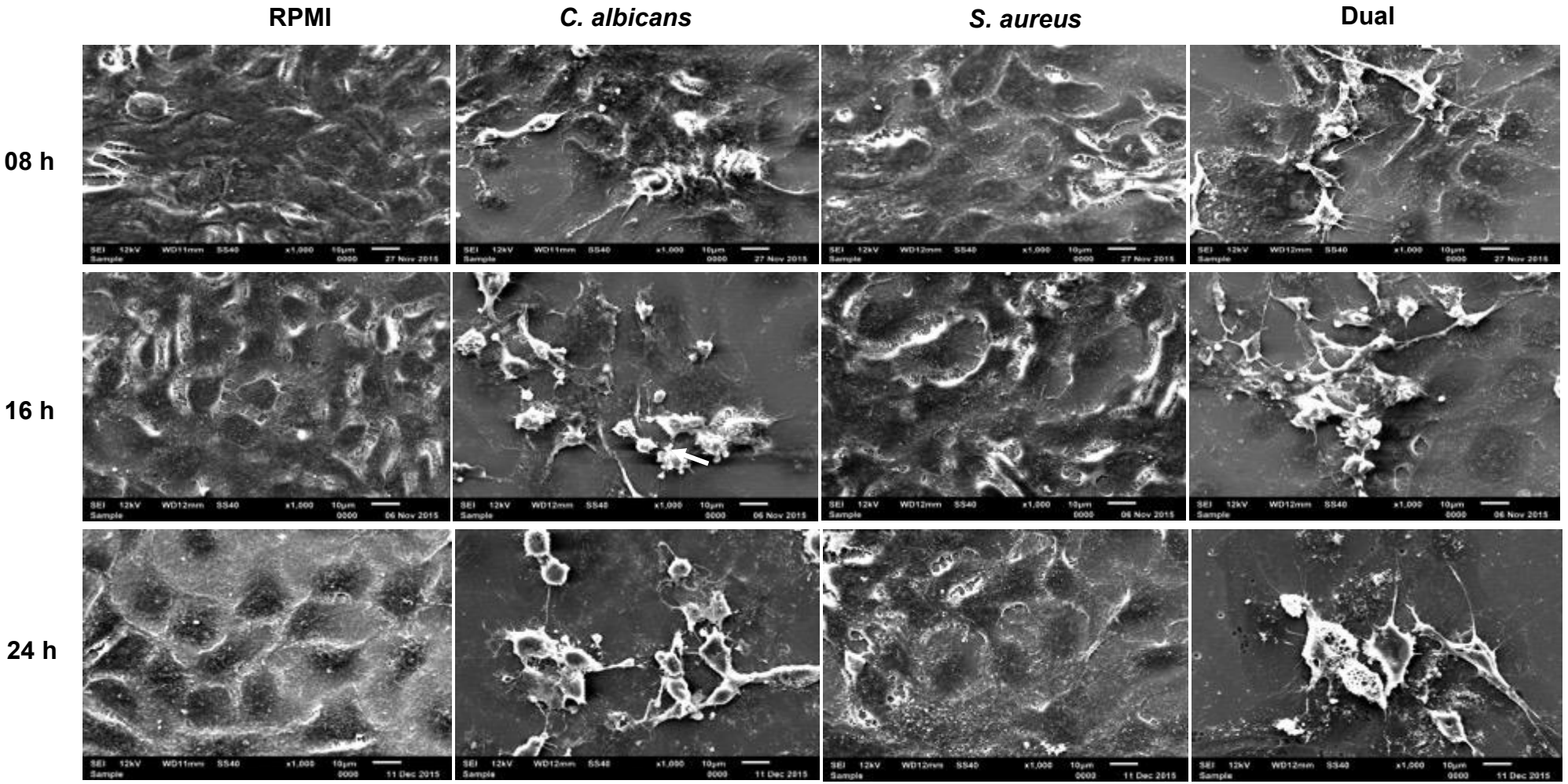

Fig. 4. SEM micrographs of NOK-si cells incubated with single and dual-species biofilms toxins in negative control group (RPMI), *C. albicans*, *S. aureus* and Dual (*C. albicans* + *S. aureus*) biofilms group; 1000 × zoom.

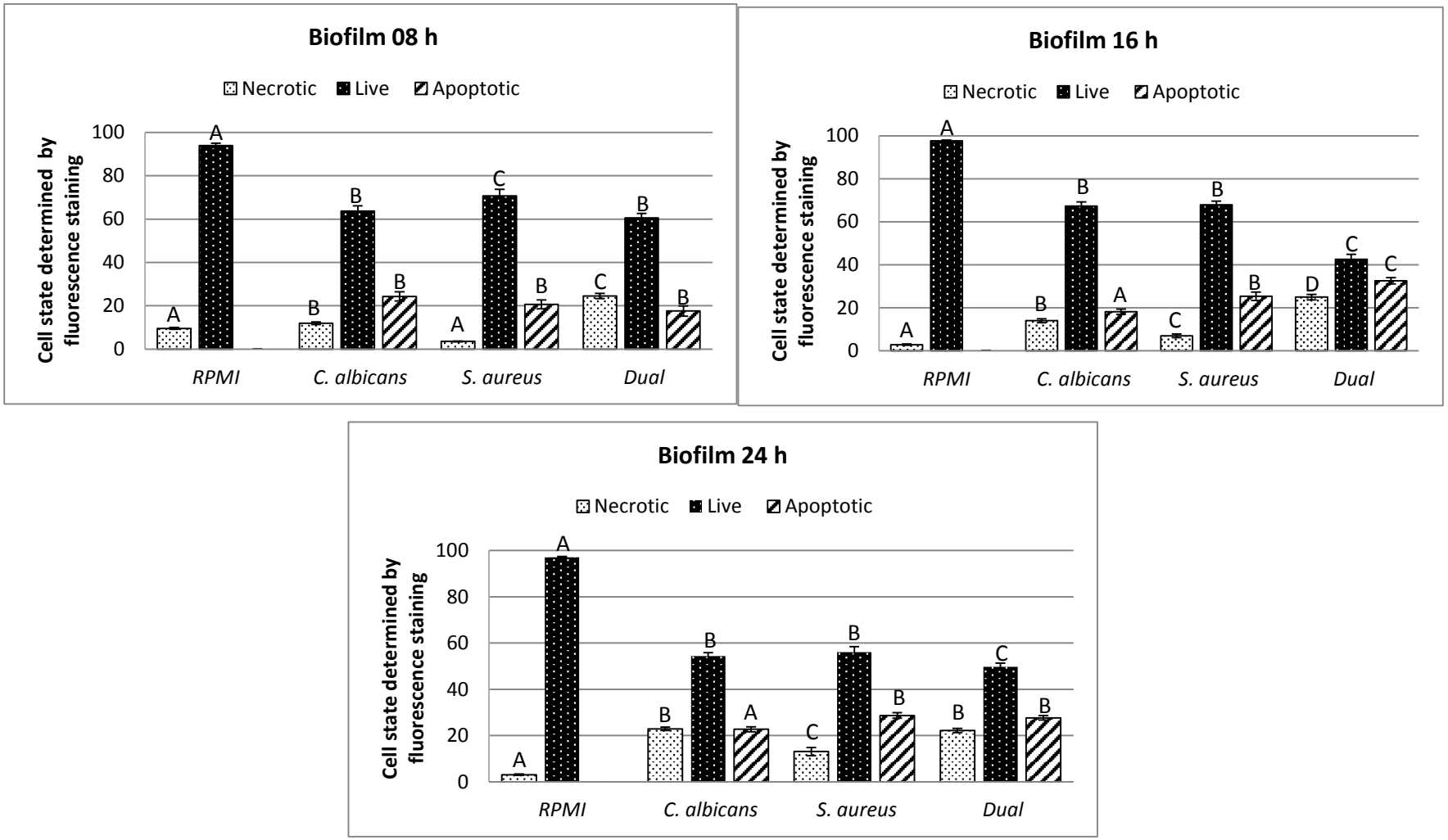

Fig. 5: Results of the In Cell analyser from NOK-si cells in contact with biofilm toxins. * different letters denote statistically significant difference ($p<0.05$) among the growth conditions.

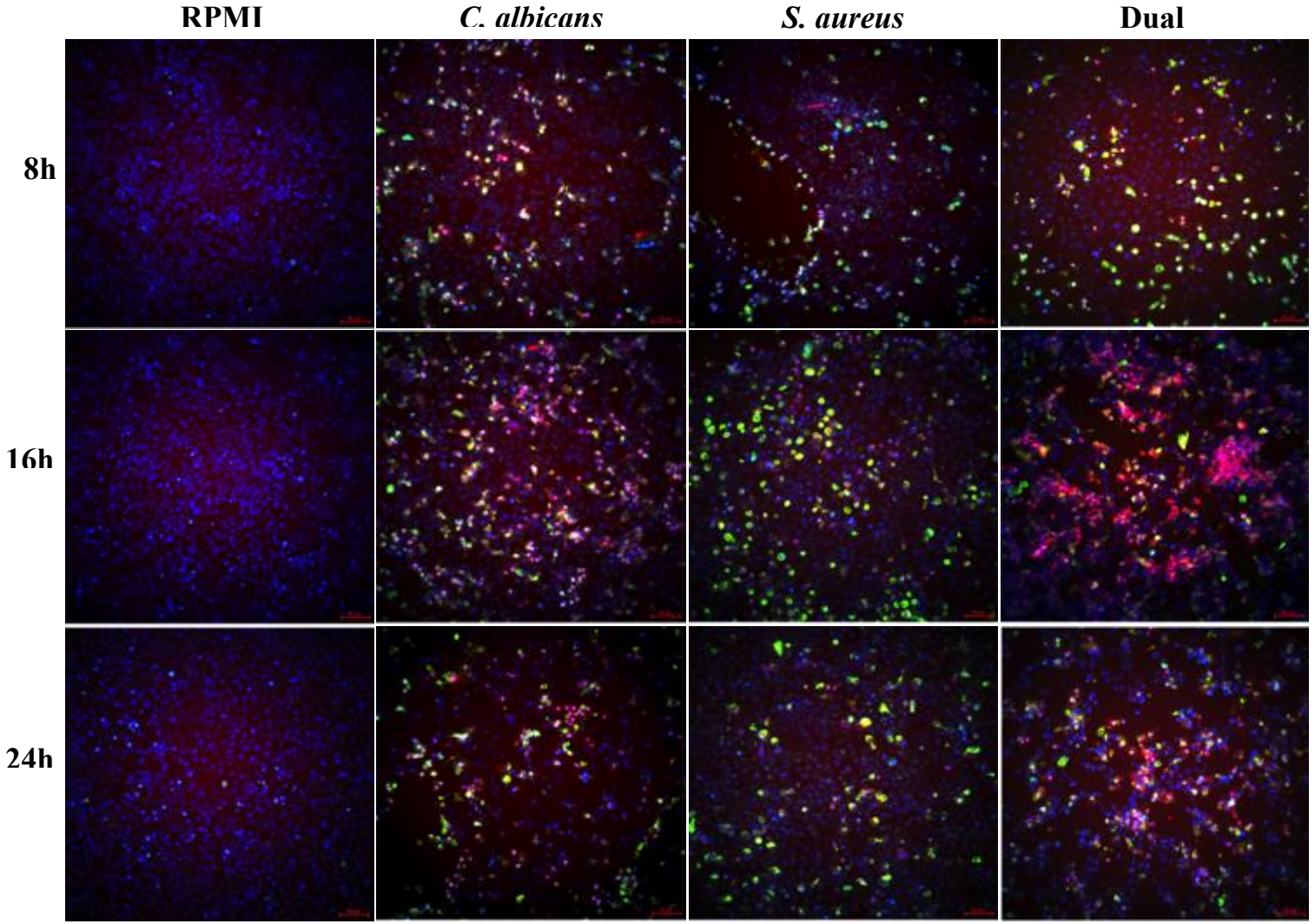

Fig. 6: The cells were then subjected to In Cell analyser after staining for annexin-V-FITC and propidium iodide. Apoptotic cells refer to those cells expressing annexin-V (green), and PI positive (necrotic; red) cells. The live cells are stained with Hoechst dye (blue).

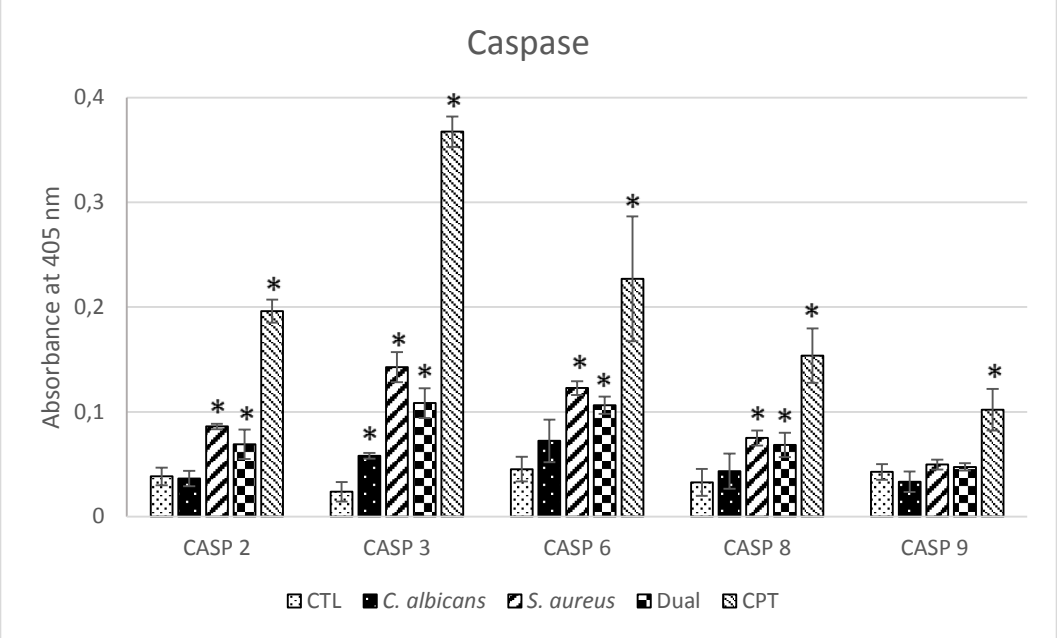

Fig. 7: Caspase activity of NOK-si cells in contact with biofilm toxins. Data from three independent replications, in triplicate for each experimental condition (n=9). Differences with $p<0.05$ (*) are considered statistically significant. CTL = negative control (RPMI); *C. albicans* = *C. albicans* biofilm toxins; *S. aureus* = *S. aureus* biofilm toxins; Dual = dual-species biofilm toxins; CPT = positive control (camptothecin).

3.4 Publicação 4

Development and characterization of a Reproducible 3D Oral Mucosa Model and its application as a model to study biofilms infections.*

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Abstract

The objectives of this study were to (i) design, develop and validate a practical and physiologically relevant reconstituted *in vitro* oral mucosa tissue model and (ii) to assess its applicability in *in vitro* infection experiments with *C. albicans* and *S. aureus* single and dual-species biofilms. Co-culture organotypic constructions were created by incorporating specific numbers of keratinocytes (NOK-si) onto cellularised, commercial collagen gel scaffolds containing human gingival fibroblasts incubated in KGM media and cultured for 14 days. The structure and detection of appropriate oral mucosa/epithelial proteins were evaluated by histology (hematoxylin and eosin (HE), periodic acid–Schiff (P.A.S.) and Picrosirius red), and immunocytochemistry (cytokeratin 13, cytokeratin 14, Ki-67 and collagen IV), and compared to a normal human gingiva. The morphology of the reconstituted tissue was analyzed by Transmission electron microscopy. To further quantitate tissue damage, lactate dehydrogenase (LDH) was measured in the culture supernatant. NOK-si grown upon a gingival scaffold provided an organotypic model in an *in vitro* setting and exhibited structural characteristics typically associated with oral mucosa tissue. Immunocytochemistry revealed detection of epithelial proteins, *e.g.* cytokeratin 13 and 14 in the reconstituted oral mucosa obtained in the *in vitro* model. Low microorganism invasion of tissue was detected after 8 h, whereas extensive tissue damage was evident after 16 h when assessed using histological examination and LDH activity determination. This study presents an *in vitro* cellularised, organotypic model of reconstituted oral mucosa, which enables close control and characterization of its structure and differentiation over a mid-length period of time in culture. The model was applied to assess infection patterns of *C. albicans* and *S. aureus* single and dual-species biofilms.

Keywords: Reconstituted human oral epithelium. Keratinocytes. Cell culture. *Candida albicans*. *Staphylococcus aureus*. Tissue engineering. Oral mucosa.

Introduction

Reconstituted oral mucosa tissue (ROMT) is the process of providing suitable matrix support structure in conjunction with viable cells coupled with an optimal growth environment that allow the development of functional tissue *in vitro*. The basic premise of ROMT is that controlled manipulation of the extracellular microenvironment can influence the ability of cells to organize, grow, differentiate, form a functional extracellular matrix (ECM) and, ultimately, generate new functional tissue (Scheller, 2009). ROMT has a broad applicability and it has been used as an alternative to human and animal testing of drugs, and for pharmacological and clinical applications (Brohem *et al.*, 2011). Moreover, three-dimensional culture exhibits cells growing in the 3D culture that closely mimics the *in vivo* environment (Edmondson *et al*., 2014).

Although the components of ROMT are basically the same, different methodologies to reconstitute epithelium and connective tissue have been reported. There is no consensus in relation to the kind of keratinocyte and fibroblast used to reconstitute epithelial- and connective-like layers, respectively, and a variety of scaffolds have been employed. The model proposed in this paper is composed by an epithelial- and connective-like layers formed by immortalized normal oral keratinocytes (NOK-si), and a collagen matrix formed by a rat tail collagen type I as scaffold. The use of established cell lines allows unlimited access by passaging and cryopreservation and may also improve the reproducibility and consistency of human skin models, thereby allowing specific pathways or variables to be identified and assessed (Boelsma *et al.*, 1999).

ROMT has been used to evaluate the potential of microorganisms to grow on, penetrate and destruct oral mucosa and to elucidate the mechanism of local infection. It has been suggested that *Candida albicans* improves its ability to penetrate across the oral mucosa and to promote tissue destruction leading to focal infection when it is associated with *Staphylococcus aureus* (Shirtliff, Peters, Jabra-Rizk; 2009). *C. albicans* can colonize the cavity alone or in combination with other microorganisms (Coronado-Castellote, 2013), and it is the most frequently isolated microorganism (64.4%) from denture bases (Ribeiro *et al*., 2011). *C. albicans* has a lot of virulence factors which makes it able to invade and to infect host cells, such as polymorphism (Berman and Sudbery, 2002; Jacobsen *et al*., 2012; Saville *et al*., 2003; Mayer *et al*., 2013), presence of adhesins (Garcia *et al.*, 2011; Verstrepen and Klis, 2006; Mayer *et al*., 2013), ability to form biofilm (Fanning and Mitchell, 2012; Finkel and Mitchell, 2011; Uppuluri 2010; Mayer et al., 2013), and phospholipase and protease activities (Calderone *et al*.,

2001; Lyon *et al.*, 2006; Pinto *et al*., 2008; Zago *et al*., 2015).

The combined effect of *C. albicans* with other microorganisms may result in synergism and increase the pathogenicity of both microorganisms (Morales, Hogan; 2010; Peters *et al*., 2012; Zago *et al*., 2015). There is evidence showing that this combination enhances the levels of local and systemic pro-inflammatory cytokines, such as interleukin-6, TNF-α, interleukin-1β, regardless of morphogenesis of *C. albicans* (Nash *et al*., 2014). It has been estimated that 27% of nosocomial *C. albicans* bloodstream infections are polymicrobial, with *S. aureus* as the third most common isolated organism (Harriot, Noverr; 2009). *S. aureus* is a Gram-positive bacterium and can be found on human skin, mucosa surfaces and several animal species (VandenBergh e*t al.*, 1999). Studies have described the high prevalence of *S. aureus* on the oral mucosa in dental prosthesis wearers, suggesting that *S. aureus* is a normal colonizer of the oral cavity (Monroy *et al*., 2005; Cuesta *et al*., 2010). In addition, it has been reported the association between *S. aureus* and *C. albicans* in the colonization of oral mucosa and dental prosthesis wearers with denture stomatitis (Monroy *et al*., 2005; Ribeiro *et al.*, 2011).

The objectives of this study were to develop and to validate a practical and physiologically relevant reconstituted oral mucosa model using immortalized cell lines as well as to evaluate the potential of single-species biofilms (*C. albicans* alone or *S. aureus* alone) and dual-species biofilms (*C. albicans* associated with *S. aureus*) to penetrate and to damage the oral mucosa model developed.

Materials and methods

ROMT construction

Fibroblasts were obtained from Rio de Janeiro Cell Bank (FGH, cod. 0089), which were derived from human primary cell line established from biopsies of normal patients' gingiva. Fibroblasts were cultivated in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, USA), antibiotic, antimycotic solution (Sigma, St. Louis, MO, USA). The cultures were used between 3rd and 8th passages. Collagen gel was produced by mixing rat tail collagen type I (First Link (UK) Ltd.) with DMEM and FBS at 4°C. The solution was neutralized with 1 M NaOH, and a fibroblasts suspension (3.0 x 10^5 cells/mL) was added in the mix. The fibroblast-containing collagen solution was placed in 24 well plates and incubated at 37°C and 5% CO_2. After polymerization, fibroblast-containing collagen gel was washed with

Hanks buffer, and keratinocytes were seeded onto the surface of the gel (2.0 x 10^6 cells/mL). NOK-si, normal oral keratinocytes that were immortalized, were a generous gift of Prof. Dr. Carlos Rossa Jr (Castilho *et al.*, 2010). NOK-si was cultivated in DMEM supplemented with 10% FBS and antibiotic antimycotic solution. The cells were used between 3rd and 8th passages. Tissues were grown until the epithelial cells reached confluence. Then they were raised to an air-liquid interface for 14 days in KGM-Gold medium (Lonza, Walkersville, MD USA) supplemented with 0.5 ml hydrocortisone, 0.5 ml transferrin, 0.25 ml epinephrine, 0.5 ml gentamicin sulfate amphotericin-B, 2.0 ml bovine pituitary extract, 0.5 ml epidermal growth factor human and 0.5 ml insulin. The culture medium was changed every other day. The tissues were prepared in duplicate on three independent occasions.

Histological evaluation

The tissues were fixed in 4% formaldehyde buffered at pH 7.2 with 0.1 M sodium phosphate for 24 h at 4°C. Subsequently, the tissues were dehydrated and embedded in paraffin. Four-micron sections were stained with hematoxylin and eosin (HE) and submitted to the periodic acid-Schiff (P.A.S.) histochemical method. Some sections were also stained with Picrosirius-red method and analyzed under light microscope BX-51 (Olympus, Japan) equipped with filters to provide polarized illumination. As control, normal human gingiva was used.

Immunohistochemical reactions

The following primary antibodies were used: rabbit anti-Ki-67 polyclonal antibody (Abcam; ab833, 1/200), rabbit anti-collagen IV polyclonal antibody (Abcam; ab6586, 1/500), rabbit anti-cytokeratin 13 polyclonal antibody (Abcam; ab154346, 1/1000) and mouse anti-cytokeratin 14 monoclonal antibody (Abcam; ab7800, 1/400) (mouse monoclonal). Immunohistochemical reactions were performed using rabbit specific HRP/DAB detection IHC kit (Abcam, ab64261) for Ki-67, collagen IV and cytokeratin 13. The sections were incubated with biotinylated secondary antibody (Dako-K0690; Dako Universal LSAB Kit) for cytokeratin 14.

Tissue sections (4 μm) were deparaffinized, rehydrated and submitted to heat-induced epitope retrieval by microwave treatment for 2 x 5 min in 0.001 M sodium citrate buffer (pH 6.0) (Ki-67, collagen IV and cytokeratin 14), or trypsin/0.1% calcium chloride (cytokeratin 13). After washing with phosphate buffered saline (PBS) (pH 7.3), sections were treated with 5% hydrogen peroxide (H_2O_2) to block endogenous peroxidase for 10 min at room temperature.

After washing, the sections were incubated for 20 min with 2% bovine serum albumin (BSA) and sodium azide/triton at room temperature. Then sections were incubated with primary antibody overnight in the humidified chamber at 4º C. Subsequently, the sections were incubated in biotinylated secondary antibody (Abcam) for 20 min at room temperature and streptavidin for 30 min. The reaction was revealed by using 3,3'-diaminobenzidine (DAB) (Dako, Carpinteria, CA, USA) for 3 min and the sections were counterstained with hematoxylin and mounted. For each sample, a negative control section was performed. In the negative controls, the primary antibody incubation step was replaced by incubation in non-immune serum.

Ultrastructural characterization

The specimens were fixed for 16 h in a mixture of 2% formaldehyde (freshly prepared from paraformaldehyde) and 2.5% glutaraldehyde buffered at pH 7.2 with 0.1 M sodium cacodylate at 4 °C. After washing with 0.1 M sodium cacodylate buffer twice for 15 min, the tissues were postfixed with sodium cacodylate-buffered 1% osmium tetroxide for 1 h. Subsequently, the specimens were washed and immersed in 2% uranyl acetate for 1 h at room temperature. After dehydration, the specimens were kept in propylene oxide for 15 min and embedded in Araldite. Ultrathin sections were collected on grids, stained with alcoholic 2% uranyl acetate and lead citrate and examined in a transmission electron microscope (TEM, JEOL JEM-1400).

Microbial strains, growth conditions, and tissue infection

C. albicans SC5314 and *S. aureus* ATCC25923 were used, as described previously (Peters *et al*., 2012; Zago *et al*., 2015). Prior to use, *C. albicans* was maintained in Yeast Peptone Glucose medium (YEPD: 1% yeast extract, 2% Bacto peptone and 2% D-glucose, 2% agar) at -80°C. To prepare stock culture, *C. albicans* was subcultured onto Sabouraud Dextrose Agar plates (SDA-Acumedia Manufactures Inc., Baltimore, MD, USA) supplemented with chloramphenicol (0.05 g/L) and incubated at 37°C for 48 h. A loop full of the agar stock culture was transferred to Yeast Nitrogen Base broth (YNB-Difco, Becton Dickinson Sparks, MD, USA) supplemented with 100 mM glucose and incubated at 37°C overnight (16 h). A 1:10 dilution of the overnight culture was made into fresh YNB supplemented with 100 mM glucose and allowed to propagate at 37°C until mid-log phase was reached (OD 540nm=0.557±0.148).

S. aureus was maintained in Tryptic Soy Broth medium (TSB- Acumedia Manufactures

Inc., Baltimore, MD, USA) and frozen at -80°C until use. To prepare stock culture, *S. aureus* was subcultured onto Tryptic Soy Broth medium, and approximately 10 colonies were inoculated into TSB liquid medium overnight (18 h) at 37°C. A 1:20 dilution of the overnight culture was made into fresh TSB and allowed to propagate at 37°C until mid-log phase was reached (OD 600nm=0.597±0.019).

Following growth, both microorganisms were washed twice in PBS by centrifugation at 5,000 x g for 5 min (rotor model A462), and re-suspended in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA). *C. albicans* and *S. aureus* suspensions were spectrophotometrically standardized at an OD_{540} nm of 1.0 and OD_{600} nm of 0.1, respectively, which corresponds to a final concentration of $1x10^7$ cells/ml of each organism. Microorganisms suspensions were placed on top of the reconstituted *in vitro* oral mucosa tissue in antibiotic-free KGM-Gold medium containing growth factors and were incubated in a CO_2 chamber at 37°C. After 8 and 16 h of incubation, biopsy specimens were collected from infected and uninfected tissues, and stained with hematoxylin and eosin (HE).

This infection protocol was compared with the supernatant protocol, which after 8 and 16 h of biofilm formation, the supernatant of biofilms was collected and filtered in low protein binding filter (0,20μm) (SFCA, Corning, Germany) and placed in contact with the tissue for 24 h, in the same conditions.

Lactate Dehydrogenase Assay

The release of lactate dehydrogenase (LDH) from tissues into the surrounding medium was monitored as a measure of epithelial cell damage. LDH release into the maintenance media of the cultures containing uninfected and infected epithelial cells (infection and supernatant protocol) was measured after 8 and 16 h of incubation. LDH activity was analyzed by measuring the rate of NADH disappearance at 540/590 nm during the LDH-catalyzed conversion of pyruvate to lactate by using the CytoTox-ONE kit (Promega, G7890) according to the manufacturer's instructions, and FluoroskanAscent FL (Thermo labsystems).

Statistical analyses

The results of each analysis were tabulated and submitted to normality tests (Shapiro-Wilk) and homogeneity of variance (Levene) to check the distribution of data. Based on the results observed through these tests, we used ANOVA with a significance level of 5%.

Results

ROMT construction and characterization

ROMT models using NOK-si cell line and FGH associated with collagen matrix allowed the establishment of a stratified epithelium tissue composed by 6-8 layers of juxtaposed cells (Fig. 1A and 1B). Although the thickness of the epithelium of ROMT has not reached that observed in the gingival mucosa, the NOK-si cells formed stratified epithelium with similar characteristics to the human gingiva (Fig. 1D and 1E). Fibroblasts and birefringent collagen fibres showed a structural arrangement forming an intricate network of supporting to the epithelium (Fig. 1A-1F).

Sections of ROMT and gingival mucosa subjected to immunohistochemistry for cytokeratin-13 and -14 detection (epithelial cells markers) exhibited positive immunolabelling in the cytoplasm of epithelial cells (Figs. 2A-2D). An enhanced cytokeratin 14 immunolabelling was observed in the epithelial cells of different layers in the ROMT (Fig. 2C). In the gingival mucosa, the positive immunoreaction for cytokeratin 14 was particularly observed in the basal and supra basal layers of epithelium (Fig. 2D). To investigate the epithelial proliferation, Ki-67 immunoexpression in the ROMT and gingival mucosa was analyzed. Ki-67-positive cells were often found in the stratum basale of the epithelium of ROMT and gingival mucosa (Figs. 2E and 2F), confirming the occurrence of epithelial cell proliferation in the ROMT.

Based in these findings, the presence of type IV collagen commonly found in the basal lamina situated in the interface between epithelium and connective tissue was investigated. In ROMT sections, a granular immunopositive material was observed in this region (Fig. 2G). In the gingival mucosa, an evident immunolabelling was detected in the basal lamina (Fig. 2H).

The ultrastructural analysis revealed epithelium composed of several layers of cells in close juxtaposition; numerous desmosomes were observed by attaching a cell to another. These cells exhibited thick tonofilament bundles distributed throughout the cytoplasm (Fig. 3A and 3B). In the subepithelial connective tissue, some collagen fibrils were also observed (Fig. 3C).

Tissue damage by biofilms ad its metabolites

The ROMT were used to evaluate the potential of single-species biofilms (*C. albicans* alone or *S. aureus* alone) and dual-species biofilms (*C. albicans* associated with *S. aureus*) to

colonize, penetrate and destruct the epithelial tissue surface after 8 and 16 h of incubation (Fig. 4). *C. albicans* and *S. aureus* were able to colonize and to destruct the ROMT. Single-species biofilms of *S. aureus* exhibited a low colonization level, as opposed to single species biofilms of *C. albicans.* Invasion of hyphae into epithelium was characterized by tissue destruction (Fig. 4A). *C albicans* alone promoted damage in the epithelium surface while the dual species invaded deeply the ROMT cultures reaching the subepithelial collagen matrix. The extent and depth of colonization were greater for dual species biofilms (*C. albicans* associated with *S. aureus*) (Fig. 4). After the contact with the supernatant of biofilms, the superficial layer of the ROMT was irregular and altered (Fig. 4).

To quantify cell injury, the levels of LDH released from ROMT cells after *C. albicans*, *S. aureus* and dual-species biofilms (microorganisms or supernatant protocol) when co-cultured with ROMT were compared. We observed that the amount of LDH released differ greatly between 8 and 16 h of infection. After 8 h of infection, LDH release was low, and single species biofilms of *S. aureus* exhibited the lowest damage. However, after 16 h of infection, a significant increase in the LDH release was found, mainly by dual-species biofilms (Fig. 5A). On the other hand, the contact with supernatant of the microorganisms caused a significant change in LDH level after 8 h, without statistical difference between 8 h and 16 h, with the exception of dual biofilms (Fig. 5B).

Discussion

The ROMT developed with NOK-si demonstrated to be a practical and stable model during histological examination and provided satisfactory results in ultrastructural analysis. Fibroblasts of lamina propria (connective tissue underlying the epithelium) and epithelial cells (NOK-si) interact to each other and reproduce an oral mucosa with a thick epithelium, which favour the epithelium differentiation. However, the ROMT models did not present flattened keratinized cells in the upper layers, as occurs *in vivo* in some stratified epithelia. Nevertheless, depending on the anatomical region of the oral mucosa, the epithelium may be no-keratinized as floor of the mouth, jugal and labial mucosa and soft palate (Squier, Kremer; 2001). Thus, the ROMT could be used to mimic these structures. In addition, the ROMT developed in this study permitted the assessment of the potential of single-species biofilms (*C. albicans* alone and *S. aureus* alone) and dual-species biofilms (*C. albicans* associated with *S. aureus*) to penetrate and to destruct the oral mucosa tissue. The advantages of the reconstituted tissue model use are

that a donor skin is not needed and interindividual differences do not influence in the experiments (Kehe *et al*., 1999).

Various studies have attempted to develop a reconstituted oral tissue as similar as human oral mucosa (Kinikoglu *et al*., 2009; Dongari-Bagtzoglou *et al*., 2006; Andrian *et al*., 2004; Yoshizawa *et al*., 2004). However, most studies have used primary fibroblasts and epithelial cells isolated from human palatal biopsy specimens. In the present study, the ROMT model is constructed by seeding NOK-si keratinocytes on gingival fibroblasts embedded in a collagen matrix and showed a well-organized and stratified tissue with a structural organization similar to human oral mucosa, which was used as a control. However, the ROMT composed by 6-8 layers of juxtaposed cells failed to promote the level of stratification of the oral mucosa found in the control tissue. This may be due to the time of tissue formation (14 days) or the availability of nutrients. NOK-si cells were used because they maintain epithelial morphology, proliferative capacity, and the expression of typical markers such as cytokeratins and Ecadherin (Castilho *et al*., 2010).

The findings showed a similar pattern of immunolabelling in the ROMT with NOK-si cells and gingival mucosa. We observed Ki-67-immunolabeled cells in the ROMT, which indicate maintenance of active cell proliferation in the basal and suprabasal layers, consistent with turnover found in the stratified epithelia (Yoshizawa *et al*., 2004; Kinikoglu *et al*., 2009; Dongari-Bagtzoglou *et al*., 2006). However, while in these studies human fibroblasts and primary oral keratinocytes were used to create a culture system to produce oral mucosa equivalents, immortalized cell lines were used in the experiments in the present investigation.

Cytokeratin 13 showed strong suprabasal immunoexpression in the control oral mucosa, with basal layers of the epithelium being negative. Similarly, the ROMT also showed expression of cytokeratin 13. These results are in agreement with those previously shown by other authors (Garzon *et al*., 2009, Sanchez-Quevedo *et al*. 2007).

Cytokeratin 14 immunoexpression is usually present in the basal and supra-basal strata of stratified epithelia (Dabija-Wolter *et al*., 2013, Rao *et al.* 2014). The strong immunolabeling for cytokeratin 14 was observed in all the layers of the stratified epithelium from ROMT may be due to an activated state of migrating epithelial cells. On the other hand, cytokeratin 14 was mainly expressed in basal epithelial cells of the control tissue, which may represent cells in a low proliferative state. These findings are in accordance with others studies that also reported the immunolabeling for cytokeratin 14 throughout the stratified epithelium in human oral mucosa models (Rouabhia *et al*., 2002; Oksanen *et al.*, 2002; Rouabhia *et al*., 2010).

The type IV collagen is a typical protein present of the basal lamina synthetized by epithelial cells (Becker *et al*., 1986). In the ROMT, the immunolabeling for collagen IV was observed between epithelium and collagen matrix, i.e., in the region correspondent to basal lamina. Although the immunolabeled material was sparsely distributed in this region, epithelial cells exhibiting immunolabeled cytoplasm were also observed, reinforcing the idea that the epithelial cells of ROMT can produce collagen IV. A weak immunoexpression for collagen IV was also found in multilayered three-dimensional organotypic culture models (Dabija-Wolter *et al*., 2013).

Ultrastructural analysis revealed the presence of bundles of tonofilaments in the cytoplasm of cells of the ROMT, as described in the epithelial cells (Cerri et al., 2009). Moreover, numerous desmosomes attaching a cell another, similar to the observed in oral epithelium was found in the epithelium of ROMT. Subjacent to the stratified epithelium, few fibroblasts and some collagen fibrils intermingle to amorphous material forming the scaffold, which allowed the epithelial cell proliferation and well-organized reconstituted epithelium tissue.

Single-species (*C. albicans* alone or *S. aureus* alone) and dual-species biofilms (*C. albicans* associated with *S. aureus*) were able to induce structural modifications at ROMT after 16 h incubation. However, the extent and depth of infection were greater for dual-species biofilms (*C. albicans* associated with *S. aureus*). Our results are consistent with literature (Schlecht *et al.*, 2015, Kong *et al*., 2015, Peters *et al*., 2012; Zago *et al*., 2015). Peters *et al*. (2012) assessed the potential implications of interaction between either species alone (*C. albicans* and *S. aureus*) or co-infected with host and a lack of inflammatory infiltrates was confirmed with a non-invasive presence of *S. aureus* on the tissue; the association of staphylococcal cells with *C. albicans* hyphae, as they penetrate host tissue, may allow *S. aureus* to gain entry into deeper tissues and initiate infection, with dire consequences for the host, particularly in critically ill patients. Evidence indicates that *S. aureus* and *C. albicans* are able to form a dense polymicrobial biofilm on the epithelial surface with or without the expression of protein agglutinin-like sequence 3 (Als3p). However, *S. aureus* is unable to enter the bloodstream and disseminate in the absence of Als3p, ostensibly due to the lack of binding to penetrating hyphae (Schlecht *et al*. 2015).

The morphological analysis revealed that *S. aureus* caused the least damage and resulted in the lowest LDH levels, although significantly greater than the uninfected control. On the other hand, the dual species biofilms yielded the highest level of the LDH and tissue damage. These findings suggest that *C. albicans* can facilitate the damaging by S. *aureus* (Schlecht *et*

al., 2015), since *S. aureus* typically requires a breach in host surface barriers to invade (Acton *et al*., 2009), which may be caused by *C. albicans*. Zago *et al*. (2015) found high production of proteinase in single *S. aureus* biofilm, while single *C. albicans* biofilm presented high phospholipase levels. However, when both microorganisms were co-cultured, both enzymes were produced. Furthermore, proteomic analysis showed that a total of 27 proteins were significantly differentially produced by *S. aureus* and *C. albicans* during co-culture biofilm growth (Peters et al, 2010).

The findings of this study indicate that a level of differentiation within the optimized reconstituted oral mucosa tissue and show that it is possible to use this tissue to investigate the mechanism of oral infection.

Conclusion

The results provide clear indications towards a reproducible and relevant *in vitro* model for investigations of oral mucosa infections and may be a useful adjunct to pre-clinical studies of oral disease progression and for studying virulence factors.

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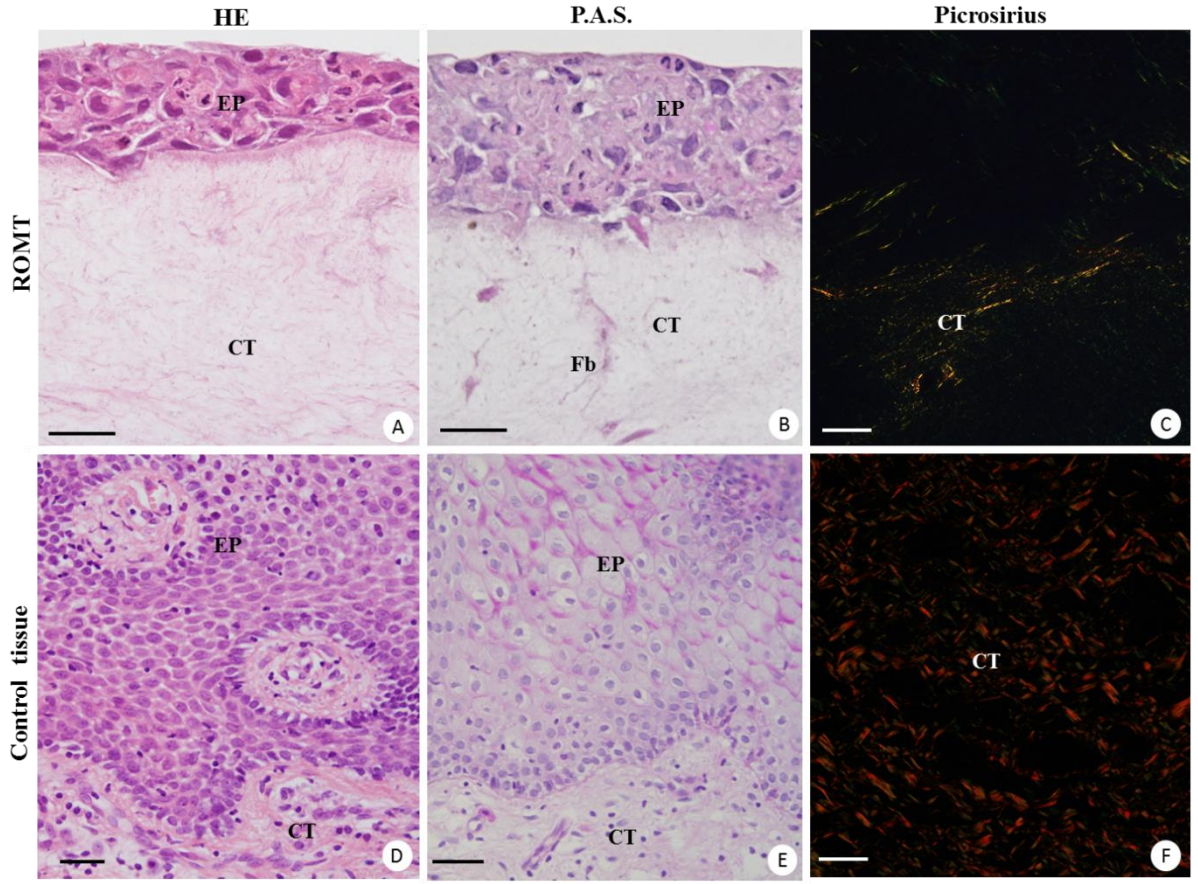


Fig 1 – Light micrographs showing portions of the sections of ROMT (A-C) and human gingival mucosa (D-F). **In A and B**, the epithelium (EP) exhibits 6-8 layers of cells in close juxtaposition. Note the thick stratified epithelial tissue of human gingiva (D and E). Subepithelial connective tissue (CT) of the ROMT shows few fibroblasts (Fb) and thin collagen fibers whereas several cells are observed in the subepithelial connective tissue (CT) from gingiva (D and E). **C and F –** sections were stained with Picrosirius-red and analyzed under polarized light. **C -** Birefringent collagen is present in the subepithelial connective tissue (CT) in the ROMT. **F -** in the human gingiva several bundles of birefringent collagen fibers are observed distributed throughout the subepithelial connective tissue (CT). Scale Bar: 30 µm.

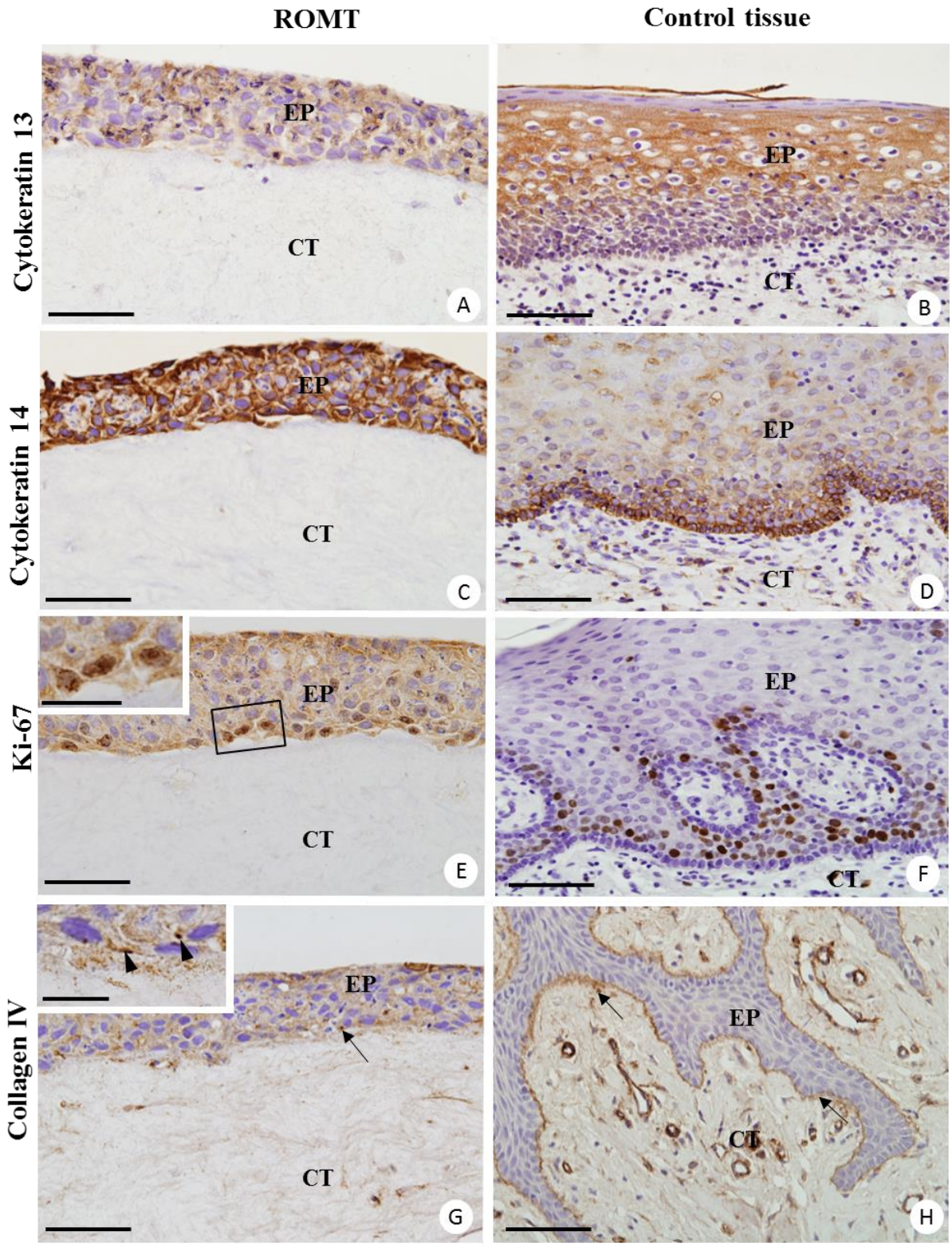


Fig 2 – Light micrographs showing portions of sections of ROMT (A, C, E and G) and human gingival mucosa (B, D, F and H). A and B images show the immunoexpression for cytokeratin 13 (brown-yellow color) in the epithelial tissues (EP). The ROMT exhibits immunolabeled cells in the different layers of the epithelium while in the human gingiva

the immunoexpression is present in the middle and in the superficial layers of epithelium (EP). **C and D** images show strong cytokeratin 14 immunolabeling in epithelial cells is observed in the ROMT. Note that in the human gingiva immunostaining is evident in the basal stratum. **E and F –** Ki-67-immunopositive nucleus are observed in the basal stratum of the epithelia of ROMT and human gingiva. The inset of the outlined area in E shows strong Ki-67-immunopositive cells in the basal stratum of ROMT. **G and H –** a delicate collagen IV immunolabeling (arrows – G) is observed between epithelial (EP) and connective tissues (CT) in the ROMT. The inset shows granular immunopositive material in the epithelium-connective interface. Epithelial cells exhibit immunopositive cytoplasm (arrowheads). **H -** A conspicuous immunostaining (arrows) is observed in the basal lamina of the human gingiva. Scale Bar: 45 µm; inset (E and G): 20 µm.

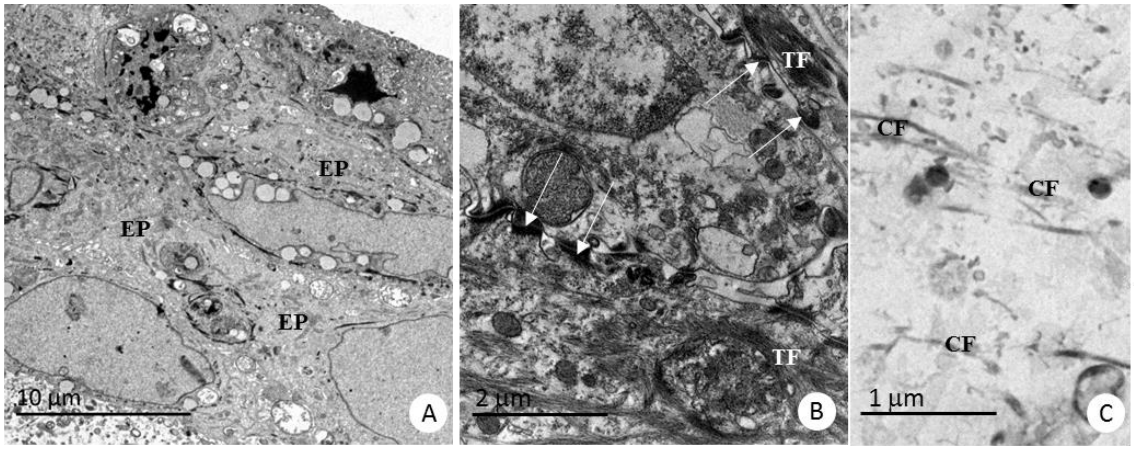
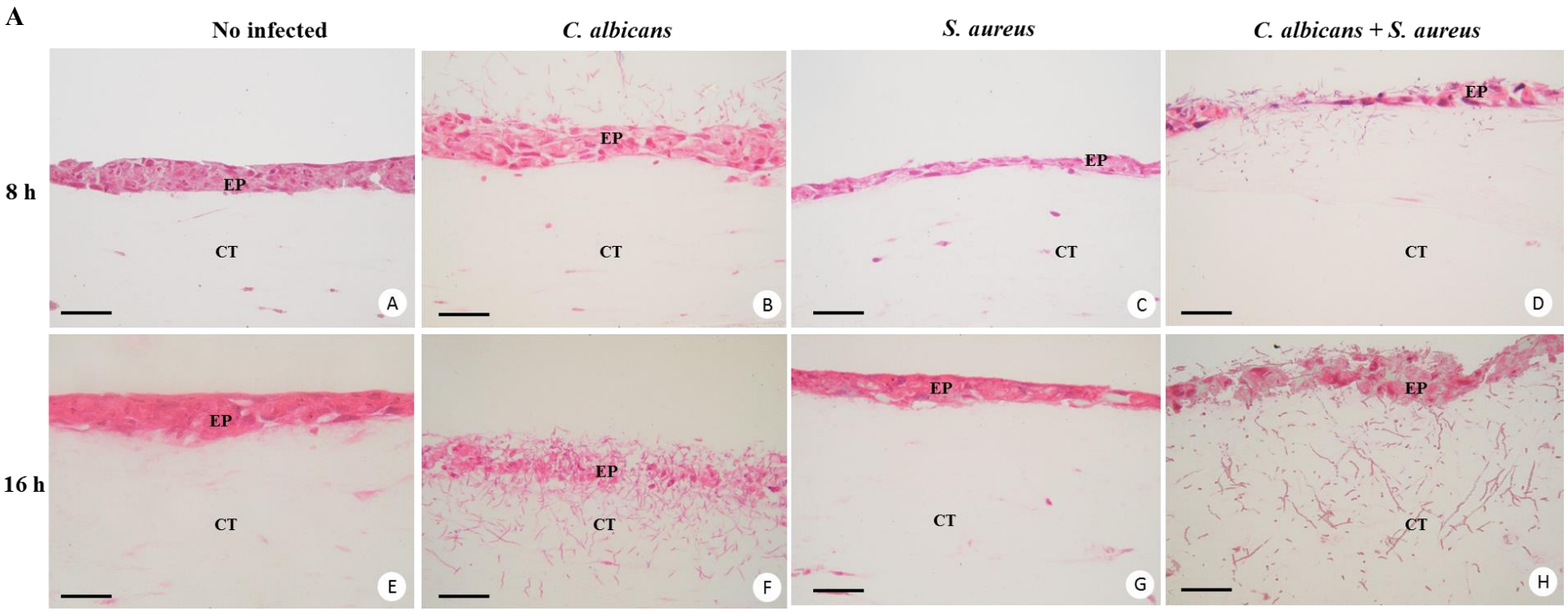


Fig 3 - Electron micrographs of ROMT. **A**) Epithelium tissue shows several layers of cells (EP) in close juxtaposition. **B)** desmosomes junctions (arrows) are observed between adjacent epithelial cells. Bundles of tonofilaments (TF) are distributed throughout the cytoplasm of epithelial cells. **C)** a portion of the connective tissue showing some collagen fibrils (CF) surrounded by amorphous material.


A
No infected
C. albicans
S. aureus
C. albicans + S. aureus
8 h
16 h
EP
CT
A
B
C
D
E
F
G
H

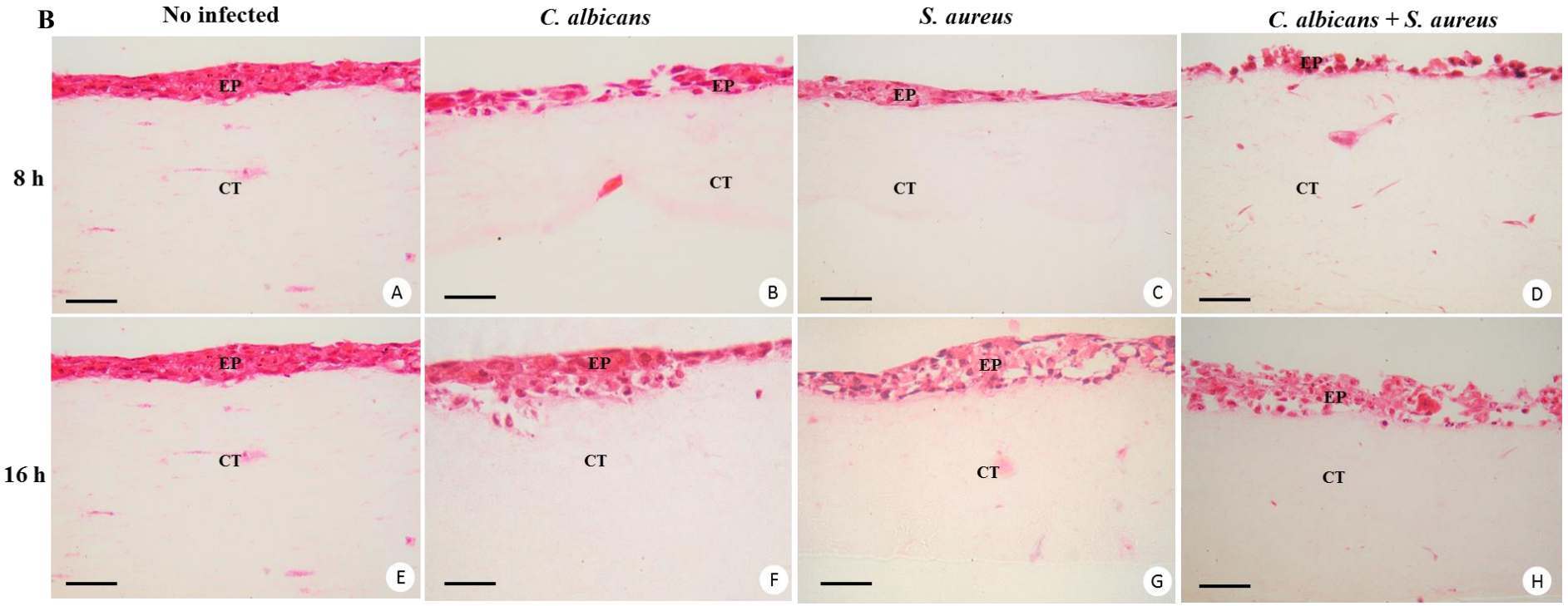


Fig 4 - **A**: Light micrographs showing sections of ROMT infected with single-species biofilms (*C. albicans* alone or *S. aureus* alone) and dual-species biofilms (*C. albicans* associated with *S. aureus*) after 8 and 16 h. EP: epithelium; CT: subepithelial connective tissue. HE. Bars: 50 µm. **B:** Light micrographs showing portions of sections of ROMT exposed to single-species biofilms metabolites (supernatant) (*C. albicans* alone or *S. aureus* alone) and dual-species biofilms metabolites (*C. albicans* associated with *S. aureus*) after 8 and 16 h exposure. EP: epithelium; CT: subepithelial connective tissue. HE. Bars: 50 µm.

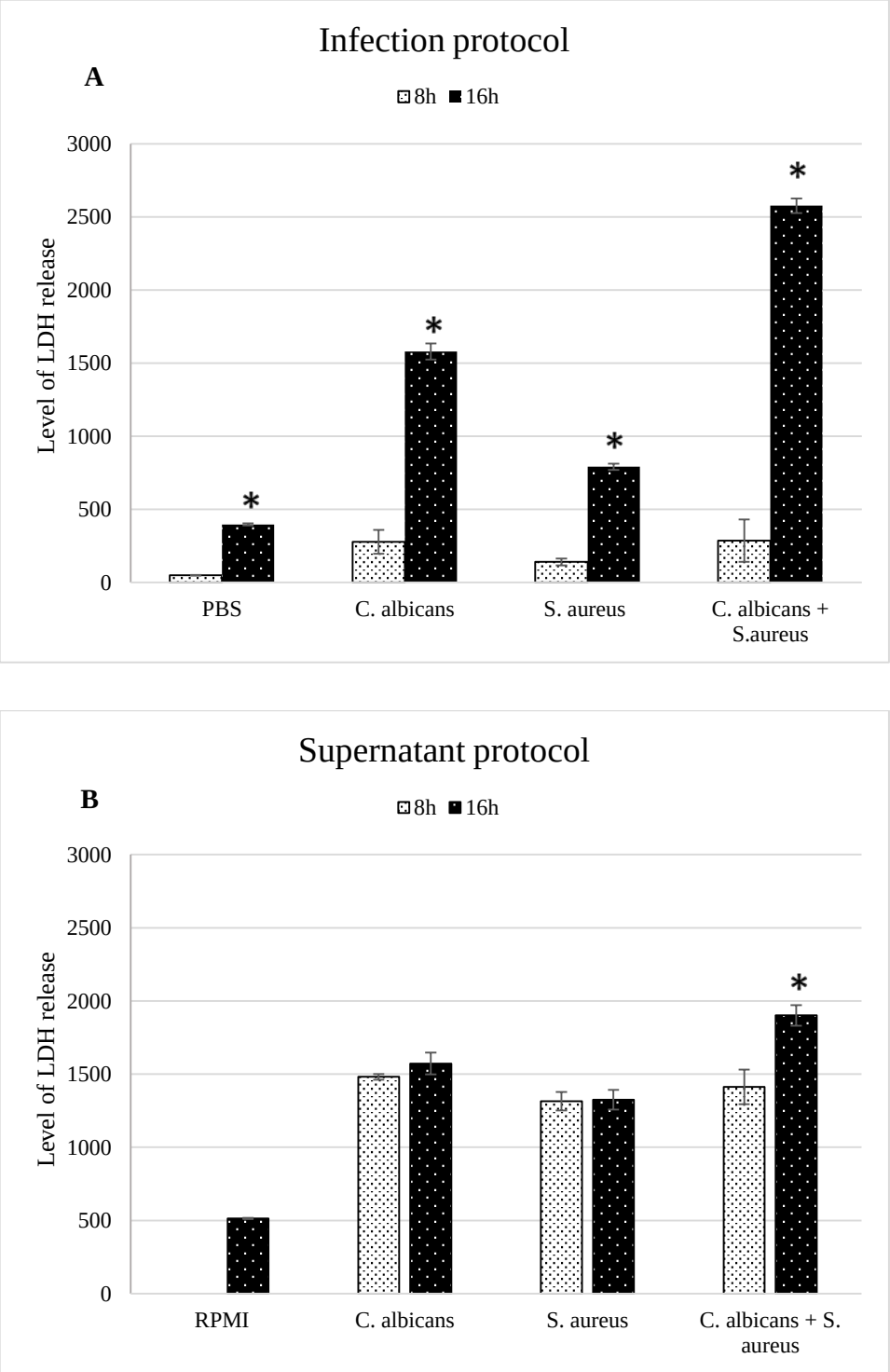


Fig 5 - LDH released from ROMT infected with single- (*C. albicans* alone and *S. aureus* alone) and dual-species biofilms (*C. albicans* associated with *S. aureus*) (A) or exposed to single- and dual-species biofilms metabolites (supernatant) (B) after 8 and 16 h. Results are the mean ± SD of three experiments, each condition set up in triplicate. Error bars represent SD (n = 9 replicates).* $p \leq 0.05$.

4 CONCLUSÃO

Diante dos objetivos propostos e dos resultados obtidos, foi possível concluir que:

1. O meio RPMI / HEPES suplementado com L-glutamina (2,0 mM) e bicarbonato de sódio (2,0 g/L) pode ser utilizado como uma alternativa viável em estudos para investigar os efeitos de biofilmes em queratinócitos ao longo do tempo;

2. Os metabólitos dos biofilmes mistos foram capazes de induzir um maior dano celular, ao passo que os metabólitos de *S. aureus* foram capazes de induzir o aumento da resposta inflamatória;

3. Os metabólitos dos biofilmes monoespécie foram mais relacionados à apoptose, enquanto que os metabólitos de biofilme misto de *C. albicans* e *S. aureus* produziram morte celular por apoptose tardia ou por necrose;

4. Os metabólitos dos biofilmes simples de *S. aureus* e misto ativaram a produção das caspases -2, -3, -6 e -8, ao passo que os metabólitos dos biofilmes simples de *C. albicans* estimularam apenas a produção da caspase -3;

5. O tecido epitelial reconstituído apresentou características histológicas e imunohistoquímicas semelhantes ao epitélio oral e pode ser utilizado para investigar padrões de infecção causada por biofilmes em culturas simples e mista de *C. albicans* e *S. aureus*;

6. O biofilme misto (*C. albicans* e *S. aureus*) foi responsável pela maior destruição tecidual e liberação da enzima lactato desidrogenase no tecido epitelial reconstituído.

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* De acordo com o Guia de Trabalhos Acadêmicos da FOAr/UNESP, adaptado das Normas Vancouver. Disponível no site da Biblioteca: http://www.foar.unesp.br/#biblioteca/manual.

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ANEXO A- Página inicial do artigo publicado

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Note

Influence of different buffers (HEPES/MOPS) on keratinocyte cell viability and microbial growth

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ABSTRACT

This study assessed the effect of the buffers 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 3-(N-morpholino) propanesulfonic acid (MOPS) on keratinocyte cell viability and microbial growth. It was observed that RPMI buffered with HEPES, supplemented with L-glutamine and sodium bicarbonate, can be used as a more suitable medium to promote co-culture.

Studies of co-culture with keratinocyte cells and microorganisms have been fundamentally important in assessing the virulence and cytotoxicity of biofilms. The loss of keratinocyte cell viability when cells are exposed to biofilms of Staphylococcus aureus can be observed after 3 h of exposure (Kirker et al., 2008). The co-culture of Candida albicans and oral epithelium cells stimulates signaling pathways that promote cell death (Villar and Zhao, 2010).

The culture medium used for biofilm growth should not interfere with cell viability. Although RPMI (Roswell Park Memorial Institute) buffered with MOPS has been used, the concentration of MOPS (Zago et al., 2015) is greater than that permitted for cell cultures (Eagle, 1971), and could compromise co-culture studies. As an alternative, HEPES could be used for biofilm formation of C. albicans and S. aureus (Peters et al., 2010). The present research compared the effect of RPMI buffered with HEPES and MOPS on keratinocyte cell viability and microbial growth.

C. albicans SC5314 and S. aureus ATCC25923 microorganisms were used to produce single and dual species biofilms, in accordance with the methodology described by Zago et al. (2015). To prepare the yeast and bacteria pre-inoculum, a loop full of agar stock cultures was transferred to 10 mL of Yeast Nitrogen Base broth (YNB — Difco, Becton Dickinson Sparks, MD, USA) supplemented with 100 mM glucose and Tryptic Soy Broth (TSB — Acumedia Manufacturers Inc., Baltimore, MD, USA), respectively, and incubated at 37 °C overnight. Thereafter, the dilution of the inoculum was performed and cultures were incubated until they reached mid-exponential phase growth. Cells of the resultant cultures were harvested and washed twice with sterile phosphate-buffered saline solution (PBS, pH 7.2) in sterile tubes at 5000 × g for 5 min.

Microorganisms were re-suspended (about 10^7 cells per mL) in culture medium RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA), using two different buffers: Buffer 1 (pH 7.0 ± 0.1, 164 mM MOPS) (Sigma-Aldrich, St. Louis, MO); Buffer 2 (pH 7.0 ± 0.1, 25 mM HEPES) (Sigma-Aldrich, St. Louis, MO) 2 mM L-glutamine (Laura, Wallerandlle, USA) and 2.0 g/L sodium bicarbonate (Synth, São Paulo, Brazil). The adhesion of microorganisms was performed on 24-well sterile plates (TPP Techno Plastic Products AG., Switzerland) at 37 °C in an orbital shaker (75 rpm) for 90 min (Pereira et al., 2011). The pH was measured using a benchtop pH meter (QX 1500 Plus-Qualxtron, São Paulo, Brazil).

Cell cultures were prepared with the Normal Oral Keratinocytes (NOK) (Castilho et al., 2010) and Human Keratinocyte Cell Line (HaCat) (BCRJ 0041). The cells were cultivated until they reached confluency (48 h), washed with 10 mM PBS, centrifuged and re-suspended in Dulbecco's Modified Eagle Medium (DMEM; (Gibco BRL, Grand Island, NJ, USA) and placed (about 4.5×10^4 cells/well) in 24 well plates. Live cells were counted in a Neubauer chamber (magnification × 10).

MTT assay [3-(4,5-dimethylthiazole-2-yl)2,5-diphenyl tetrazolium bromide] (Sigma-Aldrich, St. Louis, MO, USA) was performed at 02, 04, 06, 08 and 12 h after incubation at 37 °C in 5% CO_2 with the RPMI/MOPS, RPMI/HEPES and DMEM (control cells). After each period of contact, the culture media were removed and 250 μL of MTT/PBS solution (5.0 mg·mL^{-1}) was added to each well and incubated for 4 h at 37 °C. Next, MTT solution was removed and the formazan crystals were

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