MARTINNA DE MENDONÇA E BERTOLINI

Condições de crescimento influenciam as características estruturais e de virulência de biofilmes de *Candida* e *Streptococcus* formados sobre modelos *in vitro* de mucosa oral humana

Growth conditions influence at structural and virulence characteristics of *Candida* and *Streptococcus* biofilms developed on *in vitro* models of human oral mucosa

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Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do título de Doutora em Clínica Odontológica, Área de Prótese Dental.

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Orientadora: Profa. Dra. Altair Antoninha Del Bel Cury

Este exemplar corresponde à versão final da tese defendida por Martinna de Mendonça e Bertolini, orientada pela Profa. Dra. Altair Antoninha Del Bel Cury

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RESUMO

O patógeno oportunista *Candida albicans* e *Streptococcus* do grupo Mitis formam comunidades complexas em múltiplos sítios da cavidade oral, nos quais o ambiente e a disponibilidade de nutrientes sofrem mudanças constantes. Objetivou-se estudar as características estruturais e de virulência de biofilmes de *Candida albicans* na presença e ausência de *S. oralis* crescendo sobre um modelo tri dimensional de mucosa oral humana, em diferentes condições: (1) umidade da superfície mucosa (molhada ou semi seca), (2) disponibilidade de nutrientes (suplementação do meio de cultura com BHI) e (3) morfotipo da hifa (hifa ou pseudo hifa). Para isso foram utilizados modelos tri dimensionais de mucosa oral humana formado por queratinócitos imortalizados (linhagens celulares OKF6-TERT2 ou SCC15) sobre uma matriz colágena com fibroblastos para o crescimento dos biofilmes. Estes foram infectados por *Streptococcus oralis* 34, e/ou *Candida albicans*, sendo uma cepa de referência e cepas mutantes para a formação de pseudo-hifas, pela deleção dos genes ndt80 ou tup1. A determinação do biovolume e estrutura do biofilme foram realizadas por microscopia confocal a laser, com os biofilmes sendo corados por imunofluorescência com anticorpo especifico para C. albicans e sonda para *Streptococcus*. Como determinante de virulência secções de tecido com 5 µm de espessura foram coradas da mesma maneira anterior ou por hematoxilina e eosina, com o intuito de se detectar a invasão de microorganismos. O dano tecidual também foi mensurado pela liberação de lactato desicrogenase no meio de cultura. Os dados foram avaliados por análises de variâncias (ANOVA) e os procedimentos para comparações múltiplas pareadas por Bonferroni t-test, com $\alpha = 5\%$. Em condições úmidas *C. albicans* estendeu hifas longas e entrelaçadas, formando um biofilme de superfície homogênea. Biofilmes mistos apresentaram uma estrutura estratificada, com *S. oralis* crescendo em contato com a mucosa e a *C. albicans* cobrindo a superfície bacteriana. Em condições de semi-secas a *C. albicans* formou densos focos de crescimento localizados a partir dos quais as hifas estenderam-se radialmente

para se entrelaçarem com hifas de focos adjacentes. Em biofilmes mistos este fenômeno provocou o acúmulo focal de *S. oralis* co-localizado com os focos de *C. albicans*. Embora o biovolume do biofilme de *C. albicans* tenha sido significativamente maior em condições úmidas (P<0,001), houve uma invasão tecidual mínima em comparação com as condições semi-secas, na qual a barreira epitelial foi completamente destruída. A suplementação do meio de cultura, em condições semi-secas não alterou a arquitetura do biofilme, mas intensificaram o crescimento, o biovolume e a invasão/dano tecidual (P<0,001), proporcionalmente as concentrações testadas. Mutantes para a formação de pseudo-hifas formaram biofilmes defeituosos, nos quais a maioria dos *S. oralis* estava em contato com a superfície epitelial, abaixo das pseudo-hifas. A presença de *S. oralis* promoveu invasão e dano tecidual em todas as condições. Conclui-se que a umidade, a disponibilidade de nutrientes, o morfotipo da Candida e a presença de *S. oralis* afetam fortemente a arquitetura e virulência de biofilmes de *C. albicans* crescidos sobre nas mucosas.

Palavras-chave: Biofilme, *Candida, Streptococcus*, infecções oportunistas, virulência.

ABSTRACT

The opportunistic pathogen *Candida albicans* and streptococci of the Mitis group form complex communities in multiple oral sites, where the environment and nutrient availability change constantly. We aimed to study structural and virulence characteristics of *Candida albicans* biofilms in the presence or absence of *S. oralis*, growing on a three-dimensional model of human oral mucosa, under different conditions: (1) moisture of mucosal surface (wet or semi dry), (2) nutrient availability (BHI supplementation on culture media) and (3) hyphal morphotype (hyphae or pseudohyphae). For this it was used a three-dimensional model of the human oral mucosa formed by immortalized oral keratinocytes (OKF6-TERT2 or SCC15 cell lines) on a fibroblast-embedded collagenous matrix to grow biofilms. Infections were carried out using *Streptococcus oralis* 34, a *C. albicans* reference strain and pseudohyphal mutants with a homozygous deletion of the ndt80, or tup1 gene. Determination of biofilm biovolume and structure was done by confocal scanning laser microscopy with biofilms stained by immunofluorescence using an anti-*Candida* antibody and a Streptococcus probe. As determinant of virulence, 5-µm-thick tissue sections were stained same way or with hematoxylin and eosin in order to detect invasion of microorganisms. Also tissue damage was measured by lactate dehydrogenase release in the culture media. Statistical analyses were performed using SigmaPlot 12 software at 5% significance level. Data were evaluated by analysis of variance (ANOVA) and when statistical significances were found, all pairwise multiple comparison procedures were performed with Bonferroni t-test, with $\alpha = 5\%$. Under wet conditions *C. albicans* extended long intertwined hyphae, forming a homogeneous surface biofilm. Mixed biofilms had a stratified structure, with *S. oralis* growing in close contact to the mucosa and *C. albicans* growing on the bacterial surface. Under semi-dry conditions, *C. albicans* formed localized foci of dense growth from which hyphae extended radially to intertwine with hyphae from adjacent foci. In mixed biofilms this promoted focal growth of *S. oralis* co-localizing with *C. albicans*. Although *Candida* biofilm biovolume was

significantly greater under wet conditions (P<0.001), there was minimal tissue invasion compared to semidry conditions where the epithelial barrier was completely destroyed. Supplementing the infection medium with nutrients under semidry conditions did not change the biofilm architecture but intensified focal growth and increased biofilm biovolume and tissue invasion/damage (P<0.001), proportionally to the tested concentrations. Pseudohyphal mutants formed defective mixed biofilms, with most *S. oralis* in contact with the epithelial surface, below the pseudohyphal mass. Interestingly, the presence of *S. oralis* promoted fungal invasion and tissue damage under all conditions. Moisture, nutrient availability, hyphal morphotype and presence of *S. oralis* strongly affect architecture and virulence of mucosal fungal biofilms.

Keywords: Biofilm, *Candida*, *Streptococcus*, opportunistic infections, virulence.

SUMÁRIO

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Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar.
Mas o mar seria menor se lhe faltasse uma gota.
Madre Teresa de Calcuta

Introdução

Candida spp. são fungos comensais que colonizam pele e mucosas de seres humanos, estando presentes em grande parte da população saudável sem causar danos (Peleg *et al*., 2010). Dentre as espécies de *Candida* mais comumente citadas como colonizadoras frequentes da cavidade oral estão presentes *C. albicans*, *C. glabrata*, *C. guillermondii*, *C. krusei*, *C. parapsilosis*, *C. pseudotropicalis*, *C. stellatoidea*, *C. tropicalis* (Dangi *et al*., 2010). É estimado que a *C. albicans* seja a mais prevalente, estando presente em cerca de 30 a 50% das pessoas saudáveis, geralmente sem causar danos aos tecidos. (Gendreau *et al*., 2011; Silva *et al*., 2012)

Entretanto, algumas condições locais e sistêmicas podem facilitar o desenvolvimento desse fungo e a transição de sua forma comensal para patogênica, aumentando sua prevalência o que, na cavidade oral, pode favorecer o quadro de candidose oral (Perezous *et al*., 2005). Como exemplo podemos citar o uso de próteses totais e parciais mal adptadas e pobremente higienizadas, que funcionam como nichos para a colonização e acúmulo deste microorganismo e demais espécies bacterianas encontradas na cavidade oral (Ramage *et al*., 2004). O uso de próteses, associadas a estes fatores, está relacionado ao aumento da prevalência de *C. albicans* e candidose oral, que pode chegar a acometer 60% dos pacientes usuários de próteses com mais de 60 anos (Singh *et al*., 2014).

A candidose é dependente ainda de outros fatores predisponentes locais como a diminuição da salivação (Guobis *et al*., 2011) principalmente relacionada a importância da presença de proteínas salivares como a histatina 5,

que apresenta atividade antifúngica (Vukosavljevic *et al*., 2012). Além de quadros com envolvimento sistêmico, principalmente nos quais os pacientes encontram-se imunossuprimidos, como transplantados e HIV-positivos (Vinh, 2011). É importante ressaltar ainda que, em pacientes hospitalizados, o fungo da espécie *Candida* é o quarto grupo de microrganismo mais isolado em infecções sistêmicas. E nesses casos de candidemia a taxa de mortalidade pode chegar a 67% em pacientes em terapia intensiva, sendo uma infecção de difícil tratamento (Kett *et al*., 2011).

Recentemente estudos vêm demostrando que não só estes fatores locais e sistêmicos influenciam na patogênese da *C. albicans*, mas que os demais microrganismos presentes na cavidade oral, também podem alterar o seu comportamento clínico. Essa interação pode levar a um aumento da habilidade da *C. albicans* de invadir o tecido e gerar maior resposta inflamatória local, aumentando ainda a expressão de alguns de seus fatores de virulência, como a expressão aumentada de aspartil proteinases (Dias *et al*., 2012; Shirtliff *et al*., 2009; Xu *et al*., 2014a; Xu *et al*., 2014b, Cavalcanti *et al*., 2015).

Biofilmes mistos compostos por *C. albicans* e bactérias estão presentes em diversos sítios do corpo humano (Peleg et al., 2010) e na cavidade oral (Zijnge *et al*., 2010) sendo sua formação possível devido à habilidade de coagregação destes microorganismos por interações de suas proteínas de superfície. A *C. albicans* é capaz de se coagregar com a maioria dos Streptococcus do grupo Mitis, principalmente representados por *Streptococcus gordonii*, *Streptococcus*

oralis, *Streptococcus mitis*, *Streptococcus parasanguinis* e *Streptococcus sanguinis* (Dias *et al*., 2012).

É importante destacar que, em alguns casos, espécies de *Streptococcus* não são capazes de sozinhos colonizarem superfícies mucosas, porém na presença de *C. albicans* passam a ser capazes de formar biofilmes extremamente robustos, como é o caso do *S. oralis* e do *S. sanguinis* (Dias *et al*., 2012). Fato este que, cada vez mais tem levantado interesse no estudo da interação *Candida-Streptococcus*. Alguns dos mecanismos responsáveis por essa coagragação já foram caracterizados como ocorrendo via interações entre adesinas presentes na superfície do *Streptococcus*, conhecidas como 'Streptococcal surface proteins' A e B (SspA e SspB), membros da família de proteínas presentes na superfície dos *Streptococcus* (Bramford *et al*., 2009), e proteínas presentes na parede de hifas de *C. albicans*, conhecidas como 'Agglutinin-like sequences', como a ALS3 (Silverman *et al*., 2010) , que serviriam como receptores.

Quando o biofilme de *C. albicans* se forma na presença de *Streptococcus* orais, observa-se uma interação sinérgica entre eles, com sérias implicações relacionadas ao potencial patogênico desse biofilme misto (Diaz *et al*., 2012, Xu et al., 2014a). Sabe-se até então que biofilmes mistos compostos por *C. albicans* na presença de espécies de *Streptococcus Actinomyces* apresentam maior expressão de genes relacionados à produção de aspartil proteinases, como SAP4 e SAP6 e das adesinas ALS3 e HWP1, quando formados sobre modelos *in vitro* de mucosa oral humana (Cavalcanti *et al*., 2015). Además tais biofilmes

exibem mais hifas e são capazes de gerar maior invasão e dano tecidual, bem como elevada produção de interleucinas inflamatórias, como a IL-8 (Cavalcanti *et al*., 2015).

Modelos *in vitro* de epitélio oral humano reconstituído (Dongari-Bagtzoglou *et al*., 2006) têm sido utilizados como ferramenta promissora para o estudo de interações entre patógeno e hospedeiro (Cavalcanti *et al*., 2015, Diaz *et al*., 2012, Villar *et al*., 2004, Xu et al., 2014a). Com eles é possível o isolamento de variáveis tais como fatores ambientais, disponibilidade de nutrientes e o morfotipo celular utilizado, uma vez que as infecções causadas por *Candida* spp. estão fortemente relacionadas com a sua transição de levedura para hifa (Villar *et al*., 2004). Essa transição é controlada por diferentes genes, tais como ndt80, tup1 rim101, entre outros, geralmente responsáveis pela separação e pela diferenciação celular em hifa, sendo esse um dos principais fatores de virulência da *C. albicans* relacionados à invasão tecidual (Sudbery, 2011).

O entendimento da influência destes fatores para o desenvolvimento estrutural do biofilme, o processo de invasão tecidual e o dano celular causado pode ter grande implicação clínica futura no tratamento da candidose, uma vez que atualmente a terapia é baseada apenas no tratamento com antifúngicos (Chen et al., 2014). Hipotetiza-se então que as características estruturais e de virulência de um biofilme de *C. albicans* formados sobre uma superfície mucosa possam ser afetadas por fatores como a umidade da superfície na qual o biofilme se desenvolve, a disponibilidade de nutrientes presentes durante a formação deste biofilme, o morfotipo da *Candida* e a presença de *S. oralis*.

Deste modo o objetivo do presente estudo foi analizar as características estruturais e de virulência de biofilmes de *C. albicans* tanto na presença como na ausência de *Streptococcus oralis* formados sobre modelos *in vitro* de epitélio oral humano, sob diferentes condições: 1) grau de umidade da mucosa; 2) disponibilidade de nutrientes, e 3) morfotipo da *C. albicans* formadora do biofilme, pela utilização de cepas mutantes desenvolvidas para a formação de pseudo-hifas.

Capítulo 01:

Candida-streptococcal mucosal biofilms display distinct structural and virulence characteristics depending on growth conditions and hyphal morphotypes

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Running head: Candida-streptococcal mucosal biofilms

Key words: *Candida*, *Streptococcus*, biofilms, infection models

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SUMMARY

Candida albicans and *Streptococci* of the Mitis group form communities in multiple oral sites, where moisture and nutrient availability can change spatially or temporally. This study evaluated structural and virulence characteristics of *Candida-streptococcal* biofilms formed on moist or semidry mucosal surfaces, and tested the effects of nutrient availability and hyphal morphotype on dual-species biofilms. Three-dimensional models of the oral mucosa formed by immortalized keratinocytes on a fibroblast-embedded collagenous matrix were used. Infections were carried out using *Streptococcus oralis* strain 34, in combination with a C. albicans wild-type strain, or pseudohyphal-forming mutant strains. Increased moisture promoted a homogeneous surface biofilm by *C. albicans*. Dual biofilms had stratified structure, with streptococci growing in close contact to the mucosa and fungi growing on the bacterial surface. Under semi-dry conditions, *Candida* formed localized foci of dense growth, which promoted focal growth of streptococci in mixed biofilms. *Candida* biofilm biovolume was greater under moist conditions, albeit with minimal tissue invasion, compared to semidry conditions. Supplementing the infection medium with nutrients under semidry conditions intensified growth, biofilm biovolume and tissue invasion/damage, without changing biofilm structure. Under these conditions, the *ndt80* and *tup1* mutants and *S. oralis* formed defective superficial biofilms, with most bacteria in contact with the epithelial surface, below a pseudohyphal mass, resembling biofilms growing in moist environment. The presence of *S. oralis* promoted fungal invasion and tissue damage under all conditions. We conclude that moisture, nutrient availability, hyphal morphotype and presence of commensal bacteria influence the architecture and virulence characteristics of mucosal fungal biofilms.

INTRODUCTION

Candida spp. are opportunistic fungal pathogens that are able to colonize diverse host niches, such as the skin and the urogenital, oral and gastrointestinal tract mucosae of humans (Peleg *et al*., 2010). At these host sites moisture and nutrient availability can change both spatially and temporally. *Candida albicans* is the most common oral mucosal fungal pathogen, with infection of otherwise healthy hosts afflicting more frequently denture wearers and the elderly (Lyon *et al*., 2006).

Reduced salivary flow under denture surfaces and xerostomia, particularly prevalent in the elderly (Johansson *et al*., 2012), can promote oral fungal infection, especially when combined with poor oral hygiene habits (Lyon *et al*., 2006) and a high carbohydrate diet (Santana IL *et al*. 2013). Xerostomia (Lyon *et al*., 2006), due to radiotherapy at the maxillofacial area, Sjögren's syndrome or the use of xerogenic medications (Guobis Ž *et al*., 2011) may also predispose the host to this oral infection. However, apart from the reduced amounts of salivary proteins with antimicrobial properties (Meiller *et al*., 2009), no other mechanisms for the increased virulence of this organism under xerostomic conditions have been proposed.

Under host-permissive conditions (Cassone & Cauda, 2012; Gavaldà *et al*., 2014; Singh *et al*., 2014), once *C. albicans* forms a mucosal biofilm it can invade the superficial strata of the oral mucosa (Williams *et al*. 2013), disperse to the esophageal mucosa, invade deeper into the submucosa, and potentially

disseminate to distant organs haematogenously (Conti *et al*., 2009). Although much more rare in humans (Lewis *et al*., 2013), this deep organ dissemination is common in mouse models of oral infection (Conti *et al*., 2009, Xu *et al*., 2014b), following the development of severe oropharyngeal and esophageal candidiasis. Germination of fungal cells to form true hyphae is a pre-requisite for epithelial invasion and damage in several experimental systems (Phan *et al*., 2000; Schlecht *et al*., 2014; Villar *et al*., 2004).

More recently, we reported that one of the factors that promote oral mucosal invasion by *C. albicans* is the presence of commensal streptococci (Diaz *et al*., 2012, Xu *et al*., 2014a). Recently, an *in vivo* study using mice (Xu et al., 2014b) and an *in vitro* study using oral and esophageal mucosal models (Diaz et al., 2012), both from our group, demonstrated that when *C. albicans* forms mucosal biofilms with oral streptococci of the mitis group, mixed biofilms acquire a more pathogenic potential, increasing *Candida* mucosal tissue invasion and amplifying the mucosal inflammatory response. Interestingly, the presence of *C. albicans* promotes an increase of the streptococcal biofilm biomass, forming communities with direct physical interactions (Diaz *et al*., 2012). These interactions are likely mediated at least in part by adhesins such as SspA and SspB on streptococci (Peleg *et al*., 2010; Silverman *et al*., 2010) and the N-terminal domain of the Als3 protein, an epithelial invasin, in *C. albicans* (Bamford *et al*., 2015).

Since interaction of *C. albicans* with streptococci of the mitis group is an important determinant of fungal pathogenicity, at least in mouse models of mucosal infection by *C. albicans*, the aim of this study was to analyze the structural and

virulence characteristics of *Candida-streptococcal* mixed biofilms forming on mucosal surfaces with different degrees of moisture or nutrients to simulate diverse mucosal environments or host colonization sites *in vivo*. For this we used three-dimensional models of the human oral mucosa formed by immortalized oral keratinocytes on a fibroblast-embedded collagenous matrix to grow mixed biofilms. Infections were carried out using a *C. albicans* wild-type reference strain, two *C. albicans* mutants with known hyphal defects (Sellam *et al*., 2010, Kadosh & Johnson, 2005, Nobile *et al*., 2012), and *Streptococcus oralis* 34, a strain with demonstrated pathogenic synergy in a mouse model of oral infection (Xu et al.,2014b). We hypothesized that *Candida*-streptococcal mucosal biofilms display distinct structural and virulence characteristics depending on growth conditions and hyphal morphotype.

MATERIALS AND METHODS

Microorganisms used and microbiological media.

The microorganisms used in the present study were *Candida albicans* wild-type reference strain SN425 which forms true hyphae (Noble *et al.,* 2010), an *ndt80* homozygous deletion mutant (CJN2412) (Nobile *et al*., 2012) and its revertant (CJN2328) (Nobile *et al*., 2012), both derived from this parental wild type strain. A tup1 homozygous deletion mutant (BCa 2-10) (Kadosh & Johnson, 2005) was also used. Strains CJN2412 and BCa 2-10 are defective in true hyphae formation, but are able to form pseudohyphal filaments under most growth

conditions (Kadosh & Johnson, 2005; Nobile *et al*., 2012; Villar *et al*., 2004), and originated from the same wild type progenitor, strain CAI4. *Streptococcus oralis* 34 (a kind gift from PE Kolenbrander) was used in these studies since it was shown to form robust mucosal biofilms in the presence of *C. albicans* (Diaz *et al*., 2012; Xu *et al*., 2014b). All *C. albicans* strains were kept in -80 °C stock cultures, and grown in yeast extract-peptone-dextrose (YPD) agar one week before each experiment. Overnight YPD broth cultures were prepared one day before each experiment, at 70 rpm agitation, aerobically, at room temperature (25°C). The YPD medium consisted of 5 g of yeast extract (Fisher Scientific, Pittsburgh, PA) liter^{-1}, 10 g of peptone (Fisher Scientific) liter^{-1}, and 20 g dextrose (Fisher Scientific) liter^{-1}. *Streptococcus oralis* was kept in -80 °C stock cultures and reactivated one day before the experiment by overnight growth in brain heart infusion (BHI) medium (Oxoid, Ltd., Cambridge, United Kingdom) under static conditions at 37°C, in a 5% CO_2 incubator.

In vitro oral mucosal tissue models

The *in vitro* oral mucosal models were described in detail previously by Dongari-Bagtzoglou & Kashleva (2006). Briefly, oral mucosa analogues are formed using trans-well inserts which allow limited diffusion of culture media from the bottom of the well. An airlifting growth phase ensures epithelial differentiation and stratification. Analogues consist of human immortalized oral keratinocytes (OKF6/TERT-2) (Wöllert *et al*., 2012) or SCC15 (ATCC) (Dongari-Bagtzoglou & Kashleva, 2006) seeded (5× 10^5 cells per well) over a collagen type I matrix,

embedded with fibroblasts (3T3 cell line, ATCC). The procedure takes approximately 2 to 3 weeks to complete resulting in a non-keratinizing stratified squamous epithelium. After tissue maturation, culture media with no antibiotics were used for 24h prior to infection with *C. albicans* and *S. oralis*.

Inoculation of mucosal tissues with *C. albicans* and *S. oralis*

Overnight cultures of *C. albicans* were washed with PBS and cells were counted on a Neubauer chamber in order to standardize the inoculum. Overnight stationary-phase cultures of *S. oralis* were inoculated in fresh BHI broth and then allowed to reach the late logarithmic phase for 3-4 h (optical density at 600 nm of 1.0, corresponding to 10^7 cells/ml). The final inoculum consisted of 10^6 cells of *C. albicans* and 10^7 cells of *S. oralis* in 20 µL of infection medium (consisting of DMEM, supplemented with L-glutamine, hydrocortisone, ITES, O-phosphorylethanolamine, adenine and triiodothyronine) pipetted in the middle of each well. After a 45 minute incubation at room temperature, infection media were added outside (semidry condition) or inside and outside (moist condition) of the well insert. To rule out the possibility that biofilms growing in a moist environment presented characteristics related to increased nutrient availability, we supplemented the inoculation media with increasingly high concentrations of BHI (0%, 5%, 10%) under semi-dry conditions. Biofilms were then allowed to develop for 16 hours at 37°C, 5% CO_2. An uninfected tissue was used as a control to evaluate tissue viability under all conditions.

Determination of biofilm biovolume and structure by confocal laser scanning microscopy

For biofilm biovolume and structure analysis, tissue samples were fixed in 4% paraformaldehyde for 2 hours. Subsequently *C. albicans* was stained using fluorescein isothiocyanate (FITC)-labeled anti-*Candida* polyclonal antibody (Meridian Life Science, Saco, ME). For biofilms containing *S. oralis*, this step was followed by FISH with the *Streptococcus*-specific oligonucleotide probe STR405 (Thurnheer *et al*., 2001), labeled with Alexa 546, as previously described (Dongari-Bagtzoglou *et al*., 2009). Biofilms were visualized using a Zeiss LSM 510 confocal scanning laser microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) with an argon laser (488- and 543-nm), using air Plan-Apochromat ×20/0.8 objective. Stacks of z-plane images from at least nine different fields of view per sample were acquired and then reconstructed into 3-D images using the IMARIS software (Bitplane, Inc., Saint Paul, MN). Isosurface reconstructions using the surpass mode were used to calculate the biovolume (in μm^3/microscopic field) of each microorganism.

Determination of mucosal invasion

Determination of tissue invasion was done in samples fixed in 4% paraformaldehyde for 2 hours, followed by a series of ethanol and xylene dehydrations before paraffin embedding. Hematoxylin and eosin stained sections were used to observe tissue architecture, and biofilm distribution and invasion through the tissue layers. To detect invasion of each organism sections were also

stained by immunofluorescence for *C. albicans* and FISH for *S. oralis* as described above, and counter-stained with nucleic acid stain Hoechst 33258 (Invitrogen, Carlsbad, CA) to visualize the epithelial layers (Diaz *et al.*, 2012). Images were obtained using a Zeiss Axio Imager M1 microscope and an EC Plan-Neofluar ×20 NA 0.5 air objective and further analyzed by the AxioVision LE64 program.

Quantification of tissue damage by lactase dehydrogenase assay

Lactate dehydrogenase (LDH) release into the basal culture media was monitored as an indicator of tissue/cell damage; media from uninfected tissues served as negative controls. The CytoTox-ONE Homogeneous Membrane Integrity Assay kit (Promega, Madison, WI, USA) was used to assay LDH activity using an Opsys MR™ Microplate Reader (Dynex Technologies Inc., Chantilly, VA, USA) and the Revelation QuickLink software (Thermo Labsystems, Chantilly, VA) according to the manufacturer's protocol. The LDH data were expressed as optical density units.

Statistical analysis

Statistical analyses were performed using SigmaPlot 12 software (SigmaPlot v. 12.3, Systat Software Inc., San Jose, USA), using a 5% significance threshold. Data were evaluated by analysis of variance (ANOVA) and when statistical significances were found, all pairwise multiple comparisons were performed with a Bonferroni t-test.

RESULTS

C. albicans-streptococcal biofilm architecture, submucosal invasion and mucosal cell damage are determined by the amount of moisture on the mucosal surface

First we compared the structural characteristics of mucosal biofilms formed on wet (media-submerged) versus semidry mucosal surfaces (media limited to inoculum). When grown under moist conditions, the wild-type *C. albicans* strain extended long intertwined hyphae, to form homogeneous surface biofilm mats in both single and mixed biofilms (Fig.1A). In mixed biofilms under these conditions, *S. oralis* also formed a relatively homogeneous biofilm mat which was located under the *Candida* biofilm in contact with the mucosa, resulting in two distinct strata (fungal biofilm apically, and bacterial biofilm basally). In contrast, when grown under semi-dry conditions, wild-type *C. albicans* formed localized foci of dense growth from which hyphae extended radially to intertwine with hyphae from adjacent foci (Fig. 1B). In mixed biofilms under semidry conditions, *S. oralis* grew in close physical contact with the fungal hyphae within the foci, as shown by the yellow co-localization pattern in the 3 dimensional reconstruction of the mucosal biofilm (Fig. 1B). Thus the degree of moisture strongly influenced the structure of the mixed biofilm. In the absence of *C. albicans*, *S. oralis* did not form a robust mucosal biofilm under the conditions tested (data not shown), consistent with previous findings (Diaz *et al*., 2012).

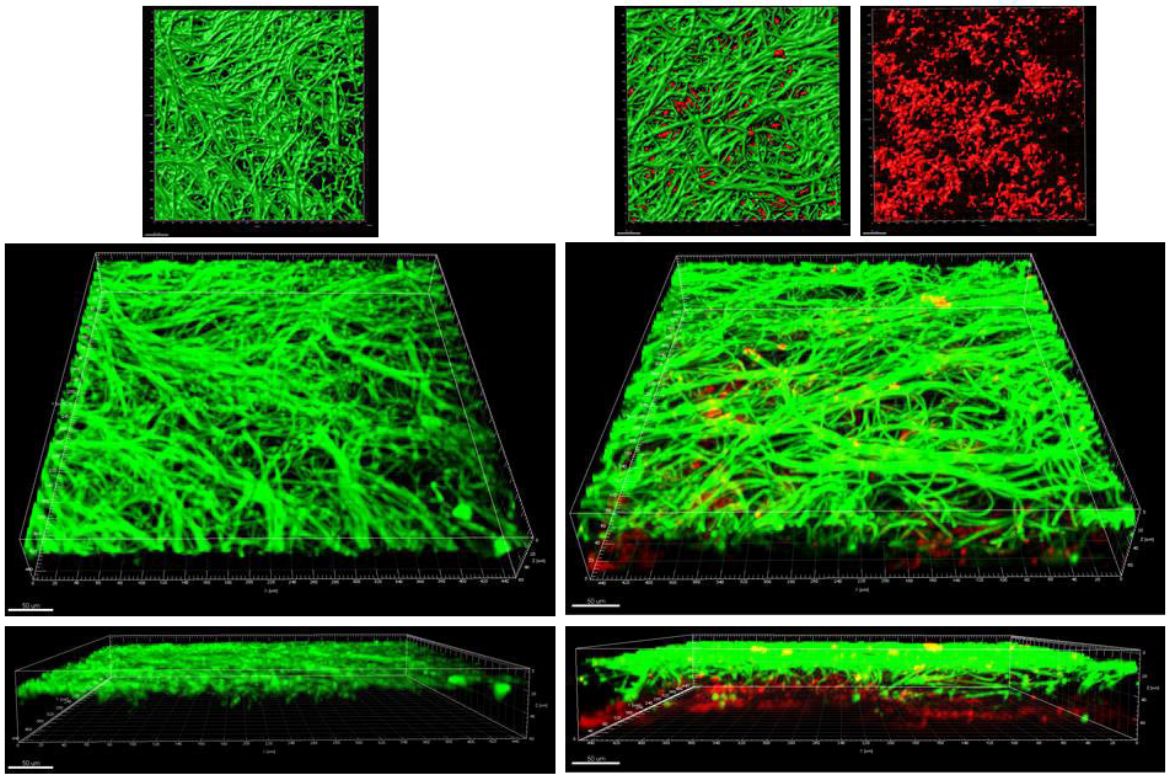

Figure 1A: Sixteen-hour mucosal biofilms of *C. albicans* monospecies (left panel) or *C. albicans*-streptococci mixed-species (right panel). Biofilms were grown on the surface of three-dimensional models of oral mucosa under wet (media-submerged) conditions. X-Y isosurfaces (top panel) and 3-D reconstructions (bottom panels) of representative confocal laser scanning microscopy images are shown. *C. albicans* (green) was visualized after staining with a FITC-conjugated anti-*Candida* antibody. *S. oralis* (red) was visualized after fluorescence *in situ* hybridization (FISH) with a Streptococcus-specific probe conjugated to Alexa 546. Scale bar = 50 µm

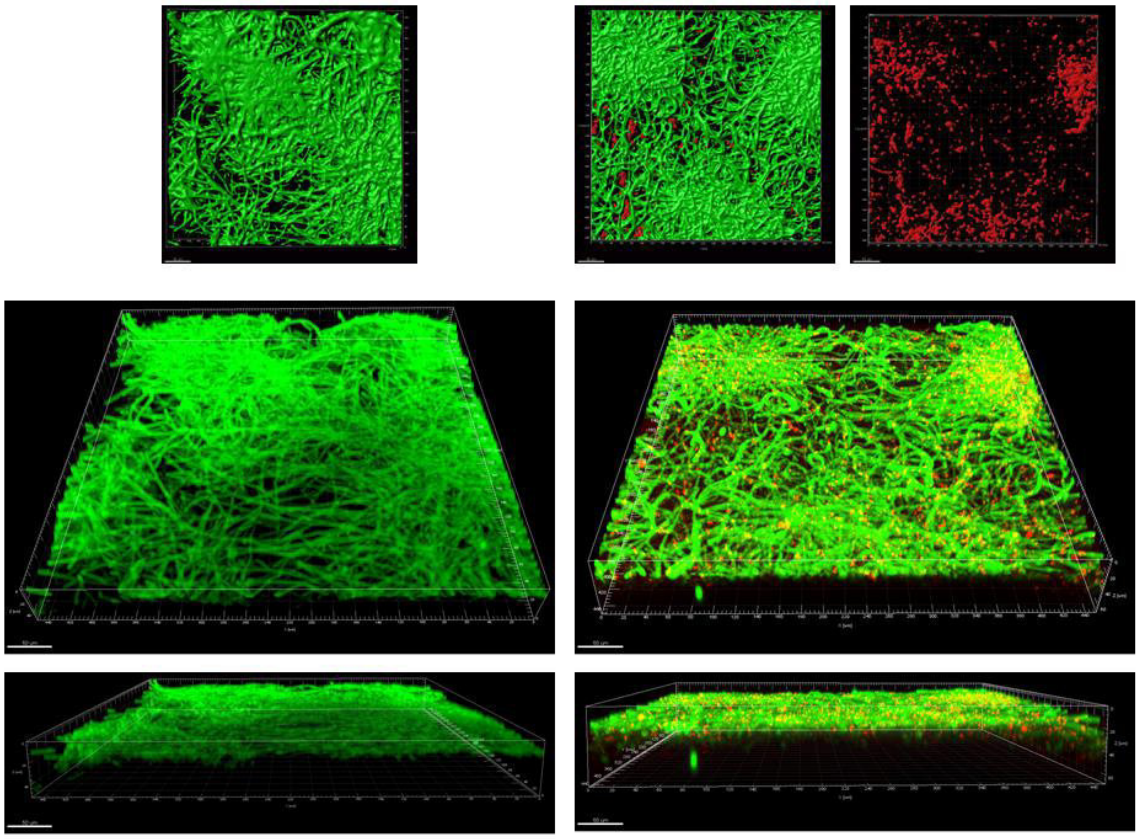

Figure 1B: Sixteen-hour mucosal biofilms of *C. albicans* monospecies (left panel) or *C. albicans*-streptococci mixed-species (right panel). Biofilms were grown on the surface of three-dimensional models of oral mucosa under semidry (media limited to inoculum) conditions. X-Y isosurfaces (top panel) and 3-D reconstructions (bottom panels) of representative confocal laser scanning microscopy images are shown. *C. albicans* (green) was visualized after staining with a FITC-conjugated anti-*Candida* antibody. *S. oralis* (red) was visualized after fluorescence *in situ* hybridization (FISH) with a Streptococcus-specific probe conjugated to Alexa 546. Scale bar = 50 μm

When biofilm biovolumes were compared between moist and semidry conditions, there was a significant increase in the biovolume of wild-type *C. albicans* in both single and dual biofilms in moist versus semidry biofilms ($p \leq 0.001$). There was also a small but statistically significant increase in wild-type

C. albicans biovolume in dual- compared to single-species *C. albicans* biofilms under wet (p≤0.001, Fig. 2), but not under semidry conditions. Finally, wet conditions favored growth of *S. oralis* in dual-species biofilms (p≤0.001) but did not increase single-species biofilm biovolume (data not shown).

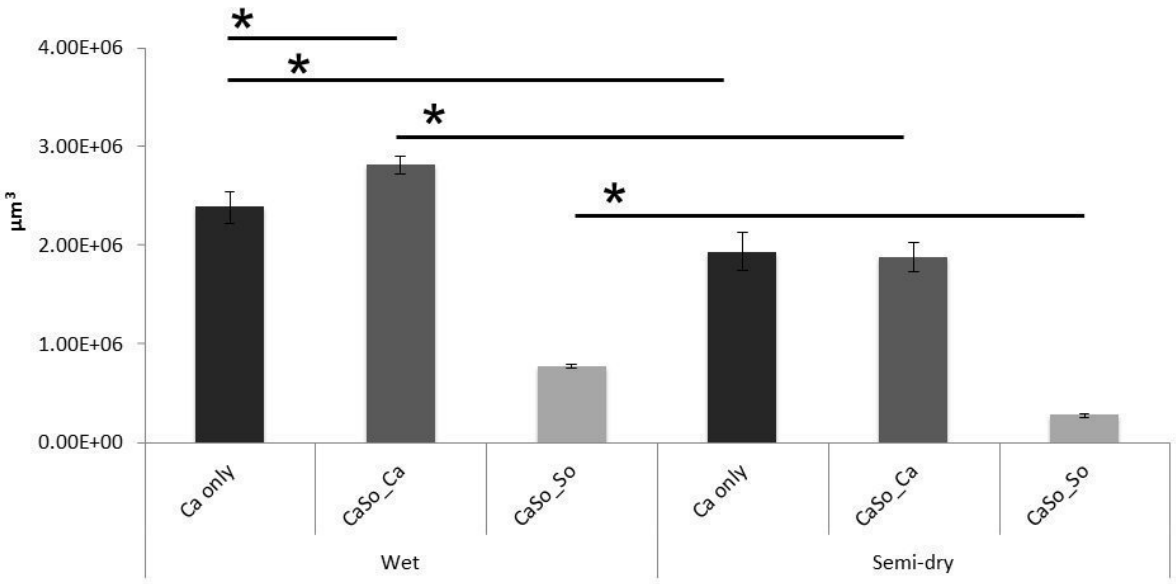

Figure 2: Biovolumes of sixteen-hour single *C. albicans* (Ca) or mixed *C. albicans-S. oralis* (CaSo) mucosal biofilms. Average biovolumes were calculated (in μm^3) for each species in eight different confocal laser scanning microscopy images using image stacks from two independent experiments. Average biovolumes of *C. albicans* in single (Ca only) or mixed (CaSo_Ca) biofilms and *S. oralis* (CaSo_So) in mixed biofilms, under wet (media-submerged) or semidry (media limited to inoculum) conditions. *P = <0.001 when monospecies biovolumes were compared to mixed-species biovolumes or between wet and semi-dry conditions, using the Bonferroni t-test. The error bars indicate one standard deviation of the mean of eight different images from two independent experiments.

Interestingly, although the wild-type *Candida* biofilm biovolume was significantly greater under moist conditions, fungal growth in these biofilms was mostly superficial and was associated with minimal tissue invasion compared to

semidry conditions where the epithelial barrier was completely breached (Fig. 3). Under wet conditions, *S. oralis*, which formed a biofilm adjacent to the mucosa layer (Fig. 1A), promoted invasion of wild-type *C. albicans* past the epithelial barrier (Fig. 3) and increased tissue damage ($p \leq 0.005$, Fig. 4). These results are in agreement with our previous findings in a saliva-supplemented mucosal flow cell system (Diaz *et al*., 2012). Under semidry conditions both single- and dual-species biofilms traversed the entire thickness of the stratified epithelium and entered the submucosal compartment (Fig. 3).

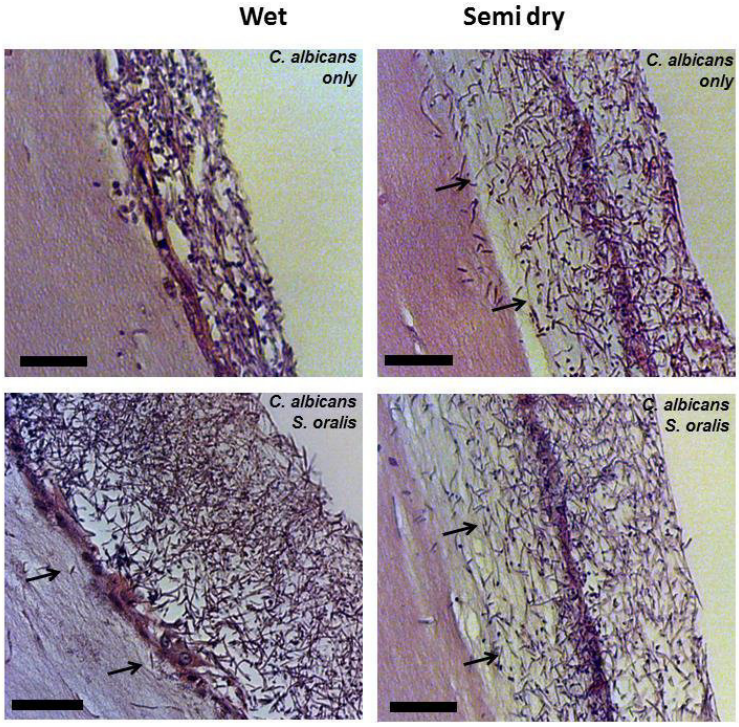

Figure 3: H&E-stained tissue sections of 16 hour *C. albicans* and *C. albicans*-streptococci mixed-species mucosal biofilms under wet (media-submerged) or semidry (media limited to inoculum) conditions. Arrows indicate invasion of *C. albicans* through the epithelial barrier formed by OKF6 cells. Scale bar = 50 µm.

Consistent with these findings, tissue damage was highest in dual-species biofilms under semidry conditions (Fig. 4).

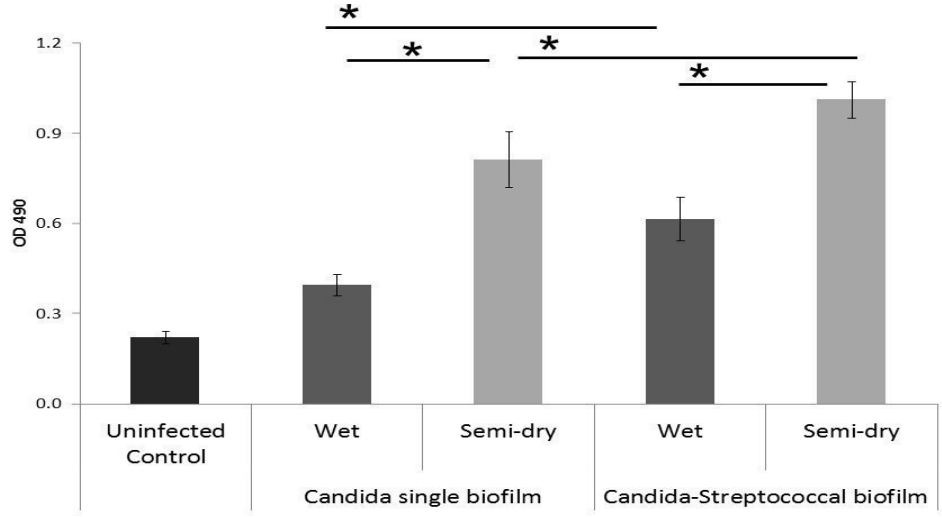

Figure 4: Lactate dehydrogenase (LDH) released by mucosal cells. Results represent the average OD490 of subnatant samples from triplicate wells in two independent experiments. *P< 0.05 for a comparison with *C. albicans* and *C. albicans*-streptococci mixed-species mucosal biofilms under wet (media-submerged) and semidry (media limited to inoculum) conditions, using the Bonferroni t-test. The error bars indicate one standard deviation of the mean of three different wells from two independent experiments.

Nutrient availability increases mucosal biofilm growth and tissue destruction but does not affect architecture of *C. albicans*-streptococcal biofilms.

To rule out the possibility that the "wet" biofilm stratified, superficial growth phenotype was attributed to increased nutrient availability we supplemented the microorganism inoculation media with increasingly high concentrations of BHI under semi-dry conditions. We hypothesized that if the "wet" growth phenotype was due to increased nutrient availability in the media, by supplementing the inoculation media with increasing amount of nutrients under semidry conditions, we could promote a more homogeneous superficial growth as opposed to focal,

invasive growth. However, supplementing the infection medium with increasing amounts of BHI under semidry conditions did not change the focal biofilm architecture but rather intensified the growth of the foci, which coalesced to form a denser biofilm mat over the entire mucosal surface at the maximum concentration of BHI tested (10%) (Fig. 5A).

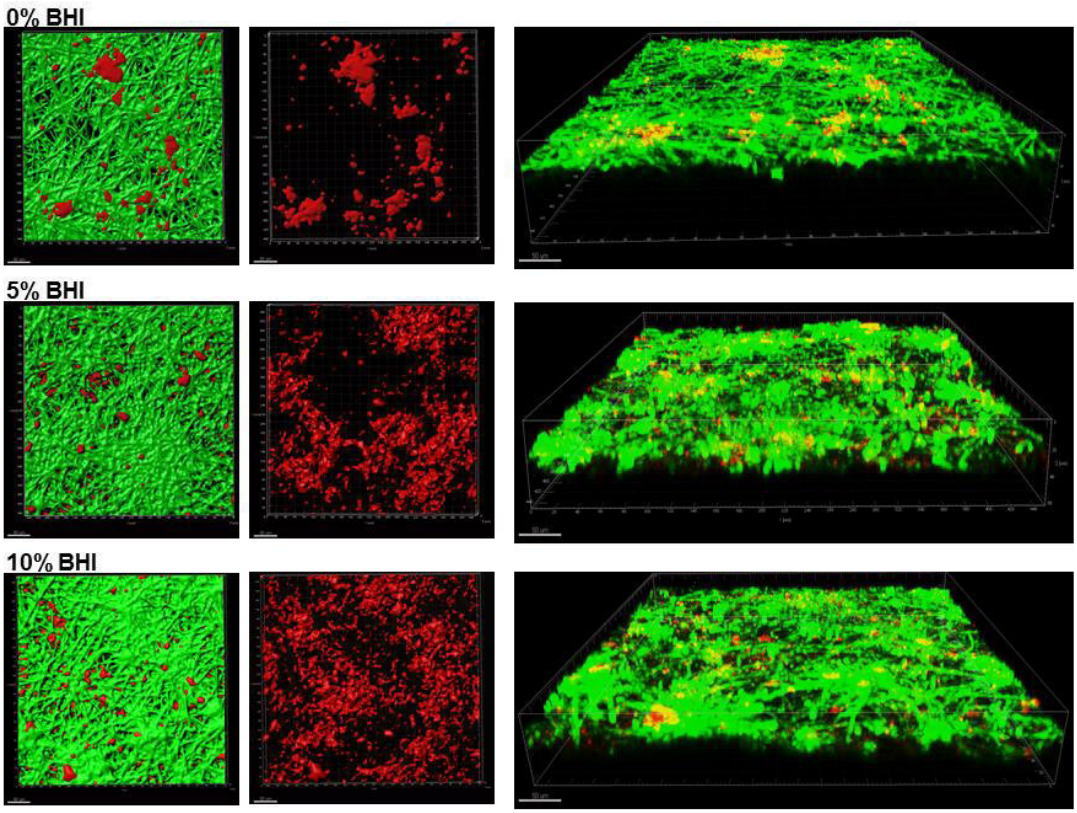

Figure 5A: Sixteen-hour *C. albicans*-streptococci mixed-species biofilms. Biofilms were grown on the surface of oral mucosa analogues under semidry (media limited to inoculum) conditions and microbial inoculation media were supplemented with 0%, 5% or 10% brain heart infusion broth (BHI). X-Y isosurfaces (left two panels) and 3-D reconstructions (right panel) of representative confocal laser scanning microscopy images of biofilms. *C. albicans* (green) was visualized after staining with a FITC-conjugated anti-*Candida* antibody. *S. oralis* (red) was visualized after fluorescence *in situ* hybridization (FISH) with a Streptococcus-specific probe conjugated to Alexa 546. Center panel images display the red channel only, showing *S. oralis* distribution in mixed-species biofilms. Scale bar = 50 µm

Increasing the BHI concentration under semidry conditions, led to a dose-dependent increase in wild-type *C. albicans* biovolumes in mixed *Candida*-streptococcal biofilms (p≤0.001). The biovolume of *S. oralis* was also significantly increased in mixed biofilms grown with 10% BHI, compared to 0 and 5% (Fig. 5B).

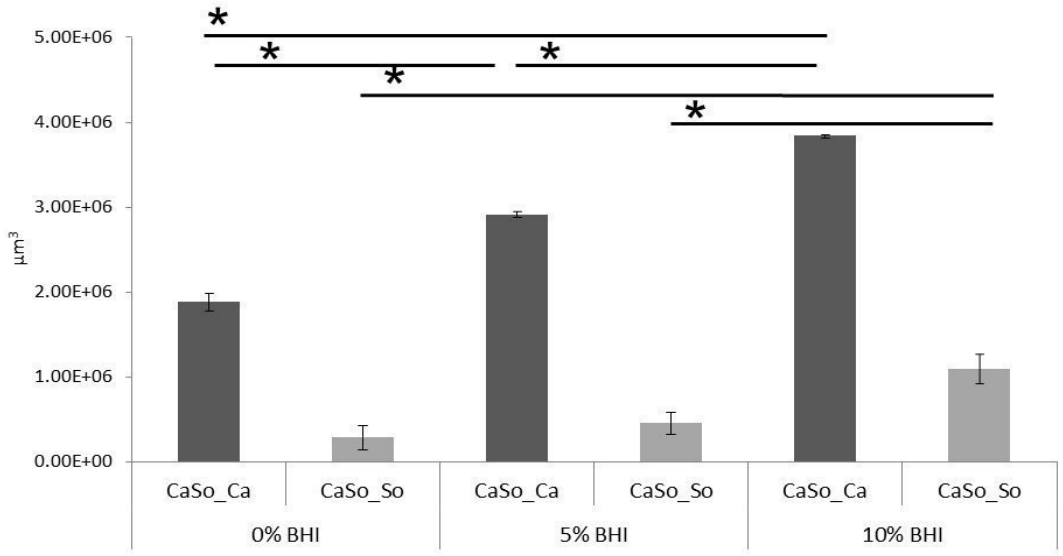

Figure 5B: Average biovolumes (in μm^3) for each species in 16h *C. albicans-S. oralis* (CaSo) mixed-species biofilms grown under the conditions shown in figure 5A. Biovolumes were measured in eight different CLSM image stacks from two independent experiments. Bars represent average biovolumes of *C. albicans* (CaSo_Ca) or *S. oralis* (CaSo_So) when grown together. *P = <0.001 when biovolumes were compared between different BHI concentrations, using the Bonferroni t-test. The error bars indicate one standard deviation of the mean of eight different images from two independent experiments.

Fungal invasion past the epithelial barrier and tissue damage were also greatest at the highest concentration of nutrients, consistent with the increased biofilm growth and tissue damage (Fig. 6A and 6B). From these experiments we also concluded that semidry conditions using inoculation media supplemented with 10% BHI were optimal for pathogenic synergy and thus these conditions were used for all subsequent experiments.

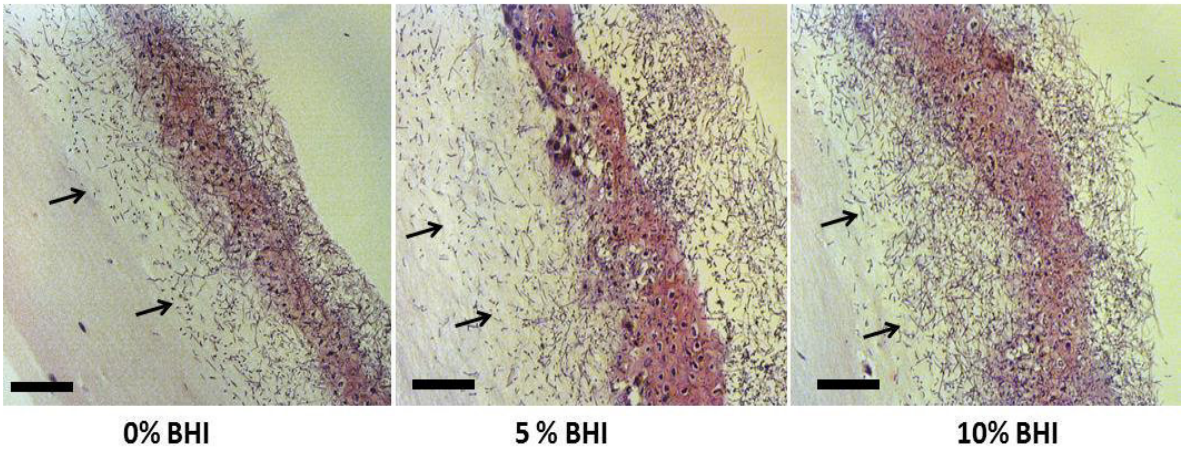

Figure 6A: H&E-stained tissue sections of 16 h *C. albicans*-streptococci mixed-species mucosal biofilms. Biofilms were grown on the surface of oral mucosa analogues under semidry (media limited to inoculum) conditions and microbial inoculation media were supplemented with 0%, 5% or 10% BHI. Arrows showing submucosal invasion through the multilayer epithelial barrier formed by SCC15 cells. Scale bar = 50 µm

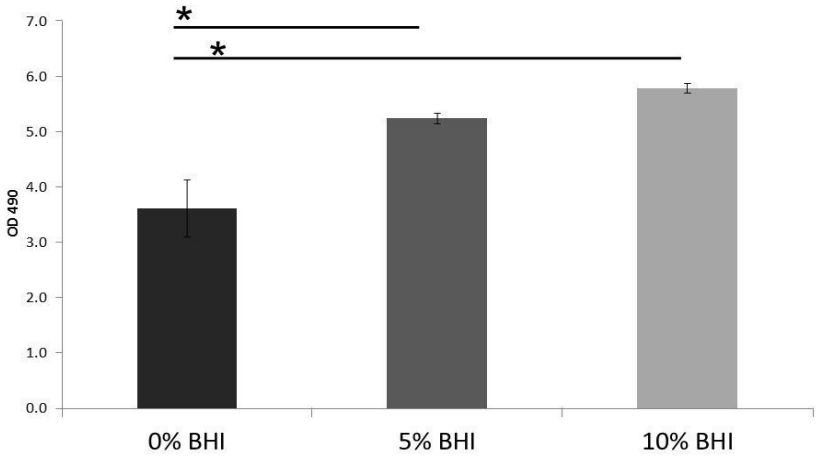

Figure 6B: LDH released by oral mucosa analogues with 16 h C. albicans-streptococci mixed-species mucosal biofilms. Biofilms were grown on the surface of oral mucosa analogues under semidry (media limited to inoculum) conditions and microbial inoculation media were supplemented with 0%, 5% or 10% BHI. Results represent the average of OD490 values in triplicate wells from two independent experiments. *P< 0.05 for a comparison with 0% BHI, using the Bonferroni t-test. The error bars indicate one standard deviation of the mean of three different wells from two independent experiments

Biofilm architecture and virulence characteristics of *Candida*-streptococcal biofilms are affected by the hyphal morphotype

A *C. albicans* homozygous *ndt80* deletion mutant forms defective abiotic surface biofilms composed of long pseudohyphae under several environmental conditions (Nobile *et al*., 2012). Given the strong co-aggregation interactions between true hyphae and oral streptococci of the Mitis group (Bamford *et al*., 2015; Silverman *et al*., 2010; Xu *et al*., 2014b), we asked whether the biofilm architecture of this strain with *S. oralis* may be affected by its pseudohyphal morphology. In mixed biofilms with the reference and *ndt80*-complemented strains, *S. oralis* grew in close contact with *Candida* hyphae and was interspersed throughout the thickness of the biofilm (Fig 7A and 7B). In contrast, when growing with the *ndt80* pseudohyphal mutant, *S. oralis* mostly grew in close contact with the mucosal surface (Fig. 7A and 7B).

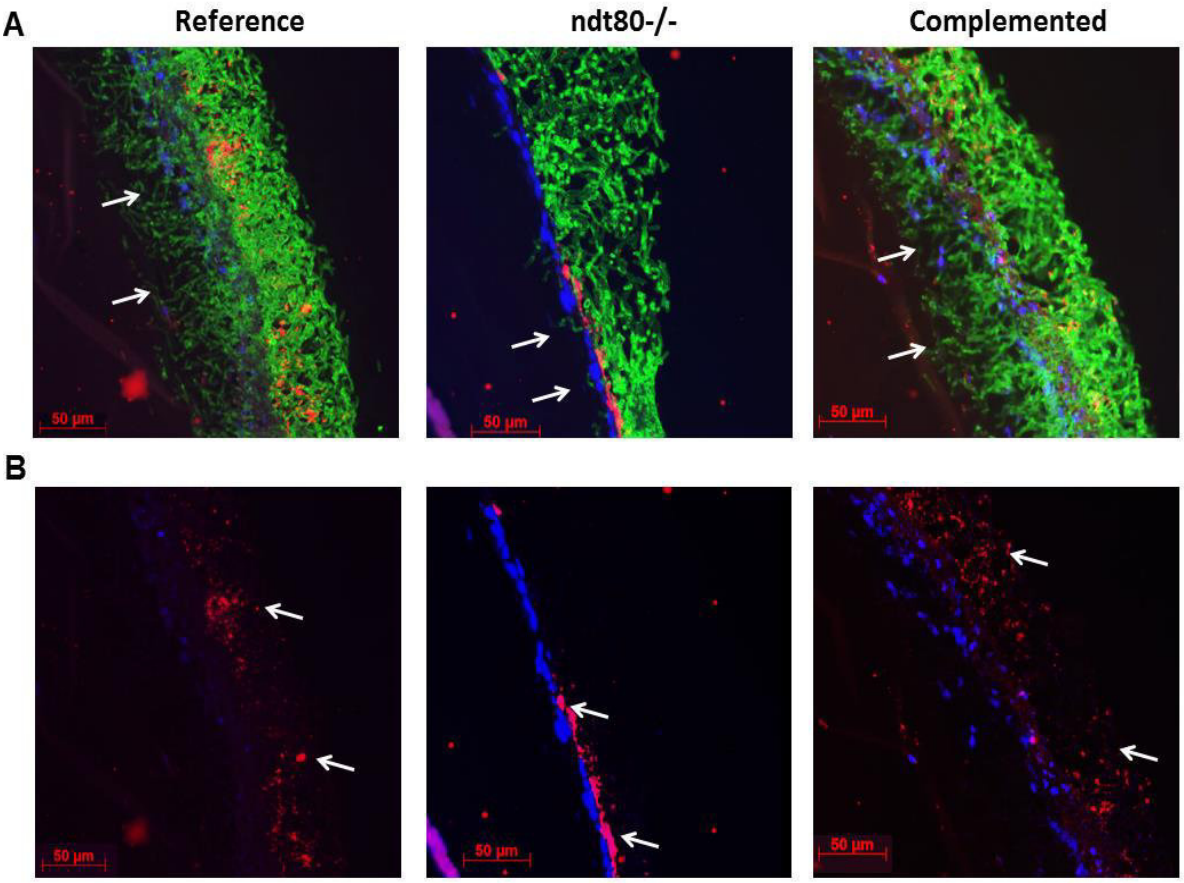

Figure 7: *C. albicans*-streptococci mixed-species mucosal biofilms grown under semidry (media limited to inoculum) conditions. *C. albicans* reference strain, ndt80 homozygous deletion mutant (ndt80-/-) and its complemented strain were grown in the presence of *S. oralis* 34 for 16h. Representative tissue sections are shown where *C. albicans* (green) was visualized after staining with a FITC-conjugated anti-*Candida* antibody and *S. oralis* (red) was visualized after fluorescence *in situ* hybridization (FISH) with a Streptococcus-specific probe conjugated to Alexa 546; Mucosal cell nuclei were counterstained with the nucleic acid stain Hoechst 33258 (blue). A: Overlay of three-color images, showing invasion of each *Candida* strain into the submucosal compartment (white arrows). B: Red and blue channel overlay, showing *S. oralis and* epithelial nuclei, respectivelly, highlighting the distinct *S. oralis* biofilm architecture in mutant vs reference and complemented strain biofilms.

Three dimensional reconstructions of dual biofilms further showed that the pseudohyphae spread on the surface of the bacterial layer, forming a biofilm with a mixed, partially stratified architecture since some bacteria also co-aggregated with pseudohyphae (please note yellow co-localization, Fig. 8A). To rule out the possibility that the mixed biofilm phenotype of the *ndt80* deletion strain was gene-specific we tested the phenotype of another *C. albicans* transcription factor mutant, *tup1*, which also forms pseudohyphae under most environmental conditions (Kadosh *et al*., 2005; Villar *et al*., 2004). As shown in fig. 8B, similar to the *ndt80* mutant, this pseudohyphal strain exhibited a partially stratified biofilm architecture with some bacteria interspersed throughout the pseudohyphal mass but most growing in contact with the epithelial surface.

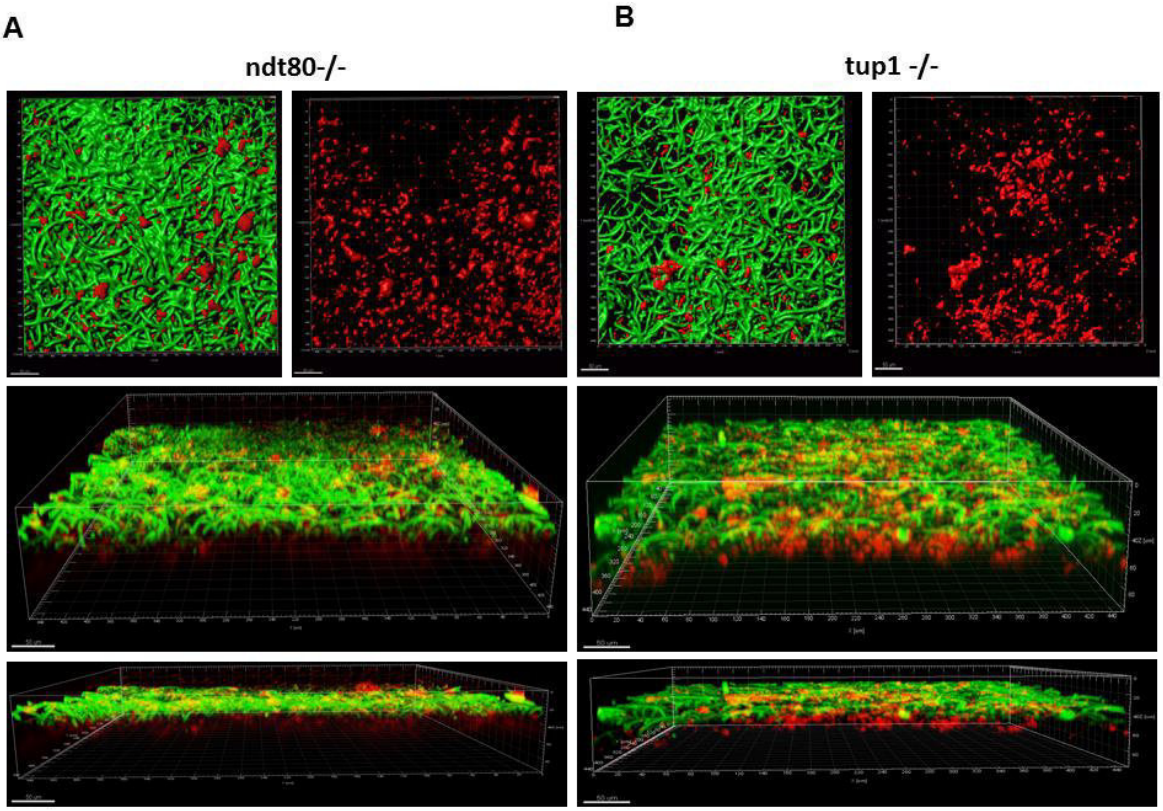

Figure 8: *C. albicans*-streptococci mixed-species mucosal biofilms grown under semidry (media limited to inoculum) conditions. C. albicans pseudohyphal strains, ndt80 homozygous deletion mutant (ndt80-/-) and tup1 homozygous deletion mutant (tup1-/-) were grown in the presence of S. oralis 34 for 16h. Representative x-y isosurfaces (top panel) and 3-D reconstructions (bottom panel) of representative CLSM images. *C. albicans* (green) was visualized after staining with a FITC-conjugated anti-*Candida* antibody. *S. oralis* (red) was visualized after fluorescence *in situ* hybridization (FISH) with a Streptococcus-specific probe conjugated to Alexa 546. Note the partially stratified structure of biofilms with bacteria co-aggregating on the surface of the oral mucosa as well co-localizing with pseudofilaments (yellow). Scale bar = 50 µm

We have previously shown that pseudohyphal organisms are deficient in invading oral epithelium and triggering cell damage (Villar *et al*., 2004), we thus wondered whether the presence of streptococci could alter this phenotype.

As shown in Fig. 9A, in the absence of *S. oralis* neither pseudohyphal mutant was able to cross the mucosal barrier. However, *S. oralis* triggered a significant crossing-over of pseudohyphae into the submucosal compartment.

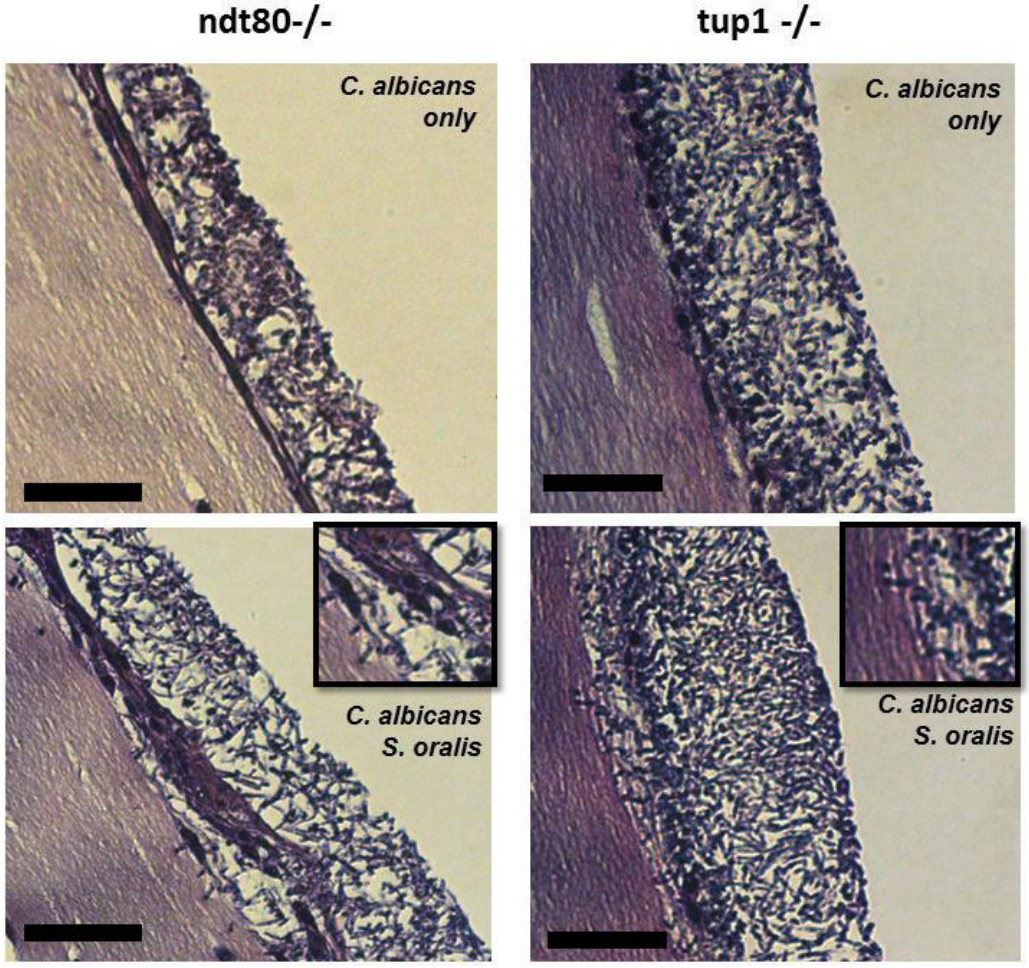

Figure 9A: H&E-stained tissue sections of biofilms shown in figure 8. Magnifying squares show invasion of C. albicans pseudohyphae through the epithelial barrier for both mutant strains. Scale bar = 50 µm

This was consistent with the increased tissue damage, as assessed by measuring LDH release, in dual- versus single-species pseudohyphal biofilms (Fig. 9B).

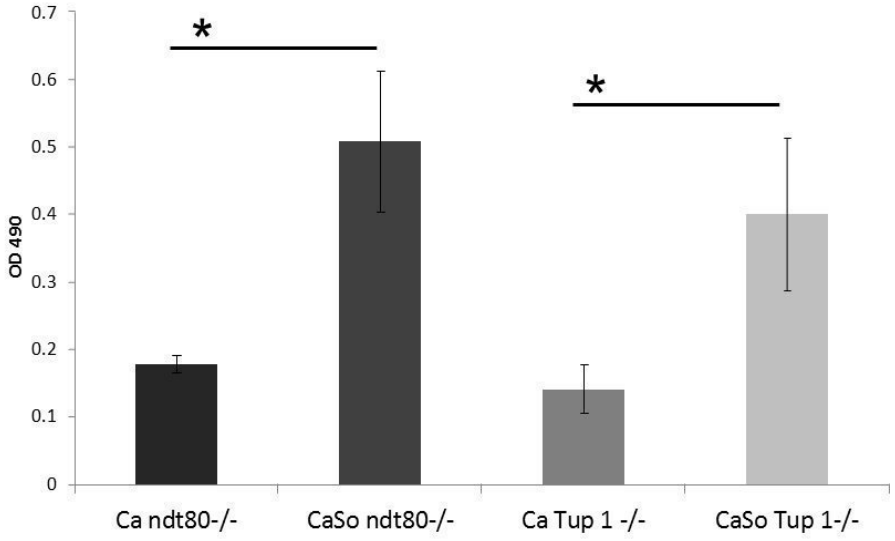

Figure 9B: LDH released by mucosal analogues inoculated under semidry conditions with pseudohyphal mutants in the presence or absence of *S. oralis* 34 for 16h. Bars represent the average of the OD490 values in triplicate wells from two independent experiments. *P< 0.05 for a comparison of *C. albicans* monospecies and *C. albicans*-streptococci mixed-species mucosal biofilms, using the Bonferroni t-test. The error bars indicate one standard deviation of the mean of three different wells from two independent experiments.

As expected, biofilm biovolume estimates showed that pseudohyphal mutants formed deficient single- and dual-species biofilms compared to the reference strain (Fig. 10). Both pseudohyphal strains were able to promote *S. oralis* biofilm growth, compared to *S. oralis* alone, albeit at lower levels compared to the reference strain ($p \leq 0.001$). However there were no significant differences in the biovolumes of *C. albicans* in a comparison between dual- and single-species biofilms in both pseudohyphal strains. This suggests that pseudohyphal invasion, which was noted only in the presence of *S. oralis,* was a consequence of the

streptococcal presence and not due to increased fungal growth in dual-species biofilms.

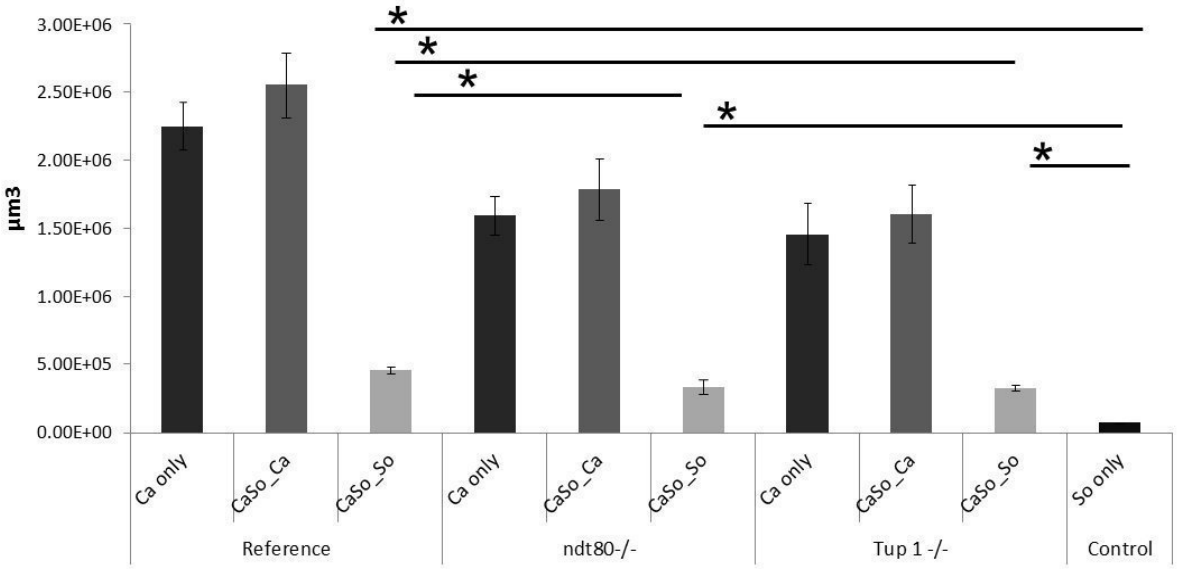

Figure 10: Average biovolumes (in μm^3) for each species in 16h C. albicans-S. oralis (CaSo) mixed-species biofilms grown under the conditions shown in figures 8-9. Biovolumes were measured in eight different CLSM image stacks from two independent experiments. Bars represent average biovolumes of C. albicans (Ca only) single biofilms, S. oralis (So only) single biofilms and C. albicans (CaSo_Ca) or S. oralis (CaSo_So) when grown together. *P = <0.001 when biovolumes were compared between different strains using the Bonferroni t-test. The error bars indicate one standard deviation of the mean of eight different images from two independent experiments.

DISCUSSION

In recent years *C. albicans*-bacterial cross-kingdom interactions have received increased attention (Shirtliff *et al*., 2009; Morales & Hogan, 2010). Synergistic interactions leading to increased virulence are complex and may be affected by specific environmental host conditions such as mucosal or salivary secretions (Kavanaugh *et al*., 2014, Edgerton & Koshlukova, 2000). For example,

in addition to providing moisture, saliva contains proteins with antifungal properties, such as histatins, that form part of the innate immune system (reviewed by Edgerton & Koshlukova, 2000).

In this work we showed that reduced moisture on oral mucosal surfaces promotes fungal invasion and tissue damage, independently of host-derived antimicrobial proteins or of the amount of nutrients present. Importantly, we showed that streptococcal growth promotes *C. albicans* mucosal invasion and damage both under moist and semi-dry conditions. Surprisingly, we also found that in the presence of streptococci, fungal invasion and tissue damage can bypass the requirement for true hyphal transformation, although the use of a yeast-locked mutant would be required to completely address this question. A certain degree of epithelial invasion by *C. albicans* has been suggested to be compatible with stable colonization and commensalism, however, significant invasion that leads to tissue damage is the hallmark of the transition of this fungus to pathogenicity (Gow & Hube, 2012). The fact that oral streptococci can trigger further fungal invasion and tissue damage under different experimental conditions is an indication that commensal bacteria may play an important role in the transition of *C. albicans* from commensalism to pathogenicity.

Presence of sufficient moisture provides adequate growth conditions for *C. albicans* on human skin without the need of exogenously added nutrients (Leyden, 1984). This also held true in our experimental mucosal system where increased moisture on the mucosal surface promoted robust fungal growth in both single- and dual-species biofilms, in the absence of added microbial nutrients. However, this is

the first report to demonstrate that lack of adequate moisture on a host surface can alter biofilm architecture and hyphal orientation, leading to increased tissue invasion. Hyphae respond to polarized environmental signals by altering their axes of growth, thus it is possible that under semi-dry conditions increased moisture within and below the tissue analogue provided a strong environmental cue for vertical hyphal orientation and tissue penetration. In support of our findings, mutants in orientation response genes sustain their hyphal phenotype but have attenuated tissue invasion and epithelial damage properties (Brand *et al*., 2008). Since physical contact with semisolid substrates triggers invasion of hyphae into these substrates (Brown *et al*., 1999), it is also reasonable to suggest that reduced hyphal-tissue physical contact in moisture-covered surfaces attenuated tissue penetration. These results provide a novel mechanistic explanation of the increased susceptibility to oral candidiasis in xerostomic patients (Lyon *et al*., 2006, Torres *et al*., 2007).

In mixed biofilms the presence of *S. oralis* promoted *C. albicans* invasion and tissue damage under both moist and semi-dry conditions, consistent with our prior findings in mucosal biofilms forming under salivary flow (Diaz *et al*., 2012). One way whereby commensal bacteria can directly increase fungal pathogenicity is by upregulating expression of hyphae-associated fungal genes that have a demonstrated role in mucosal adhesion, invasion and damage. Such candidate genes include the epithelial adhesins hwp1 and als3 as well as secreted aspartyl proteases involved in degradation of epithelial tight junction proteins (Staab *et al*., 1999; Villar *et al*., 2007; Cavalcanti *et al*., 2015).

Pseudohyphal mutants, such as the *ndt80* and *tup1* transcription factor homozygous deletion mutants, are deficient in the expression of many epithelial adhesins and invasins. This is because transcriptional regulators that control hyphal morphogenesis are also master regulators of most hyphae-associated virulence genes (Sellam *et al*., 2010, Kadosh & Johnson, 2005). Thus it was not surprising that the *ndt80* and *tup1* homozygous deletion mutants were deficient in mucosal invasion and damage. However, to our surprise, these pseudohyphal strains acquired a small but significant invasive and tissue damage phenotype in the presence of *S. oralis*. Since considerable functional redundancy exists among transcriptional regulators in *Candida*, it is reasonable to suggest that *S. oralis* may upregulate another transcriptional regulator in the *ndt80* or *tup1* deletion backgrounds affecting virulence gene expression. A reasonable candidate transcriptional regulator, is Rim101, as we and others have shown that *C. albicans* utilizes the Rim101 transcription pathway to invade the oral and corneal mucosa (Villar *et al*., 2005, Villar *et al*., 2007, Yuan et al., 2010).

Pseudo-hyphal mutants also displayed a different biofilm architecture with *S. oralis* compared to the reference or complemented strains. Interestingly, when co-cultured with pseudohyphal mutants, *S. oralis* grew mostly in contact with the mucosal surface, and fewer bacteria co-aggregated with pseudohyphal filaments. This might be due to the fact that these mutants are deficient in activating hypha specific genes encoding for cell wall proteins involved in the recognition of and coaggregation with *Streptococcus spp.,* such as the Als3 or Hwp1 proteins (Conti *et al*., 2009, Mao et al., 2008, Sellam et al., 2010). More specifically, it has been

shown that the *ndt80* mutant, is unable to activate both Hwp1 and Als3 genes (Sellam *et al*., 2010), whereas the *tup1* mutant has a two- to three-fold reduction of Hwp1, and almost no Als3 transcribed (Mao *et al*., 2008). The partially stratified biofilm structure may be explained either by the weak expression of these genes on the surface of the pseudofilaments or by the weak upregulation of an alternative functionally redundant transcriptional regulator triggered by *S. oralis* in these mutants.

The significant increase of *S. oralis* biovolume when co-cultured with *C. albicans* may also suggest a more direct effect of these bacteria on mucosal cells, which could facilitate tissue penetration by hyphae or pseudohyphae. In a mouse *S. oralis* - *C. albicans* co-infection model, we have previously shown that Toll-like receptor 2 expression (TLR2) is significantly increased in the oral mucosa and that *S. oralis* can signal via this receptor to activate a neutrophilic inflammatory response (Xu *et al*., 2014b). Signaling via TLR2 may also trigger activation of mucosal cell calpains, Ca^{2+}-dependent cysteine proteases which can cleave the epithelial junctional proteins E-cadherin and occludin (Chun & Prince, 2009), thus facilitating filament penetration between cells.

Taken together our data show that moisture, nutrient availability, hyphal morphotype and presence of commensal streptococci strongly affect the architecture and virulence characteristics of mucosal fungal biofilms. Future studies are needed to fully elucidate the specific mechanisms that mediate the changes in the fungal biofilm phenotype triggered by oral streptococci of the Mitis group, which were observed under all environmental conditions tested.

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Conclusão

A umidade, a disponibilidade de nutrientes, o morfotipo da *Candida* e a presença de *S. oralis* afetam fortemente a arquitetura, a capacidade de invasão tecidual e o dano celular gerado ao epitélio quando estes biofilmes são crescidos sobre modelos *in vitro* de mucosal oral humana.

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[1] De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors. Abreviatura dos periódicos em conformidade com o Medline.

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Anexo 1: Confirmação de submissão do artigo

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Submission Confirmation

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Manuscript ID:	MOM-01-15-0605
Title:	Candida-streptococcal mucosal biofilms display distinct structural and virulence characteristics depending on growth conditions and hyphal morphotypes
Authors:	Bertolini, Martinna Xu, Hongbin Sobue, Takanori Nobile, Clarissa Del Bel Cury, Altair Dongari-Bagtzoglou, Anna
Date Submitted:	27-Jan-2015

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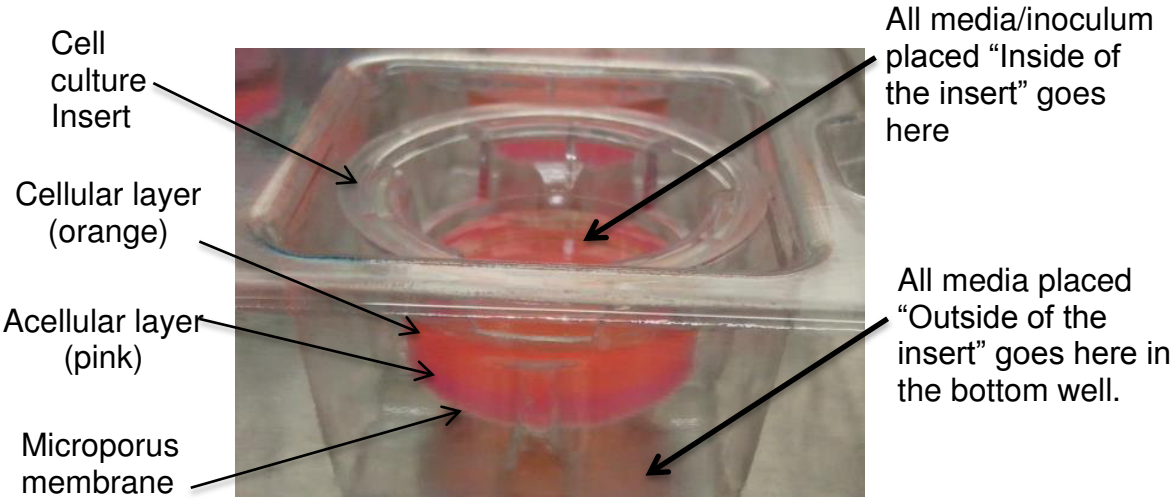
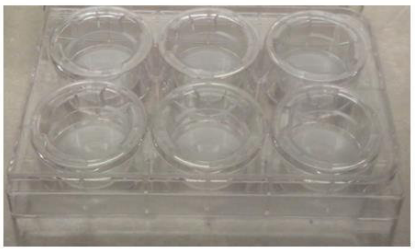
Anexo 2: Protocolo original (em inglês, da UConn Health Center, combinado com o protocolo da UPenn e revisado por Dr. Takanori Sobue), ilustrado, relativo a confecção do modelo tri dimensional de mucosa oral humana



MATERIAL:

Plates/Inserts

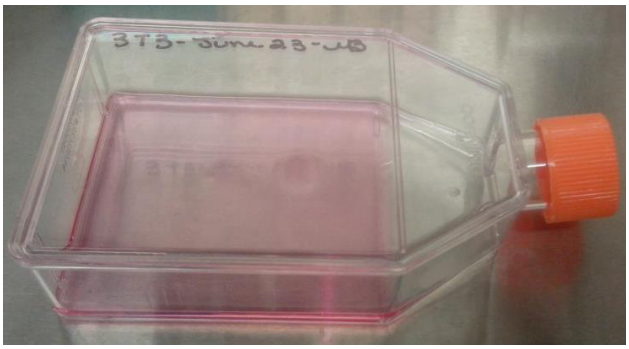
1. Plates: 6-well Transwell Carrier (Organogenesis, TS01-001) Organogenesis, Inc. 150 Dan Road, Canton, MA 02021.
2. Corning Costar Transwell 3414 (24 mm diameter inserts, 3.0 µm pore size)(6 per plate)


Corning Costar Transwell 3414


Transwell Carrier


24 mm diameter inserts, 3.0 µm pore size

Fibroblast: 3T3 cells in T-75 flasks
T-75 flask: Corning® CellBIND® Surface cell culture flasks
CellBIND 75cm2 rectangular canted neck cell culture flask w/ Vent Cap, PS, sterile



Fibroblast Culture Media: DMEM-10% FBS-1% P/S

1. DMEM: Dulbecco's Modified Eagle Medium (DMEM)(1X) liquid (high glucose) without Pyruvate: 1 litter DMEM (-)(Gibco #11965-084)(4°C)
2. FBS: fetal bovine serum (-20°C)
3. Pen Strep: Penicillin-Streptomycin (10,000 U/mL) (Gibco®)

Epithelial cells: OKF6/TERT2 cells

- Use fresh stock from Liquid N2
- Never over growth, Need to sub-culture when cells are 70-80% confluent.
- Even not fully confluent, need to sub-culture 1 week after starting culture.

Epithelial Cells Culture Media: KSFM (Invitrogen, #17005-042) with BPE (1 tube/each bottle), hEGF (0.2ng/ml), $CaCl_2$ (0.4mM) and Pen/Strep (1:100).

- Bovine Pituitary Extract (BPE) 1 tube/500ml, comes as a supplement with KSFM
- hEGF 0.1 ng/mL, comes as a supplement with KSFM (We make at 0.2 ng/mL) – Make intermediate stock of hEGF 0.1 mkg/mL (500X) by adding 10 mkl of the original hEGF to 3.2 mL or so of culture medium (it depends on the lot as different lots of hRGF have different concentrations)

- $CaCl_2$ 0.4 mM final, add 0.5 mL of 0.3 M CaCl2 per 500 mL of medium so that the final concentration is 0.1mM
- Antibiotics (Pen/Strep - Gibco, Life Technologies – ref. 15140-122)

Matrix and EP media componentes:

1. **DMEM** (-)(Gibco #11965-084)(4°C)
2. **Ham's F12** (Gibco #11765-054 or Mediatech 10-080-CV)(4°C)
3. **10x EMEM** (BioWhittaker #12-684F)(room temperature)
4. **7.5% Na-Bicarbonate** (BioWhittaker #17-613E)(room temperature)
5. **FBS** (Hyclone from Cell Center)(stored as an ~10 ml aliquot, -20°C)
6. **L-Glutamine** (Cellgro #25-005CI)(-20°C)
7. **Collagen type 1** (Organogenesis #200/50)(Stored as ~50 ml aliquots at 4°C)
8. **Matrigel** (BD Biosciences #354234)(-20°C)
9. **Hydrocortisone** (Sigma #H0888): MW = 362.46
    - Dissolve 0.0269 g in 2.5 ml EtOH
    - Add into 97.5 ml DMEM (-): 0.74 mM
    - Filter-sterilize, dispense into aliquots and store at -20°C.
10. **ITES** (BioWhittaker #17839Z)(-20°C)
11. **O-phosphorylethanolamine** (Sigma #P0503) MW = 141.06
    - Dissolve 0.705 g in 100 ml DMEM (-): 50 mM
    - Filter-sterilize, dispense into aliquots and store at -20°C.
12. **Adenine** (Sigma #A9795) MW = 171.59
    - Dissolve 1.55 g in 100 ml warm (37°C) ddH_2O: 0.09 M
    - Filter-sterilize, dispense into aliquots and store at -20°C.
13. **Progesterone** (Sigma #P8783) MW = 314.46
    - Dissolve 1 mg in 1 ml ETOH
    - Add 14.7 ml ddH2O.
    - Dilute 1ml of that in 100ml DMEM (-): 2.0 µM
    - Filter-sterilize, dispense into aliquots and store at -20°C.
14. **Triiodothyronine** (Sigma #T5516) MW = 672.96
    - Dissolve 1 mg in 1 ml 1N NaOH.
    - Add 19 ml of DMEM (-).
    - Dilute 4 µl of that in 31 ml plain DMEM (-): 1 nM
    - Filter-sterilize, dispense into aliquots and store at -20°C.
15. **Newborn Calf Serum** (Hyclone #SH 3011802) – aliquots 15 mL, -20 °C
16. **Gentamicin, 50 mg/ml** (Cellgro #MT30-0005-CR) – store Room temp

OTC matrix (stromal compartment) preparation

Start growing 3T3 and OKF6/TERT2cells **(2 or 3 weeks ahead)**

1. Thaw a frozen vial, put into 10ml medium (DMEM or KSFM), centrifuge 3min, 2000rpm.
2. After spin down, aspirate supernatant. Re-suspend with 10ml media. Seed cells in the T-75 cell culture flask.
3. Grow until they reach 80% confluence. Change media every 2-3 days.

Day 0 : The day before matrix preparation:

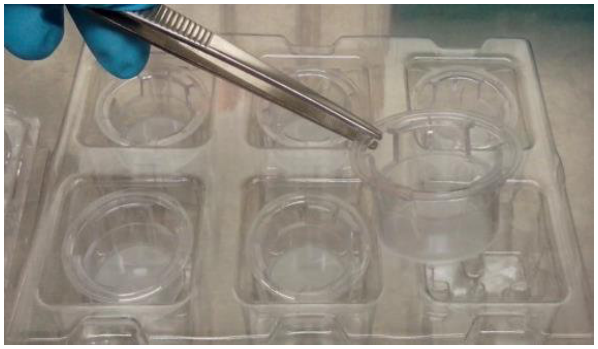
Thaw Matrigel overnight (one vial for 2-3 OTC plates) at 4°C.

Day 1

To do 15-30 min prior to making matrix:

1. Thaw FBS and L-Glutamine, clean the tube with 70% EtOH, place on ice.
2. Place tubes containing 10X EMEM and 7.5% Na-bicarbonate on ice.
3. Place Matrigel and type I collagen on ice
4. Pre-chill empty 50-ml tubes on ice for Acellular layer and Cellular layer.

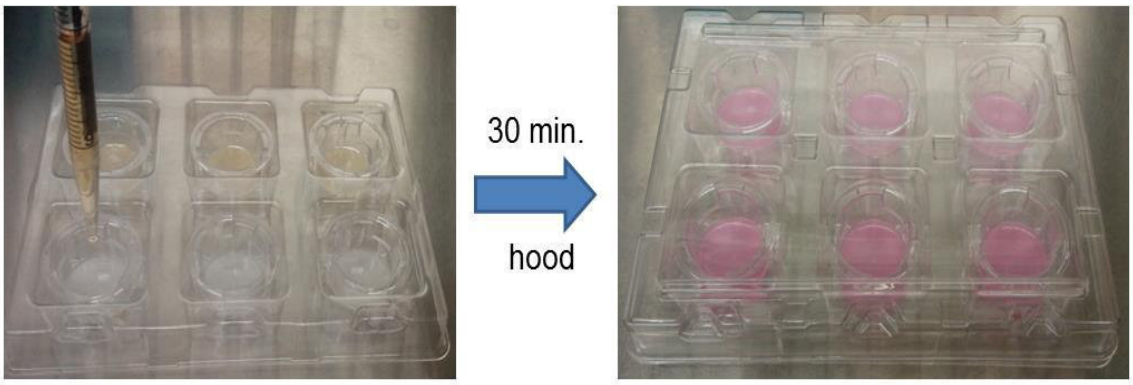
Place inserts (Corning Costar Transwell 3414) into each well (6 per plate) inside the tissue culture hood.



Making acellular layer:

1. Add 10x EMEM, FBS, L-Glutamine, N-bicarbonate and type I collagen in this order (see Table below for the volume needed).
2. Mix gently using a 25-ml pipette to avoid bubbles in a 50-ml tube on ice.
3. Pour 1 ml per insert using a 10-ml pipette.
4. Leave still inside the tissue culture hood while preparing cellular layer.

Acellular layer	1 plate	2 plates
10x EMEM	690 µl (x 1)	690 µl (**x 2**)
FBS	700 µl (x 1)	700 µl (**x 2**)
L-Glutamine	60 µl (x 1)	120 µl (x 1)
Na-bicarbonate	140 µl (x 1)	280 µl (x 1)
Type I collagen	5.6 ml (x 1)	11.2 ml (x 1)



Prepare 6x10^5/ml Fibroblast cell suspension:

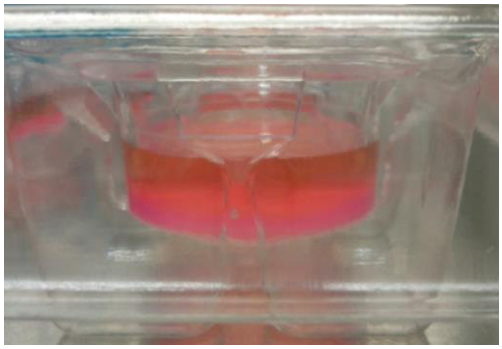
1. Trypsinize, pellet, and resuspend in DMEM-10% FBS-1% P/S.
2. Take 10 µl to count with coulter counter (1000x dilution)
3. Prepare 4 ml of 6 x 10^5/ml fibroblast cell suspension

Making cellular layer:

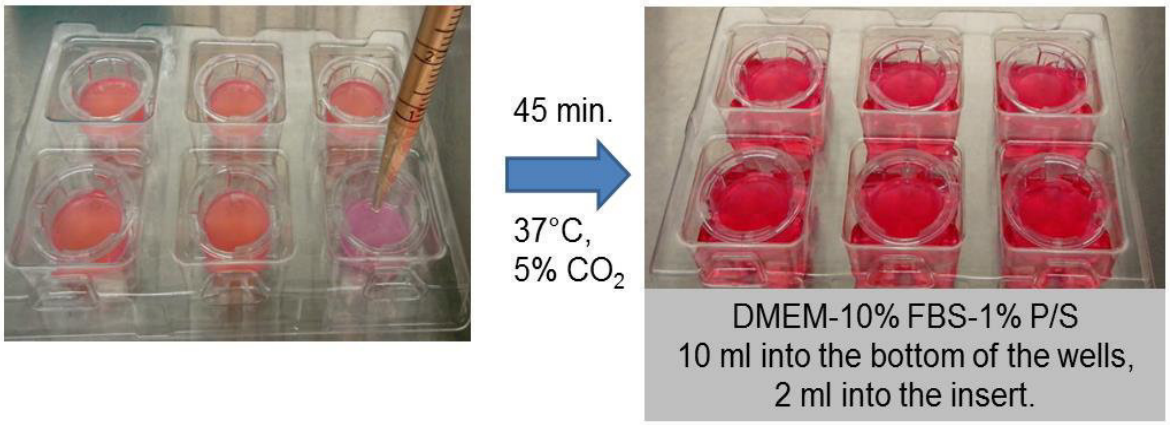
1. Add into a 50-ml tube on ice 10x EMEM, FBS, L-Glutamine, N-bicarbonate, type I collagen, Matrigel and fibroblasts in this order (Follow the Table below).
2. Mix gently using a 25-ml pipette to avoid making bubbles.
3. Pour 3 ml per insert using a 10-ml pipette.
4. Incubate for 30-45 min in the tissue culture incubator (37ºC, 5% CO2).
5. Add DMEM-10% FBS-1% P/S: 10 ml otside of the insert (in the bottom of the wells), 2 ml inside the insert.

Cellular layer	**1 plate**	**2 plates**
10x EMEM	1.8 ml (x 1)	3.6 ml (x 1)
FBS	2 ml (x 1)	4 ml (x 1)
L-Glutamine	160 µl (x 1)	320 µl (x 1)
Na-bicarbonate	380 µl (x 1)	760 µl (x 1)
Type I collagen	11.4 ml (x 1)	11.4 ml (**x 2**)
Matrigel	3.8 ml (x 1)	7.6 ml (x 1)
$6x10^5$/ml fibroblasts	1.6 ml (x 1)	3.2 ml (x 1)

Acellular layer only



Acellular layer + Cellular layer



<u>Day 2</u> – Dislodge matrix and add 2 ml of DMEM-10% FBS-1% P/S into the insert.

- Use a sterile glass Pasteur pipette to go around (2-3 times) the matrix along with the inner wall of the insert. Matrix will contract during the next days.

<u>Day 5</u> or later: Seeding epithelial cells

1. Make DMEM (-)/F12 (3:1)(See table below) to pre-saturate the OTC matrix.

DMEM/F12 Media (3:1)	2 Plates (144ml+)	3 Plates (216ml+)
DMEM(-)	120ml	180ml
Ham's F12	40ml	60ml

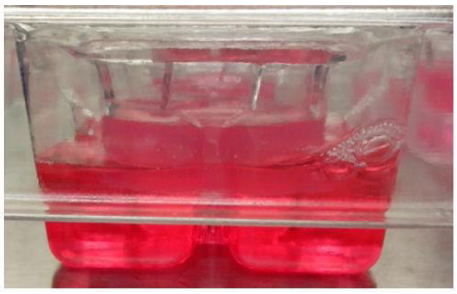
2. Remove old medium from the OTC plates/inserts.
3. Add DMEM+F-12 (3:1) – add 10 ml to the bottom, 2 ml into the insert.
4. Make **1x10^7/ml** epithelial cell suspension in the regular medium
5. Remove DMEM+F-12 (3:1) after 1 hour incubation
6. Seed epithelial cells by applying **50µl** (5x10^5) inside of the insert
7. Incubate for 2 hrs in the tissue culture incubator without medium.
8. Make EP2 (see table below). Store at 4°C. This will be used also for Day 7.
9. Add EP2 to plate - 10 ml into the bottom well and 2 ml into the insert.

EP2	2 plates (300ml)	3 plates (450ml)
DMEM (-)	218 ml	327 ml
F12	72 ml	108 ml
L-Glutamine (LQ)	6 ml	9 ml
Hydrocortisone (H)	600 µl	900 µl
ITES	600 µl	900 µl
O-phosphorylethanolamine (O)	600 µl	900 µl
Adenine (A)	600 µl	900 µl
Progesterone (P)	600 µl	900 µl
Triiodothyronine (T)	600 µl	900 µl
Newborn Calf Serum (NBCS)	300 µl	450 µl
Gentamycin	300 µl	450 µl

<u>Day 7</u> (or 2 days after seeding epithelial cells): Medium change with EP2.

<u>Day 9</u> (or 4 days after seeding epithelial cells): medium change with EP3.
Make EP3 (see table below). Store at 4°C. This will be used also for Day 11.
Remove old medium from both inserts and the bottom well.
Add **7.5 ml of EP3** into the bottom wells only.

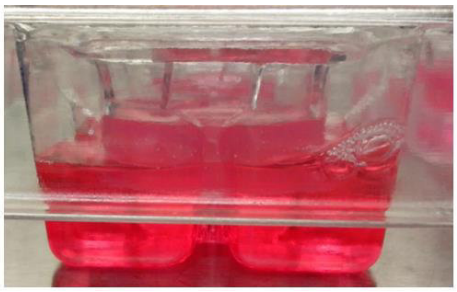
Add 7.5 ml of EP3 into the bottom wells only.



EP3	2 plates (200ml)	3 plates (300ml)
DMEM (-)	95 ml	142.5 ml
F12	95 ml	142.5 ml
L-Glutamine (LQ)	4 ml	6 ml
Hydrocortisone (H)	400 µl	600 µl
ITES	400 µl	600 µl
O-phosphorylethanolamine (O)	400 µl	600 µl
Adenine (A)	400 µl	600 µl
Triiodothyronine (T)	400 µl	600 µl
Newborn Calf Serum (NBCS)	4 ml	6 ml
Gentamycin	200 µl	300 µl

<u>Day 11</u> (or 6 days after seeding epithelial cells): medium change with EP3.
Remove old medium from both inserts and the bottom well.
Add 7.5 ml of EP3 into the bottom wells only.

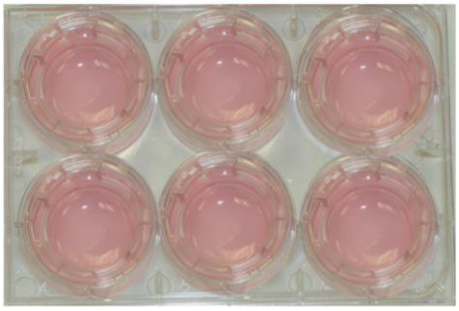
Add 7.5 ml of EP3 into the bottom wells only.



<u>Day 13</u>(or 8 days after seeding epithelial cells): medium change with EP4 (infection medium).

Remove old medium from both inserts and the bottom well.
Transfer inserts to 6 wells dish (Corning 3516) with tweezers
Add 1.5ml of EP4 into both inserts and the bottom well.

EP4 (Infection medium)	3 plates (196ml)
DMEM (-)	95 ml
F12	95 ml
L-Glutamine (LQ)	4 ml
Hydrocortisone (H)	400 µl
ITES	400 µl
O-phosphorylethanolamine (O)	400 µl
Adenine (A)	400 µl
Triiodothyronine (T)	400 µl



<u>Day 14</u> (or 9 days after seeding epithelial cells): Infection

Remove old medium from both inserts and the bottom well.
Add inoculum (Ex. $1x10^6$/20µl *Candida albicans* and $1x10^7$/20µl streptococci).
Keep dishes in the hood 45min.
Add 1.5ml EP4 medium bottom well only and move dishes into the incubator
After 2 hrs Add 0.5ml EP4 medium inside the inserts.
Culture 16 hours in the incubator at 37°C and 5% CO_2

<u>Day 15:</u> Harvesting OTC 3D culture

Collect tissue samples and supernatants for analysis