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

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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 a 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 pseudohyphal 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.

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Keywords

Candida; *Streptococcus*; biofilms; infection models

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).

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 ($p \leq 0.001$, Fig. 2), but not under semidry conditions. Finally,

wet conditions favored growth of *S. oralis* in dual-species biofilms ($p \leq 0.001$) but did not increase single-species biofilm biovolume (data not shown).

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). Consistent with these findings, tissue damage was highest in dual-species biofilms under semidry conditions (Fig. 4). These findings are also summarized in Table 1.

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

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). 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.

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

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. These findings are also summarized in Table 2.

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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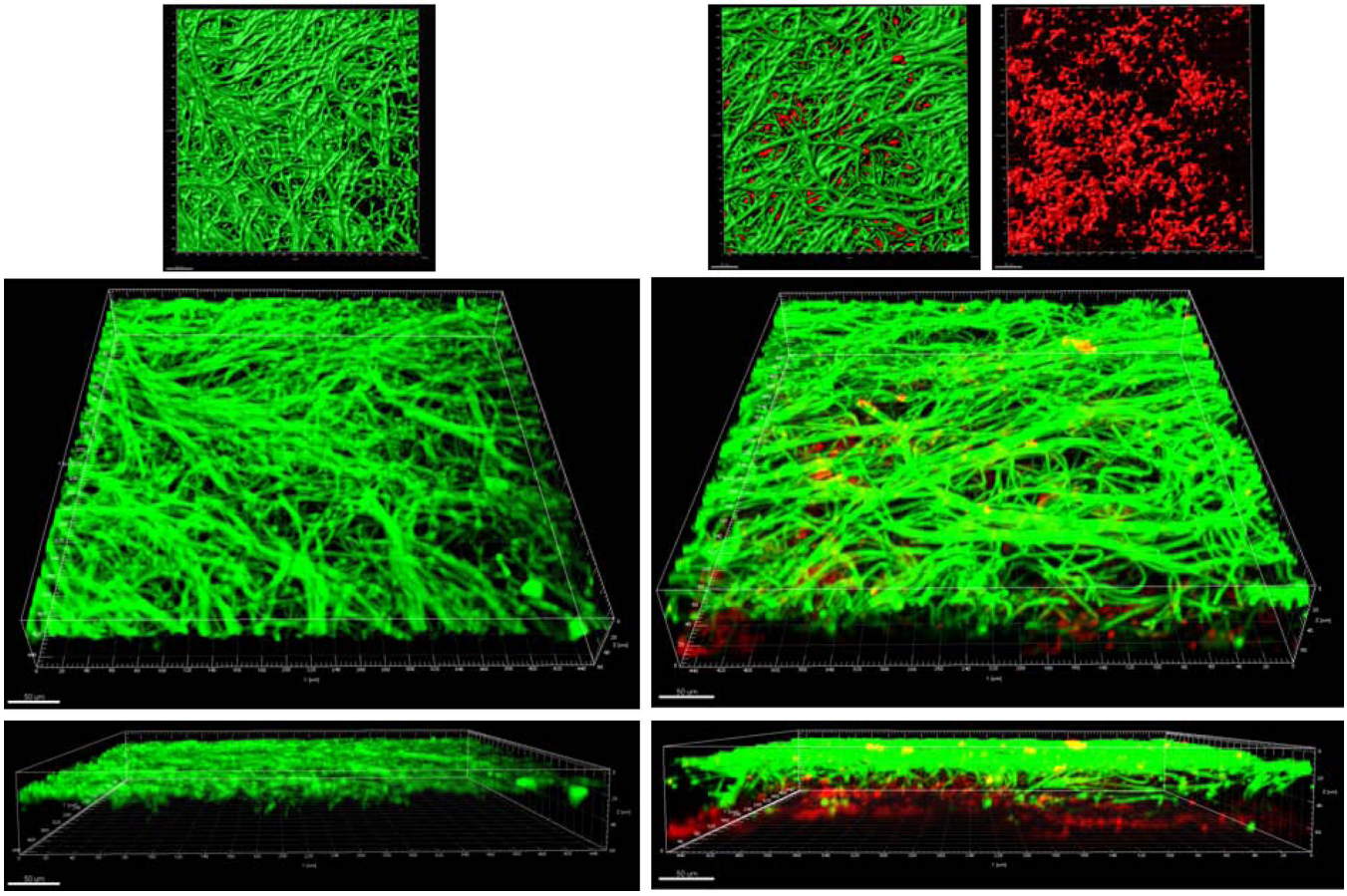


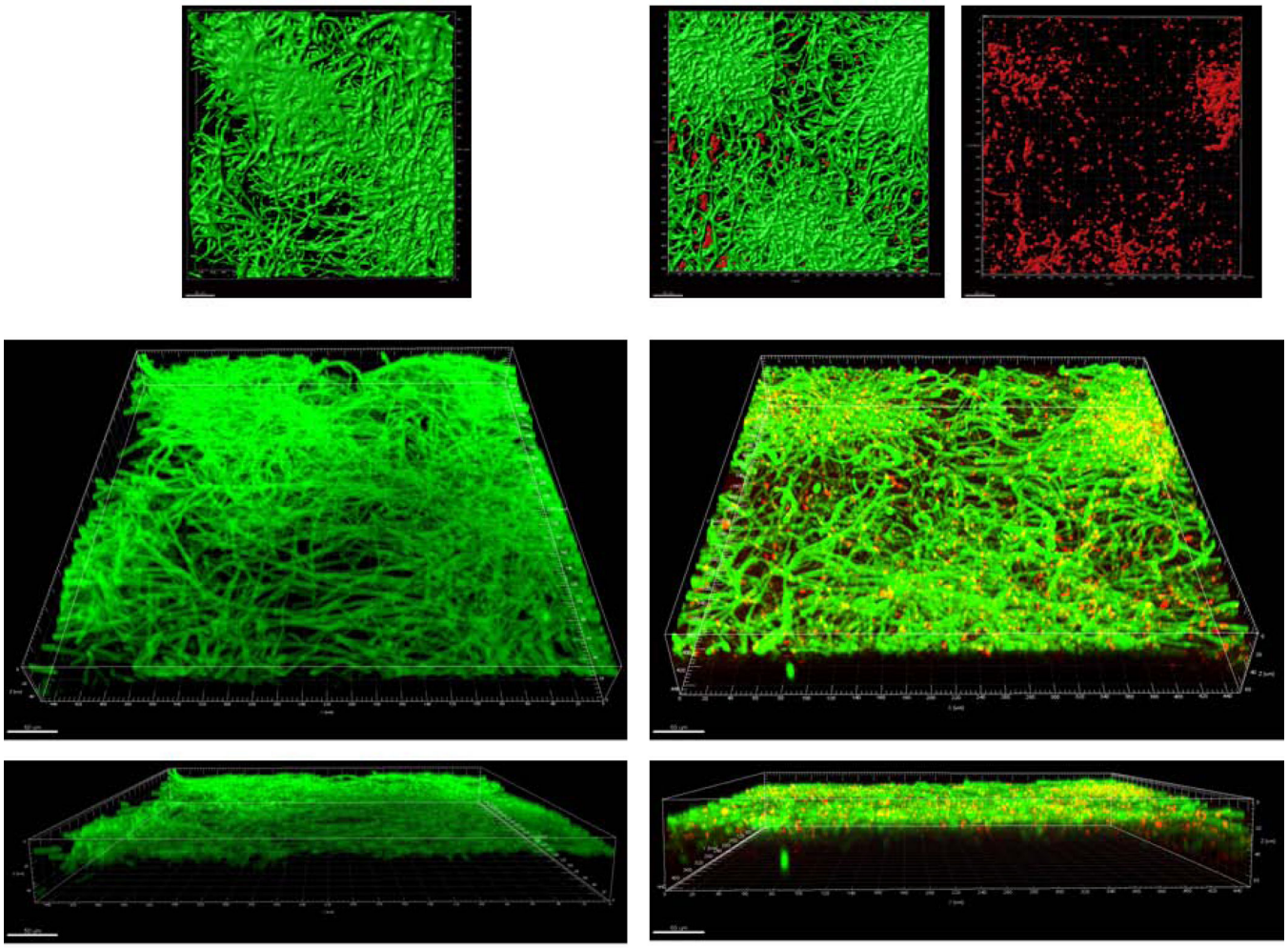


Figure 1.

A: 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.

B: 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.

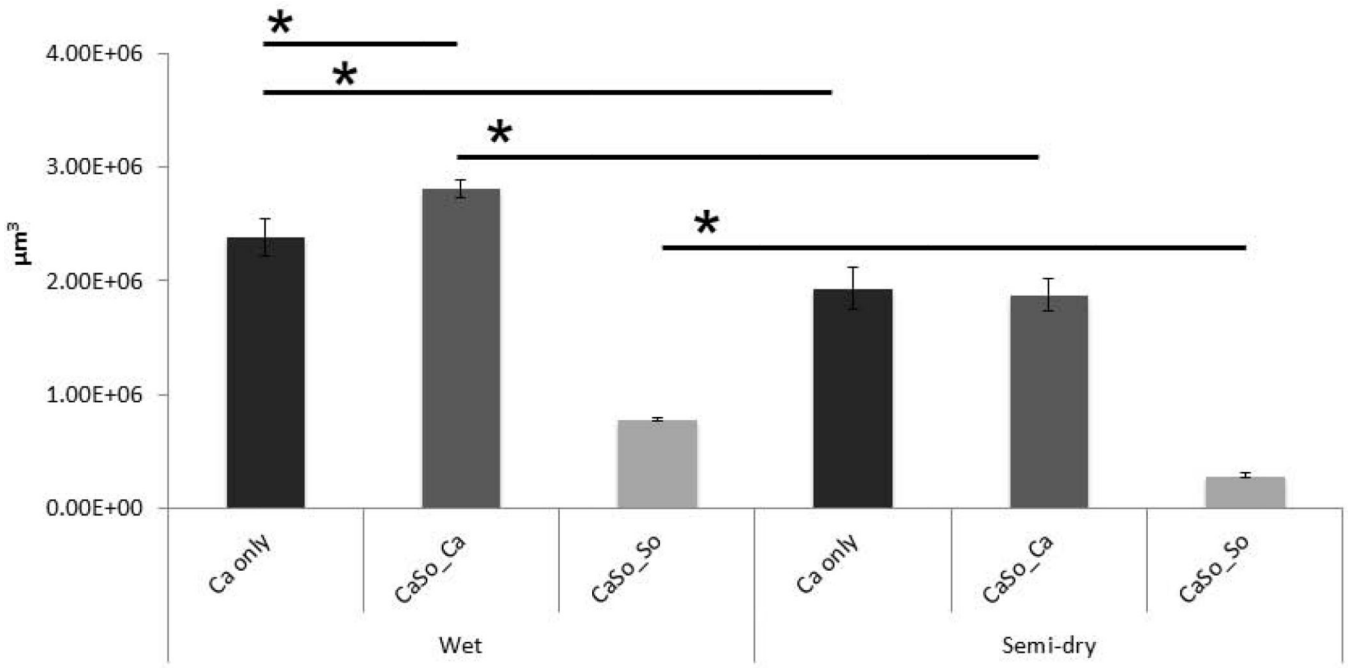


Figure 2.

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.

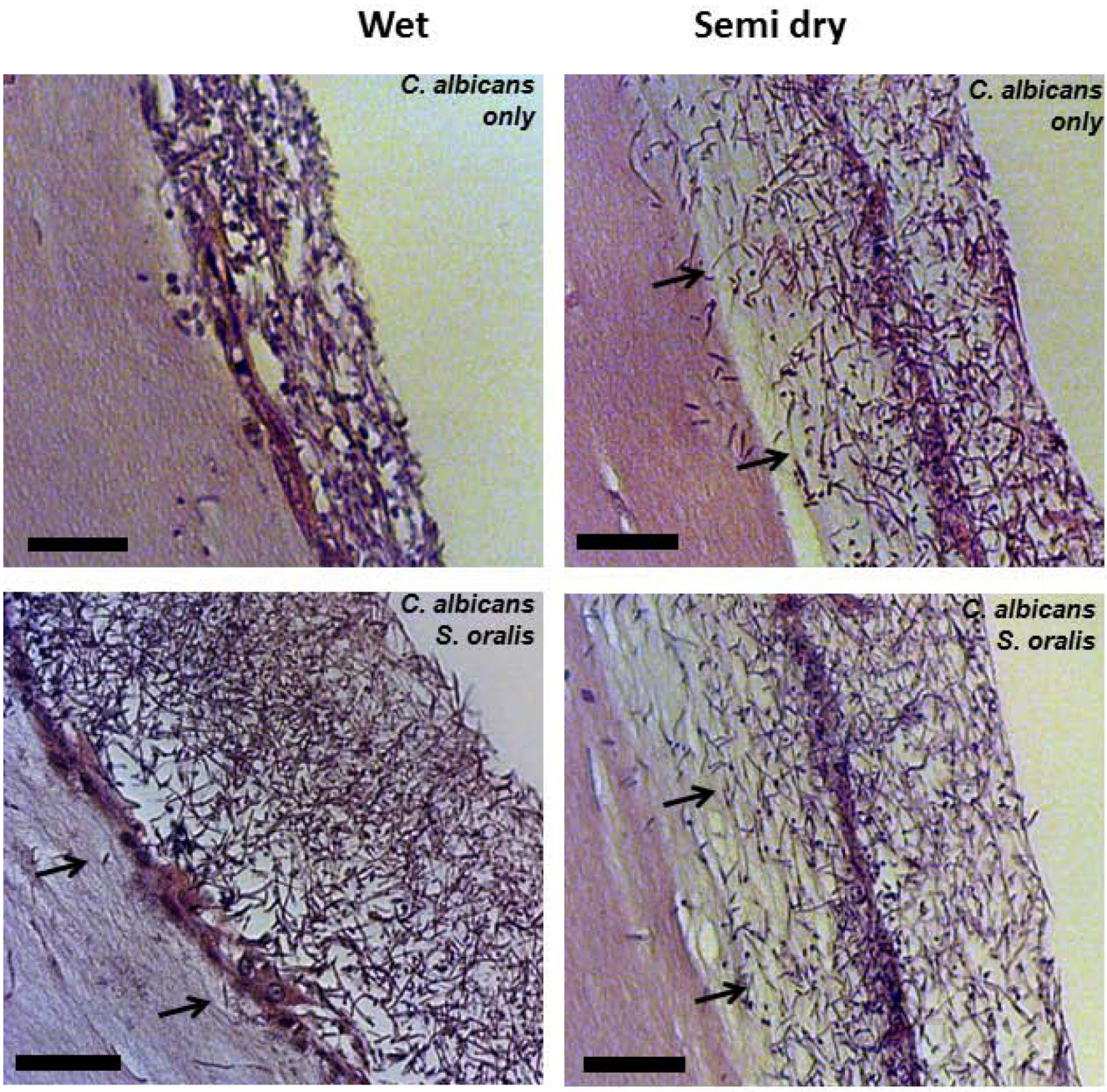


Figure 3.

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.

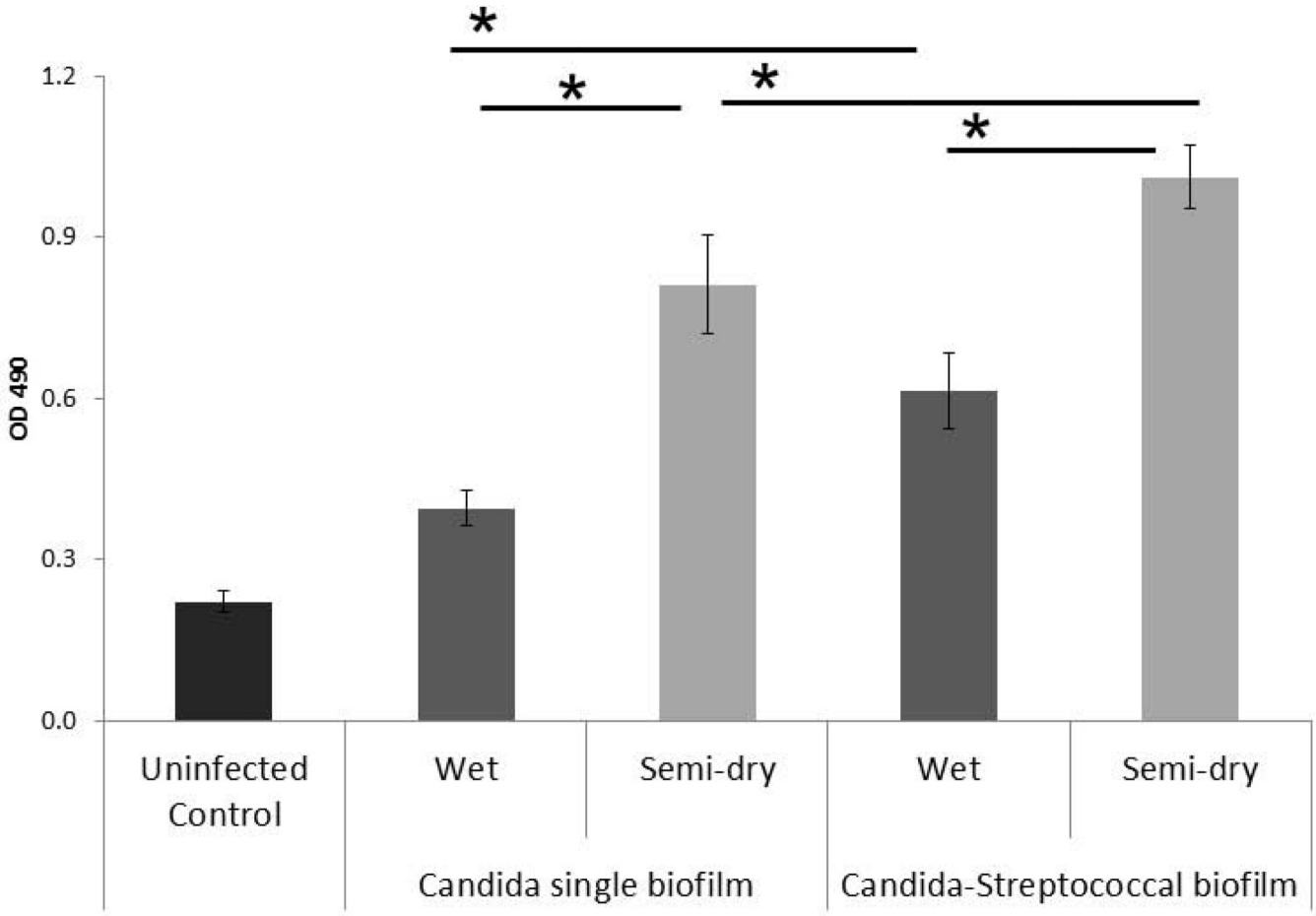
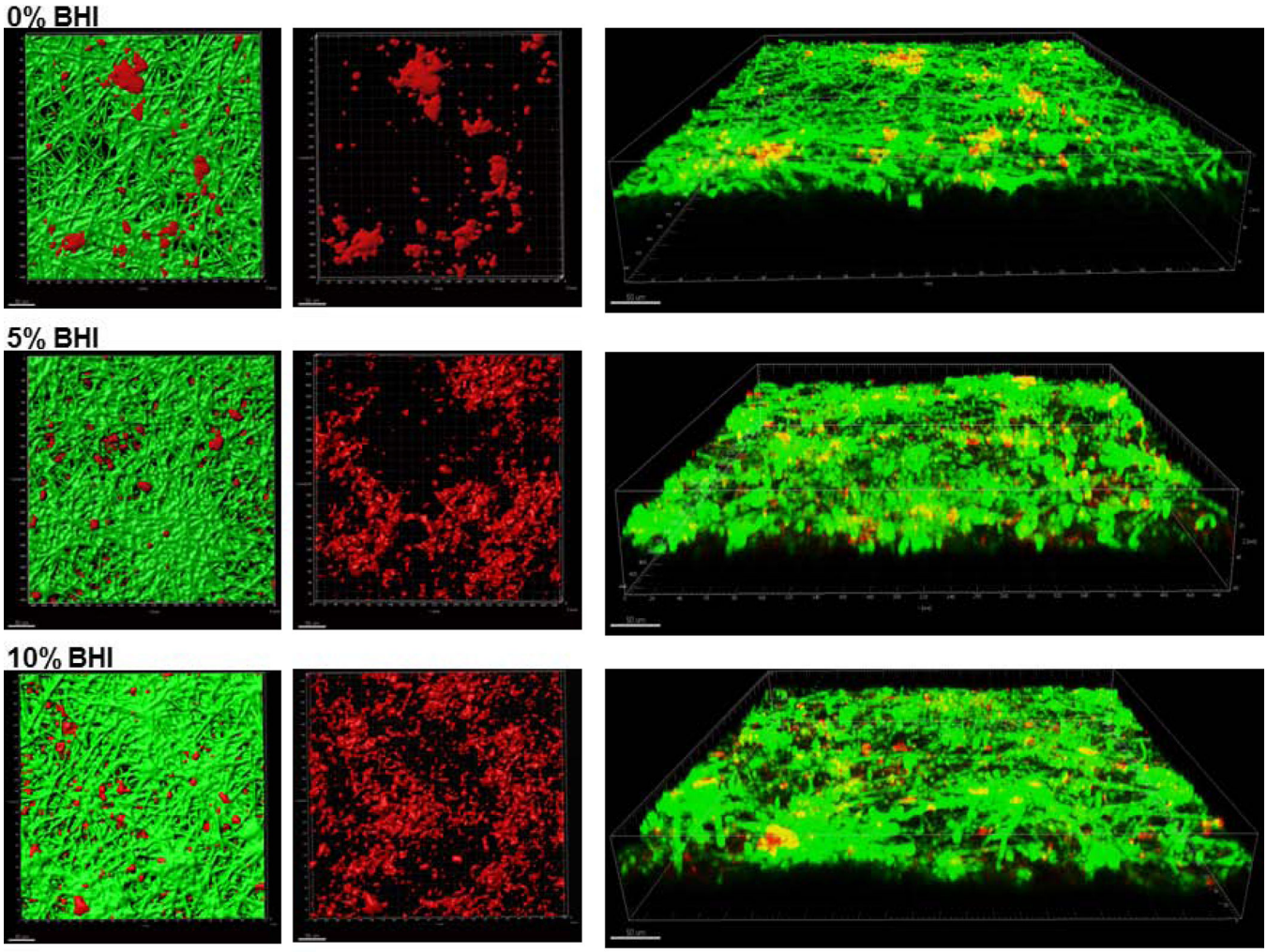


Figure 4.

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.



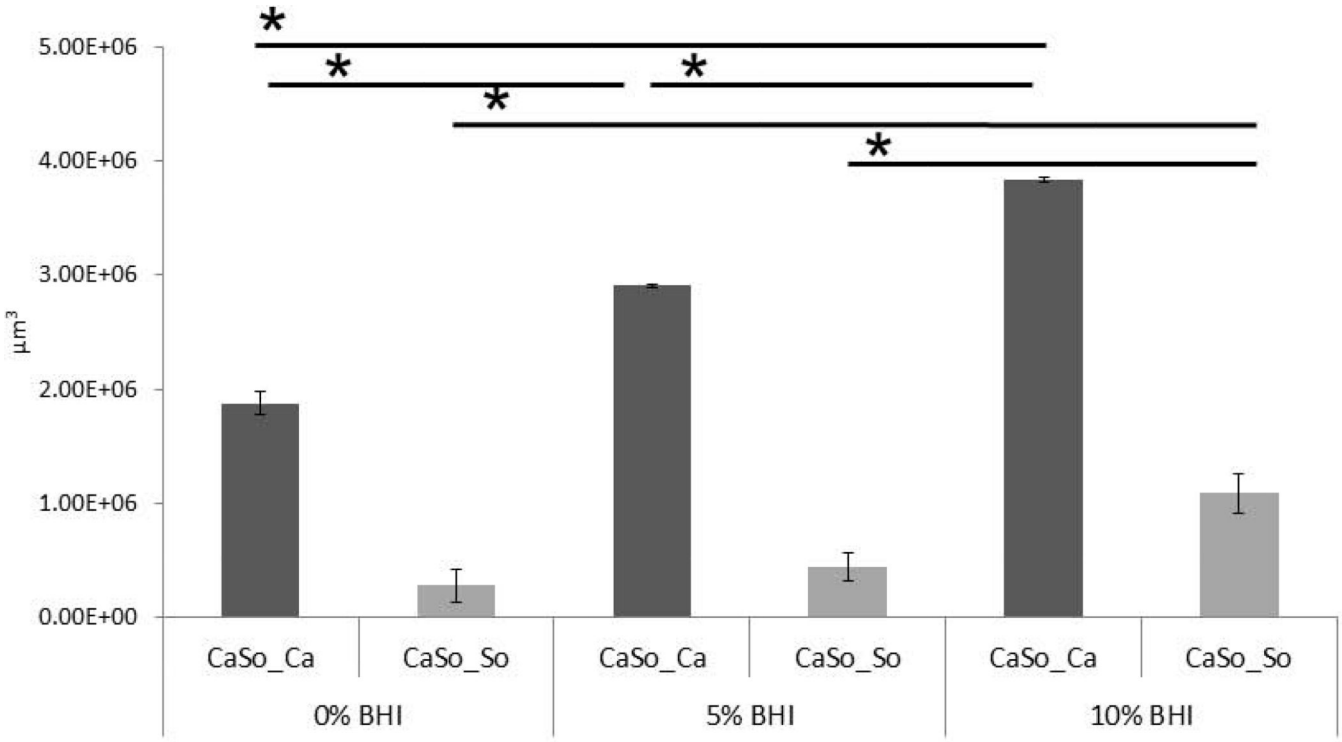


Figure 5.

A: 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.

B: 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.

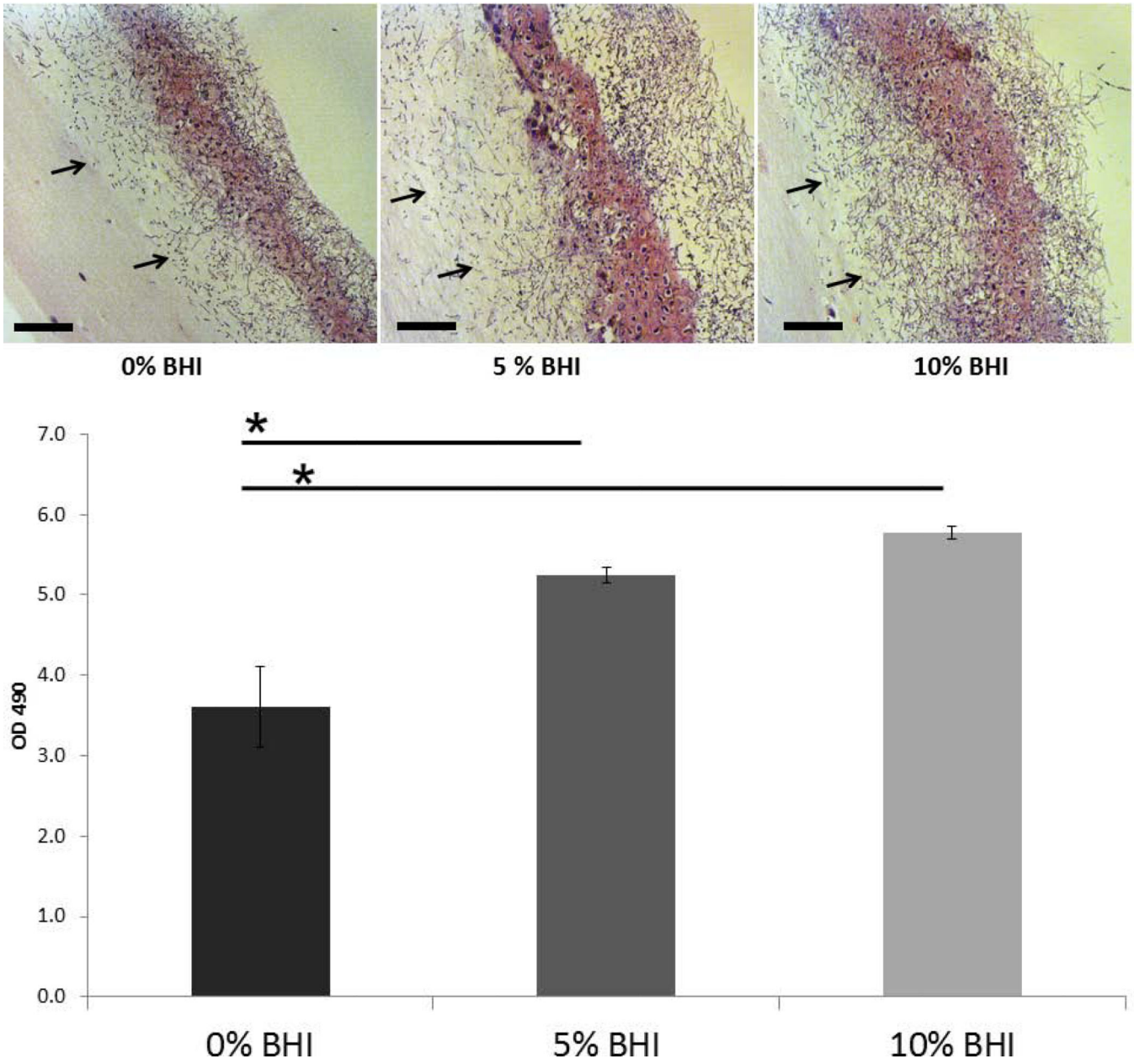


Figure 6.

A: 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.

B: 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.

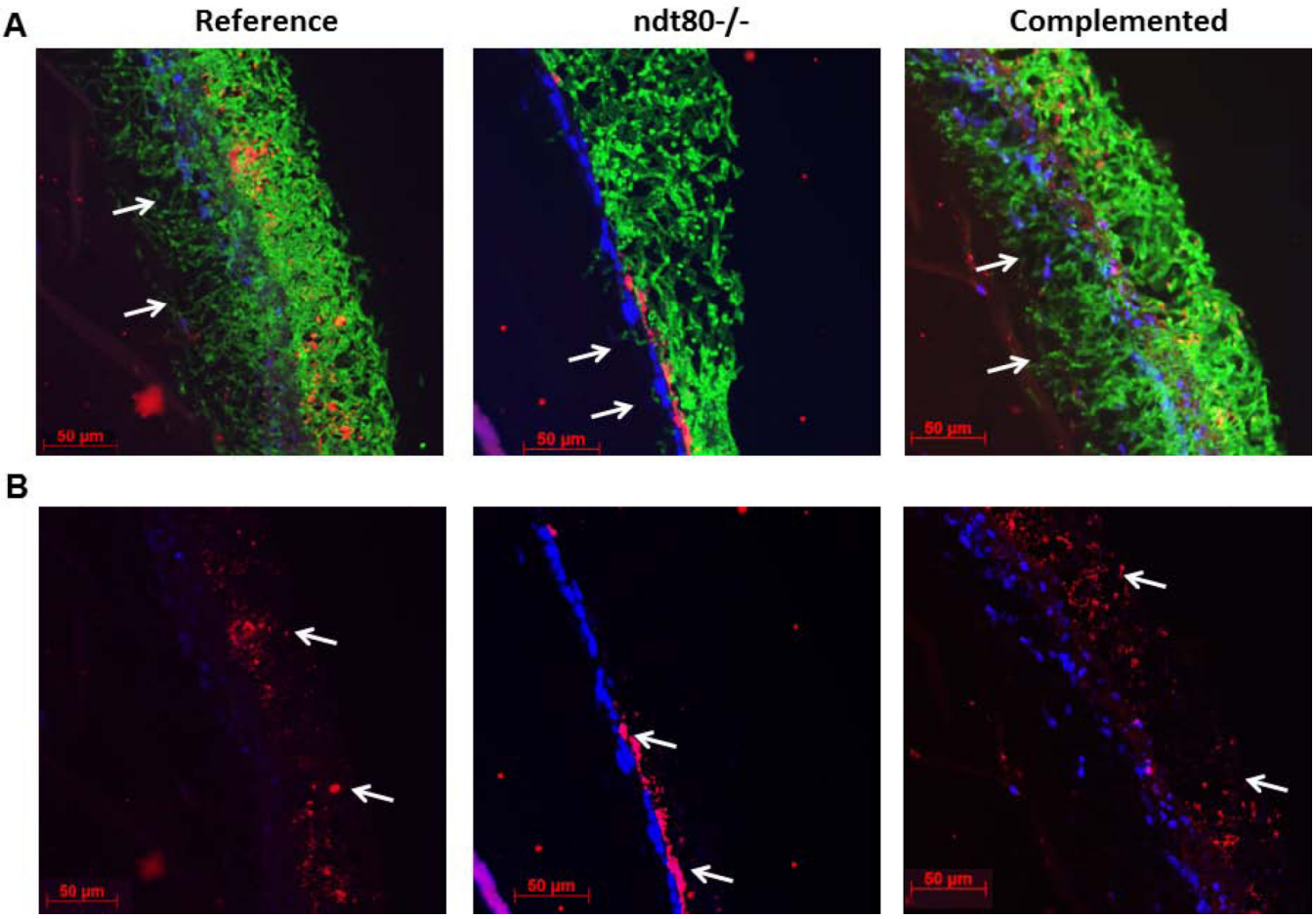


Figure 7.

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.

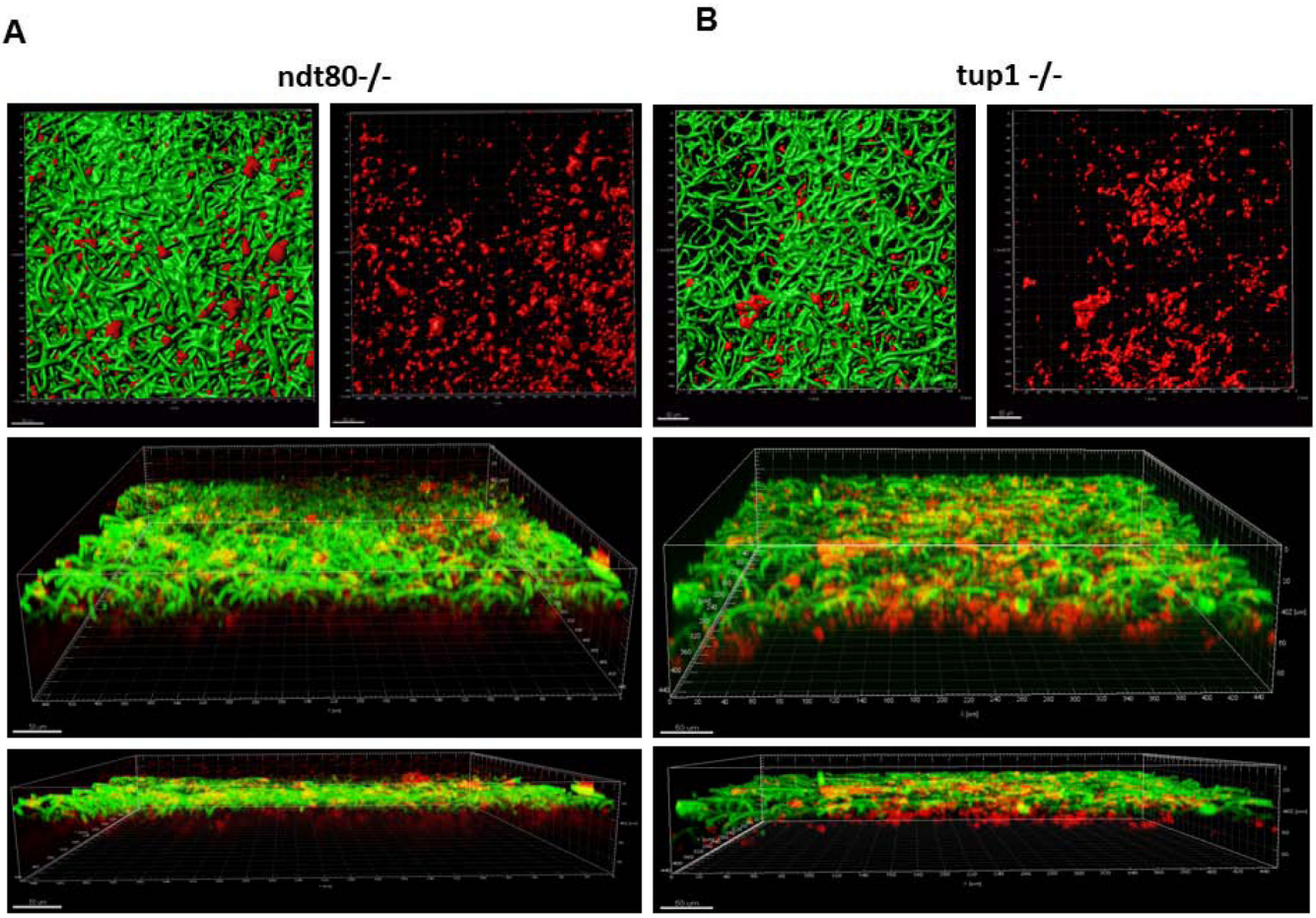
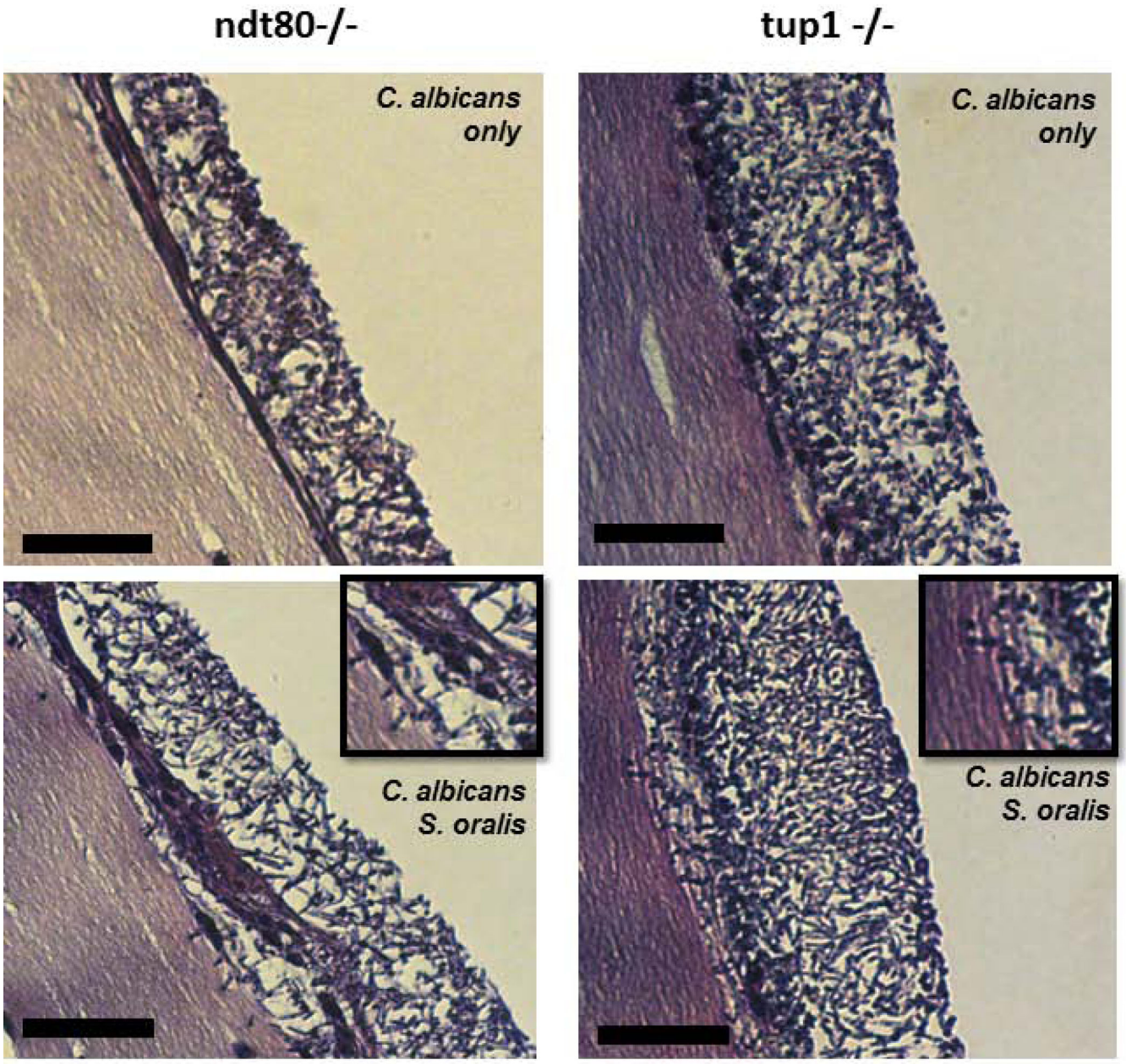


Figure 8.

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.



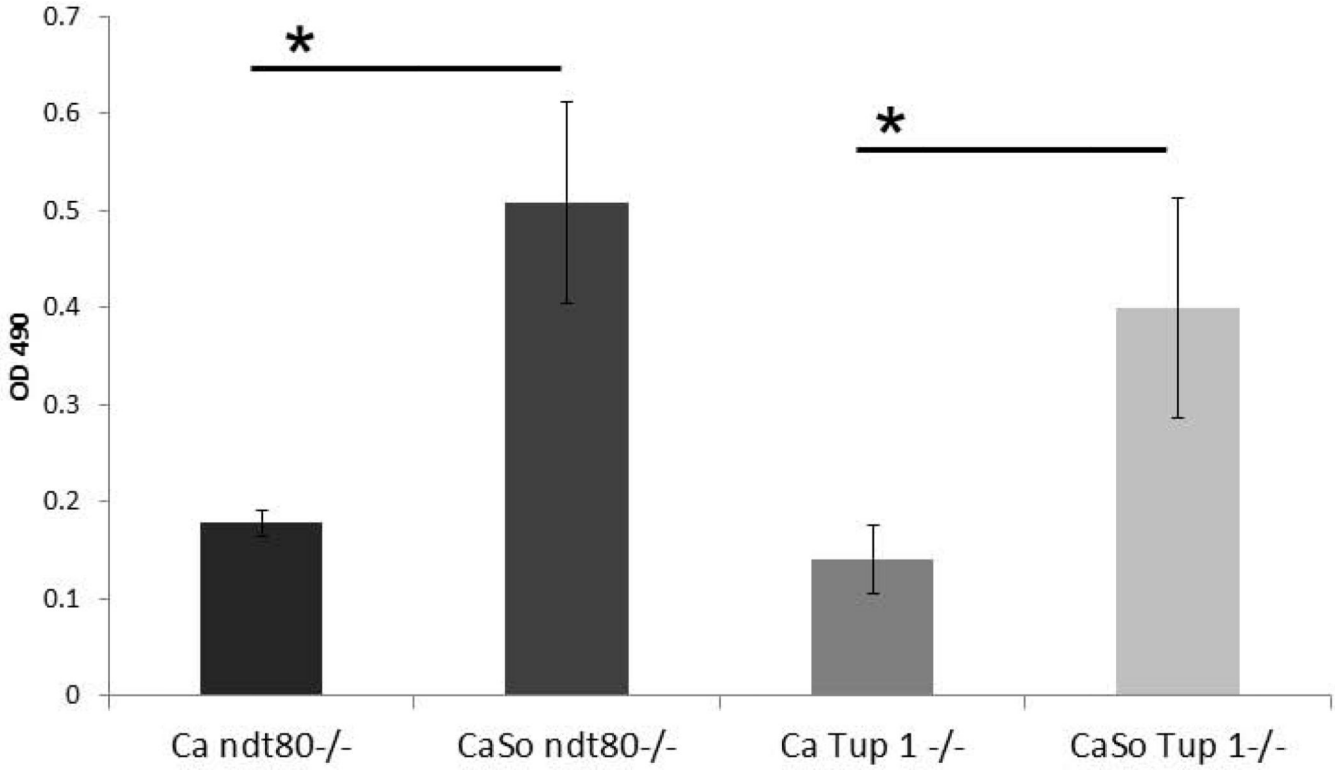


Figure 9.

A: 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.

B: 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.

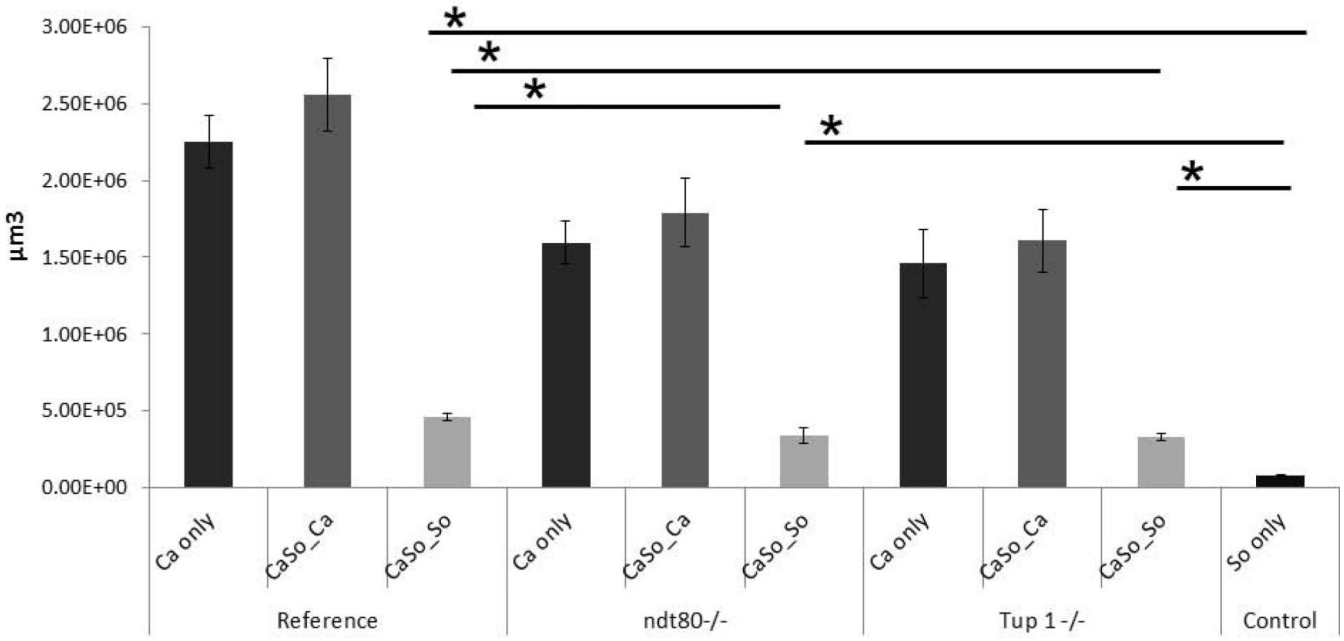


Figure 10.

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.

Table 1

Candida biofilm growth conditions	Biofilm architecture	Dual biofilm biovolume	Tissue invasion and damage
Wet	Long intertwined hyphae forming a homogeneous surface biofilm	+ + +	+
Wet, plus S. oralis	S. oralis formed a biofilm mat adjacent to the mucosa under C. albicans	+ + + +	++
Semi-dry	Localized foci of dense hyphal growth	+ + +	+ + +
Semi-dry, plus S. oralis	S. oralis in close physical proximity with hyphae within foci	+ + +	+ + + +
Semi-dry, plus S. oralis, 5% BHI	S. oralis in close physical proximity with hyphae within foci	+ + + +	+ + + + +
Semi-dry, plus S. oralis, 10% BHI	S. oralis in close physical proximity with hyphae within foci	+ + + + +	+ + + + + +

Table 2

Strain/Hyphal morphotype	Dual biofilm architecture	Dual biofilm biovolume	Tissue invasion and damage
Wild type/hyphae	S. oralis in close proximity to hyphae, interspersed throughout the thickness of the biofilm	+ + + + +	+ + + + + +
ndt80 $^{+/+}$/hyphae	S. oralis in close proximity to hyphae. Interspersed throughout the thickness of the biofilm	+ + + + +	+ + + + + +
ndt80$^{-/-}$/pseudohyphae	Most S. oralis cells localized adjacent to the mucosal surface, some also co-aggregated with pseudohyphae	+ +	*C. albicans* only + *C. albicans*+*S. oralis* + + +
tup1$^{-/-}$/pseudohyphae	Most S. oralis cells localized adjacent to the mucosal surface, some also co-aggregated with pseudohyphae	+ +	*C. albicans* only + *C. albicans*+*S. oralis* + + +