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Virulence and pathogenicity of *Candida albicans* is enhanced in biofilms containing oral bacteria

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This study examined the influence of bacteria on the virulence and pathogenicity of candidal biofilms. Mature biofilms (*Candida albicans*-only, bacteria-only, *C. albicans* with bacteria) were generated on acrylic and either analysed directly, or used to infect a reconstituted human oral epithelium (RHOE). Analyses included *Candida* hyphae enumeration and assessment of *Candida* virulence gene expression. Lactate dehydrogenase (LDH) activity and *Candida* tissue invasion following biofilm infection of the RHOE were also measured. *Candida* hyphae were more prevalent ($p < 0.05$) in acrylic biofilms also containing bacteria, with genes encoding secreted aspartyl-proteinases (SAP4/SAP6) and hyphal-wall protein (HWP1) up-regulated ($p < 0.05$). *Candida* adhesin genes (ALS3/EPA1), SAP6 and HWP1 were up-regulated in mixed-species biofilm infections of RHOE. Multi-species infections exhibited higher hyphal proportions ($p < 0.05$), up-regulation of IL-18, higher LDH activity and tissue invasion. As the presence of bacteria in acrylic biofilms promoted *Candida* virulence, consideration should be given to the bacterial component when managing denture biofilm associated candidoses.

Keywords: biofilm; *Candida albicans*; candidosis; co-infection; RHOE; virulence

Introduction

The fungal genus *Candida* contains over 150 species, several of which are frequent commensal colonisers of humans. Most often, commensal carriage of *Candida* occurs at moist mucosal surfaces, such as those of the oral cavity and vagina (Peleg et al. 2010; Gow et al. 2011). *Candida albicans* is the species most frequently encountered, growing as yeast, pseudohyphal or hyphal forms. In cases where there is host debilitation, or where there is a change in the local environment promoting *Candida* overgrowth, infection (referred to as candidosis) may follow (Peleg et al. 2010; Gow et al. 2011). Candidoses are the most prevalent human fungal infections, and whilst systemic infection can occur, most manifest superficially on the oral and vaginal mucosa (Peleg et al. 2010; Gow et al. 2011).

There are several putative *C. albicans* virulence factors, including secreted aspartyl proteinases (SAPs), phospholipases, expression of surface adhesins and the ability to grow as hyphae (Gow et al. 2011). Hyphal growth protects *C. albicans* from phagocytosis and promotes invasion of host epithelial surfaces. *Candida* species are also adept at growing as biofilms on oral surfaces including those of the oral mucosa and prosthetic biomaterials, and these biofilms are resistant to removal by the action of salivary flow and host defence molecules.

Candida-associated denture stomatitis is the most prevalent form of oral candidosis, occurring in > 65% of denture wearers (Williams et al. 2011). The infection occurs after the formation of biofilms on poorly cleansed, denture acrylic surfaces (Pereira-Cenci et al. 2010; Williams et al. 2011; Williams & Lewis 2011; Teles et al. 2012). Denture stomatitis presents as areas of erythema on the palatal mucosa overlying the denture. *Candida* biofilms serve as reservoirs of infectious agents, largely protected from host removal mechanisms (Williams et al. 2011; Williams & Lewis 2011). This protected environment for denture biofilms arises from poor salivary flow over the upper denture surface and the nature of the non-shedding acrylic material leading to colonising organisms not being removed by the sloughing action, which would occur with epithelial surfaces.

Although most attention in denture stomatitis has been given to the *Candida* component of the biofilm, and particularly to *C. albicans*, bacteria also co-exist with *Candida* within denture biofilms (Morales & Hogan 2010; Peleg et al. 2010; Pereira-Cenci et al. 2010; Teles et al. 2012; Xu et al. 2014). However, the role of bacteria in denture stomatitis remains uncertain and has received little attention. The fact that bacteria will undoubtedly alter the local environment, as well as modulate host immune responses towards *Candida* (Morales

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& Hogan 2010; Peleg et al. 2010), suggests their presence could both directly and indirectly effect progression of denture stomatitis. Evidence exists for bacterial influence in other forms of oral candidosis, most notably in acute erythematous candidosis, where receipt of broad-spectrum antibiotic therapy is a known risk factor (Morales & Hogan 2010; Peleg et al. 2010; Williams & Lewis 2011).

Bacteria could be directly involved in the infection, or could modulate the pathogenicity of *Candida*. Indeed, *Streptococcus gordonii* has recently been found to trigger the virulence attributes of *Candida* (Bamford et al. 2009; Shirtliff et al. 2009; Diaz, Xie, et al. 2012; Ricker et al. 2014), and other streptococci could act similarly (Xu et al. 2014). It is also known that receipt of broad-spectrum antibiotics predisposes to oral candidosis, and whilst this may be due to reduced microbial competition allowing the proliferation of *Candida* (Juárez Tomás et al. 2011; Purschke et al. 2012), it may also relate to changes in immune responses by the host as a result of a reduced bacterial load.

Candida–host interactions have been investigated using different model systems (Green et al. 2004; Negri et al. 2011; Xu et al. 2014). A commercially available reconstituted human oral epithelium (RHOE; SkinEthic Laboratories, Nice, France) represents a reproducible and standardised tissue suitable for modelling the interaction between *Candida* and epithelial cells (Silva et al. 2011; Yadev et al. 2011). However, the RHOE has not previously been used to model mature biofilm interactions.

The aim of this study was to examine the effect of a bacterial component on the virulence and pathogenicity of *Candida* biofilms. In addition, the study assessed epithelial cell responses to the different biofilm compositions. The intention was to gain insight into the importance of bacteria within *Candida* biofilm-mediated infections, such as denture stomatitis.

Materials and methods

Microorganisms and culture preparation

C. albicans ATCC 90028, *Streptococcus mutans* ATCC 25175, *Streptococcus sanguinis* ATCC 10556, *Actinomyces viscosus* ATCC 15987 and *Actinomyces odontolyticus* NCTC 9935 were used to develop biofilms on acrylic coupons. *C. albicans* was maintained on Sabouraud's Dextrose Agar (SDA; Oxoid, Basingstoke, UK) and bacterial species were cultured on Blood Agar (BA) (blood agar base; Oxoid) supplemented with 5% (v/v) defibrinated horse blood (TCS Biosciences, Buckingham, UK). Isolates were subcultured in Brain Heart Infusion liquid medium (BHI; Oxoid) for 24 h at 37°C. *C. albicans*, *S. mutans* and *S. sanguinis* were cultured aerobically, whilst *A. viscosus* and *A. odontolyticus* were cultured anaerobically. Microbial cells were harvested by centrifugation (3,000 × *g* for 5 min) and the resulting cell pellets washed (× 2) in phosphate buffered saline (PBS; pH 7.0). Cell pellets were resuspended in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Paisley, UK) supplemented with 10% (v/v) Foetal Bovine Serum (FBS; Life Technologies) and 50 mM glucose. The cell density was adjusted to an optical density of 1.0 at 600 nm using a spectrophotometer (DiluPhotometer™; Implen, Westlake Village, CA, USA). To produce inocula for biofilms on acrylic coupons, a 200-fold dilution of each standardised suspension was prepared in sterile culture medium (DMEM supplemented with 10% (v/v) FBS). The concentration of microorganisms in each inoculum corresponded to ~1×10^5 colony forming units (CFU) ml^{-1} of *C. albicans* and ~1×10^7 CFU ml^{-1} of the bacteria.

Preparation of acrylic coupons for biofilm development

Heat-polymerised acrylic resin (QC-20; Dentsply Int, Inc., Weybridge, UK) was prepared according to the manufacturer's instructions and fabricated into disc-shaped (10 mm diameter, 2 mm thickness) coupons. Acrylic coupons were finished using a horizontal polisher (model APL-4; Arotec, São Paulo, Brazil) with progressively finer aluminium oxide papers (320-, 400-, and 600-grit). The surface roughness was standardised to 0.30 ± 0.02 µm. Prior to use, acrylic coupons were ultrasonically cleaned with 70% (v/v) alcohol and sterile ultra-purified water (20 min) to remove surface debris. The coupons were immersed in sodium hypochlorite solution (2 g l^{-1}) for 5 min and then thoroughly rinsed with sterile water. Coupons were maintained in sterile distilled water until use. Acrylic coupons were then immersed in artificial saliva (2.5 g l^{-1} mucin, 0.25 g l^{-1} sodium chloride, 0.2 g l^{-1} potassium chloride, 0.2 g l^{-1} calcium chloride, 2.0 g l^{-1} yeast extract, 5.0 g l^{-1} protease peptone and 1.25 ml l^{-1} of 40% urea) overnight at 37°C under gentle agitation (75 rpm).

Development of in vitro biofilms on acrylic coupons

The biofilms generated consisted of *C. albicans* (single species biofilms), *C. albicans* with the four different bacterial species (mixed-species biofilms), and bacteria-only biofilms. To produce these biofilms, acrylic coupons were placed in the wells of a 24-well plate and overlaid with 2 ml of microbial inoculum. After an initial adherence period of 2 h, the culture medium was removed and 2 ml of fresh culture medium (DMEM supplemented with 10% (v/v) FBS and 50 mM glucose) added to each well. The coupons were then incubated aerobically for 72 h at 37°C in an orbital shaking incubator (75 rpm). The culture medium was changed every 24 h to maintain biofilm growth. After 72 h, acrylic biofilms were collected for

analysis, or used to overlay specimens of RHOE (SkinEthic Laboratories, Nice, France). Analysis of biofilms on acrylic coupons (n = 12) was performed in three independent experiments, using four samples per group on each occasion.

Biofilm infection of reconstituted human oral epithelium (RHOE)

Commercially available RHOE was used in combination with the biofilms on acrylic coupons to produce an *in vitro* model of denture stomatitis. The RHOE provided the contact epithelium for previously developed single (*C. albicans*), mixed-species (*C. albicans* and bacteria) and bacteria-only biofilms on acrylic coupons. Acrylic coupons without biofilm served as non-infected controls.

Acrylic coupons, with or without biofilm, were overlaid on the surface of RHOE (n = 6) and incubated for 12 h at 37°C, in a 5% (v/v) CO_2 atmosphere, under saturated humidity. After incubation, the acrylic was removed and the tissues bisected. Total RNA was extracted from one half of the tissue, with the remainder processed for histological analysis. Culture medium (Maintenance Medium without antibiotics, SkinEthic Laboratories) was also retained and used to measure lactate dehydrogenase (LDH) activity originating from the RHOE. Analysis of RHOE (n = 6) was performed on two independent occasions, using three samples per group.

Analysis of acrylic biofilms

Prior to undertaking experiments with RHOE, initial studies directly examined biofilms developed on the acrylic coupons. Biofilms generated over 72 h were recovered from acrylic surfaces (n = 12) by vigorous vortex mixing for 60 s. Biofilm cells were pelleted by centrifugation and then resuspended in 1 ml of PBS. Serial decimal dilutions of the suspensions were cultured on SDA and BA using a drop-counting technique. SDA and BA plates were incubated aerobically at 37°C for 24 h and 48 h, respectively, and the resulting CFU ml^{-1} determined.

An 800-µl volume of the biofilm suspension was also centrifuged and the cell pellet resuspended in RNALater (Life Technologies) and stored at –20°C prior to RNA extraction and qPCR analysis.

In situ biofilms on acrylic coupons (n = 6 for each group, analysed on two independent occasions) were also examined by confocal laser-scanning microscopy (CLSM), using a Leica TCS SP2 AOBS spectral confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). CLSM was used to assess the quantity and viability of biofilms, as well as the proportion of *C. albicans* hyphae. Biofilms were stained with 10 µl of SYTO-9 (25 µM; Molecular Probes, Paisley, UK) to assess cell viability, and 10 µl of concanavalin-A conjugated with Alexa Fluor® 594 (25 µM; Molecular Probes) to detect *Candida*.

Representative images (125 µm × 125 µm) of both dye-channels, SYTO-9 (green) and concanavalin-A (red), were obtained from a minimum of five different fields of view for each of three replicate specimens in two independent experiments. Images were analysed using ImageJ 1.46r (Wayne Rasband, National Institute of Health, Bethesda, MD, USA). The red channel, representative of *C. albicans*, was used to quantify yeast and hyphae. The green channel was used to ascertain cell viability within the biofilms. Images were first adjusted using the function of threshold intensity and *C. albicans* yeast and hyphal forms separately quantified using the 'Analyze Particles' tool of the software.

RNA extraction and synthesis of cDNA for qPCR

RNA was extracted from both biofilms on acrylic coupons (n = 12) and the RHOE (n = 6) that had been overlaid with biofilms and controls. Extraction of total RNA involved initial resuspended cells from biofilms and/or RHOE in lysis buffer (RLT Buffer, QIAGEN, Crawley, UK) containing 1% (v/v) β-mercaptoethanol. Cells were disrupted by high-speed homogenisation with glass beads in a Mini-Bead-Beater-8 (Stratech Scientific, Soham, UK). Total nucleic acid was obtained after separation with phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich, Poole, UK) and total RNA was recovered after DNAse I (QIAGEN) treatment using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. The integrity and purity of the total RNA was assessed by gel electrophoresis, and the total RNA concentration was spectrophotometrically determined by measuring absorbance ratio at $260/280_{nm}$ (NanoVue, GE Healthcare, Little Chalfont, UK) and standardised to 200 ng ml^{-1}. Reverse transcription reactions for cDNA synthesis included 5 µl of total RNA (200 ng $µl^{-1}$) template, 1 µl of 10 mM dNTPs, 1 µl of 50 µg ml^{-1} random primers, 1 µl of 25 U $µl^{-1}$ RNasin RNase inhibitor, 5 µl of M-MLV reaction buffer (× 5) and 1 µl of 200 U $µl^{-1}$ M-MLV (Promega, Southampton, UK). Molecular grade water was added to give a final reaction volume of 25 µl. The reaction mix was incubated at 70°C for 5 min, followed by incubation at 37°C for 60 min. The resulting cDNA was stored at –20°C prior to use for qPCR.

qPCR analysis of acrylic biofilms and infected RHOE

Primers used in qPCR analysis are presented in Table 1 and designed from full-length gene sequences obtained from the nucleotide platform in PubMed using Primer3 software (Koressaar & Remm 2007; Untergasser et al.

Table 1. Forward (FW) and reverse (RV) primers used for qPCR.

Target gene	Sequence (5′ → 3′)
ACT1 – *C. albicans* actin housekeeping gene (*C. albicans*)	FW – TGCTGAACGTATGCAAAAGG RV – TGAACAATGGATGGACCAGA
ALS1 – Human β-actin agglutinin-like sequence (*C. albicans*)	FW – CCCAACTTGGAATGCTGTTT RV – TTTCAAAGCGTCGTTCACAG
ALS3 – Agglutinin-like sequence (*C. albicans*)	FW – CTGGACCACCAGGAAACACT RV – GGTGGAGCGGTGACAGTAGT
EPA1 – Epithelial adhesin (*C. albicans*)	FW – ATGTGGCTCTGGGTTTTACG RV – TGGTCCGTATGGGCTAGGTA
SAP4 – Secreted aspartyl proteinase (*C. albicans*)	FW – GTCAATGTCAACGCTGGTGTCC RV – ATTCCGAAGCAGGAACGGTGTCC
SAP6 – Secreted aspartyl proteinase (*C. albicans*)	FW – AAAATGGCGTGGTGACAGAGGT RV – CGTTGGCTTGGAAACCAATACC
HWP1 – Hyphal wall protein (*C. albicans*)	FW – TCTACTGCTCCAGCCACTGA RV – CCAGCAGGAATTGTTTCCAT
PLD1 – Phospholipase D (*C. albicans*)	FW – GCCAAGAGAGCAAGGGTTAGCA RV – CGGATTCGTCATCCATTTCTCC
β-actin – Housekeeping gene (human cells)	FW – GAGCACAGAGCCTCGCCTTTGCCGAT RV – ATCCTTCTGACCCATGCCCACCATCACG
IL-18 – Interleukin 18 (human cells)	FW – CCTTCCAGATCGCTTCCTCTCGCAACAA RV – CAAGCTTGCCAAAGTAATCTGATTCCAGGT
Dectin1 – Cellular membrane receptor (human cells)	FW – ACAGCAATGAGGCGCCAAGGAGGAGATG RV – GGAGCAGAAAGAAAAGAGCTCCCAAATGCT

2012). The specificity of each primer was confirmed using primer-BLAST (Ye et al. 2012), which compared the respective sequences with databases of *C. albicans* and human genomes. In addition, primer specificity for qPCR was confirmed in preliminary studies involving extracted genomic DNA. The targeted putative virulence genes of *C. albicans* were ALS1 and ALS3 (agglutinin-like sequence), EPA1 (epithelial adhesin), SAP4 and SAP6 (secreted aspartyl proteinases), HWP1 (hyphae wall protein) and PLD1 (phospholipase D). In the case of RHOE, expression of genes encoding IL-18 (an interleukin) and Dectin-1 (a *Candida* receptor) was determined. ACT1 and β-actin served as reference genes for *C. albicans* and human cells, respectively.

Triplicate qPCRs were performed in 96-well plates in an ABI Prism 7000 instrument (Life Technologies). Each 20-µl reaction comprised 2 µl of cDNA, 10 µl (×2) of SYBR-Green PCR Master Mix (Precision Master Mix; Primer Design, Southampton, UK), 1 µl of each primer (10 mM), and 6 µl of molecular biology grade water. The thermal cycle profile comprised of initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s, primer annealing at 58°C for 30 s and primer extension at 72°C for 30 s. A final extension at 72°C for 2 min was performed, followed by cooling at 4°C. A dissociation stage at 60°C was used to generate a melting curve for verification of the amplified product.

After qPCR, the threshold was adjusted according to the amplification curves of all evaluated genes. Comparison between groups was made based on the cycle number at which both the target and reference genes reached threshold cycle (Ct) fluorescence. Analysis of relative gene expression was achieved according to the $^{\Delta\Delta}$Ct method (Bustin et al. 2009).

Lactate dehydrogenase (LDH) activity in the culture medium of infected RHOE

LDH activity was measured in RHOE culture medium (n = 6, for each group) using an LDH Cytotoxicity Assay kit as recommended by the manufacturer (Thermo Fisher Scientific, Cramlington, UK). Experiments were performed in triplicate, with control samples for normalisation purposes. The LDH activity of RHOE infected with biofilms was compared to uninfected controls.

Histological assessment of damage and candidal invasion of infected RHOE

RHOE damage and invasion of the epithelium by *C. albicans* was determined by light microscopy and CLSM. RHOE for histological analysis was initially fixed in 10% (v/v) neutral buffered formalin (Leica Biosystems, Newcastle-upon-Tyne, UK) for 48 h. Tissues were dehydrated through an increasing alcohol concentration series and then infiltrated with paraffin wax. Sections (20 µm) were placed on microscope slides and de-waxed in xylene, with subsequent rehydration using water. Selected sections for light microscopy were stained using the Periodic Acid-Schiff (PAS) technique.

For fluorescence *in situ* hybridisation (FISH) and CLSM analysis, sections were treated with 10 mg ml^{-1} lysozyme (30 min, 37°C) and incubated in 1 × citrate buffer (pH 6.0, 30 min, 55°C; Sigma-Aldrich). Sections were then exposed to fluorescent probes specific either for *C. albicans*, bacteria or epithelial cells at 55°C for 60 min. *Candida* was detected using the Yeast Traffic Light® PNA FISH® kit (AdvanDx, Vedbaek, Denmark) in combination with concanavalin-A conjugated with Alexa Fluor® 594 (1 µg $µl^{-1}$; Life Technologies) (Malic et al. 2007). Bacteria were detected using the universal bacterial Cy3 labelled peptide nucleic acid (PNA) probe (Bac-Uni1CY3; 300 nM; probe sequence: CTGCCTCCCGTAGGA) specific for bacterial 16S rRNA (Malic et al. 2009). Epithelial cells were treated with 20 µg ml^{-1} of pan-cytokeratin antibody (C11) (sc8018, Santa Cruz Biotechnology, Heidelberg, Germany) followed by an Alexa Fluor® 488-labelled goat anti-Mouse IgG (H+L) antibody (5 µg $µl^{-1}$; Life Technologies) plus a nucleic acid dye (Hoechst 33342; 1 µg $µl^{-1}$) (trihydrochloride-trihydrate; Life Technologies) (Malic et al. 2007). Tissue sections were mounted using Vectashield (H-1000, Vector Laboratories, Peterborough, UK) fade-retarding mount. The CLSM parameters were as described earlier.

Statistical analysis

Statistical analysis was undertaken using Sigma Plot v. 11.3 (Systat Software Inc., London, UK) at 5% significance. The assumptions of equality of variances and normal distribution of errors were evaluated for each variable. When data were not normally distributed, they were logarithmically transformed. Comparison between parameters (viable cells number, hyphae enumeration and gene expression) for single and mixed-species biofilms, as well as candidal gene expression was by independent *t*-test ($\alpha < .05$). LDH activity and epithelial cell gene expression were analysed by one-way ANOVA. *Post hoc* comparisons of one-way ANOVA tests were performed using Tukey's Honestly Significant Difference (HSD) test.

Results

Analysis of biofilms on acrylic coupons and infected RHOE

Both *C. albicans*-only and mixed-species biofilms extensively colonised acrylic surfaces after 72 h growth (Figure 1). The number of viable *C. albicans* in single-species biofilms (3.7×10^7 CFU ml^{-1}) was not statistically different ($p > 0.05$) from those in mixed-species biofilms, where *C. albicans* appeared as the predominant organism (1.6×10^8 CFU ml^{-1}).

CLSM (Figures 2 and 3) confirmed the findings of culture and interestingly revealed higher proportions of hyphae in mixed-species acrylic biofilms compared with *C. albicans*-only biofilms. Significantly ($p < 0.01$) higher hyphal proportions (Figure 3) were seen in both mixed species biofilms colonising the acrylic (5.6%) and infecting RHOE (13.4%). Hyphal elements in the mixed-species biofilms also appeared more developed than those in *C. albicans*-only biofilms (Figure 2).

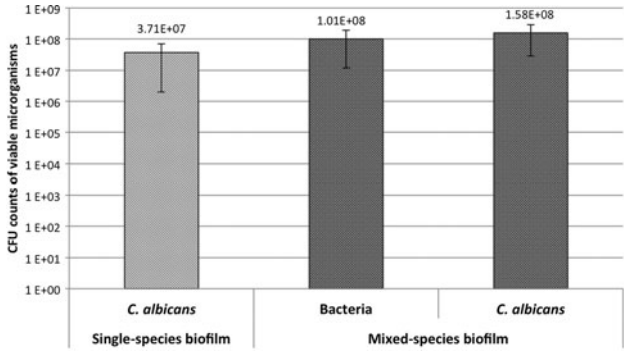

Figure 1. CFU ml^{-1} in single-species (*C. albicans* only, $n = 12$) and mixed-species (*S. mutans*, *S. sanguinis*, *A. viscosus*, *A. odontolyticus* and *C. albicans*, $n = 12$) biofilms. Bars show averages and SDs within each group.

Gene expression of *C. albicans* adhesins differed between biofilms developed on acrylic and infected RHOE (Figure 4A), with higher expression of both ALS1 and ALS3 evident in *C. albicans*-only biofilms on acrylic ($p < 0.05$). Interestingly, significantly ($p < 0.01$) higher expression of *C. albicans* ALS3 and EPA1 genes occurred in RHOE infected by mixed-species biofilms compared with RHOE infected by *C. albicans*-only biofilms.

Similarly, expression of *C. albicans* SAP4 and SAP6 genes (Figure 4B) was significantly ($p < 0.05$) higher in mixed-species biofilms on acrylic compared with *C. albicans*-only biofilms. For infected RHOE, expression of SAP4 was similar for *C. albicans*-only biofilms and mixed-species biofilms, whilst SAP6 expression was significantly ($p < 0.05$) up-regulated in the latter.

Expression of *C. albicans* PLD1 (Figure 4C) was equivalent for all biofilm types. However, expression of the *C. albicans* hyphal wall protein gene (HWP1) (Figure 4C) was significantly ($p < 0.01$) higher in mixed-species biofilms on acrylic and RHOE mixed infections, compared with *C. albicans*-only biofilms and associated infections.

With regards to the responses of RHOE to biofilm infection (Figure 4D), expression of human interleukin-18 (IL-18) and Dectin-1 (a cell receptor for *C. albicans* primarily associated with immune cells) were analysed. Expression of IL-18 was up-regulated for RHOE infected by mixed-species biofilms, relative to other biofilms types ($p < 0.05$). Dectin-1 expression was equivalent for RHOE infected by mixed species and *C. albicans*-only biofilms ($p < 0.05$).

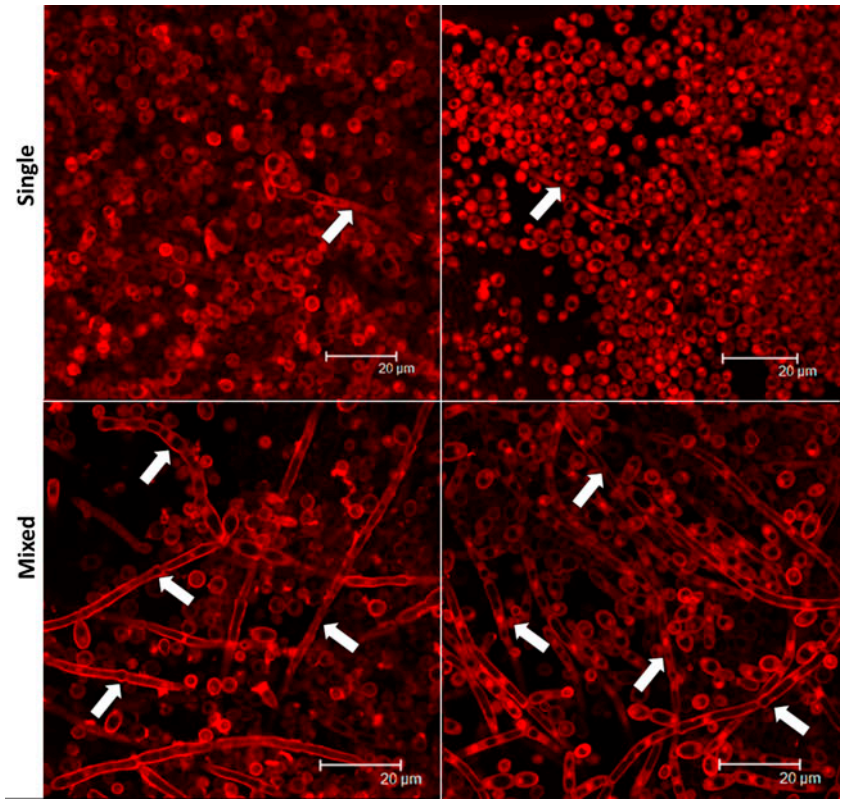

Figure 2. Representative CLSM images of single-species (*C. albicans*-only) biofilms (upper) and mixed-species (*S. mutans*, *S. sanguinis*, *A. viscosus*, *A. odontolyticus* and *C. albicans*) biofilms (lower) on an acrylic surface. *C. albicans* was stained with concanavalin-A conjugated with Alexa Fluor® 594. The white arrows highlight the presence of hyphae in both biofilm types. Note that hyphae in mixed-species biofilms appear more developed than those in single-species biofilms.

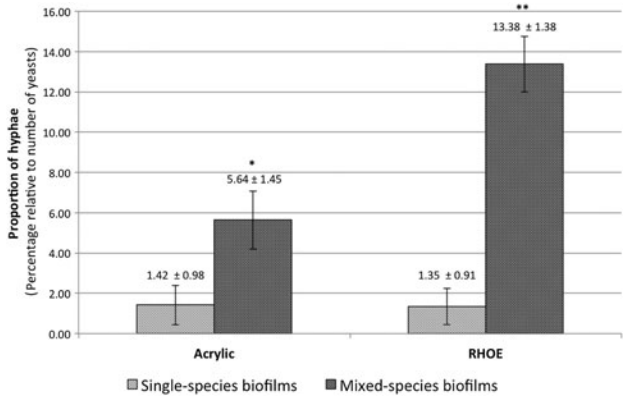

Figure 3. Proportion (%) of hyphae relative to the total number of *C. albicans* yeast in single and mixed-species biofilms (n = 6, per group) on acrylic and in infected RHOE. Bars show averages and SDs within each group. *Shows a statistically higher proportion of hyphae relative to single-species biofilms. **Shows a statistically higher proportion of hyphae relative to single-species biofilms and to mixed-species biofilm developed on acrylic.

Analysis of RHOE damage based on LDH activity (Figure 5), revealed that both *C. albicans*-only, and mixed-species biofilms resulted in significantly ($p < 0.05$) higher tissue damage compared with uninfected and bacteria-only biofilms. The tissue damage caused by mixed-species biofilms was significantly ($p < 0.01$) higher than for all other infection types.

The increased damage caused by mixed-species biofilms was also histologically confirmed using light microscopy (Figure 6) and CLSM analysis (Figure 7). Microscopy revealed higher numbers of *C. albicans* hyphae (proportion of *C. albicans* in hyphal form: 13.4%) in mixed-species biofilm infections, which extensively invaded the epithelial tissue. Although *C. albicans*-only biofilms demonstrated lower invasion and reduced proportions of hyphae compared to mixed-species biofilms (Figures 6 and 7), the damage observed for this group was significantly higher than that for the bacteria-only infection and uninfected controls. It was apparent that bacteria-only infections produced limited tissue damage compared to uninfected controls.

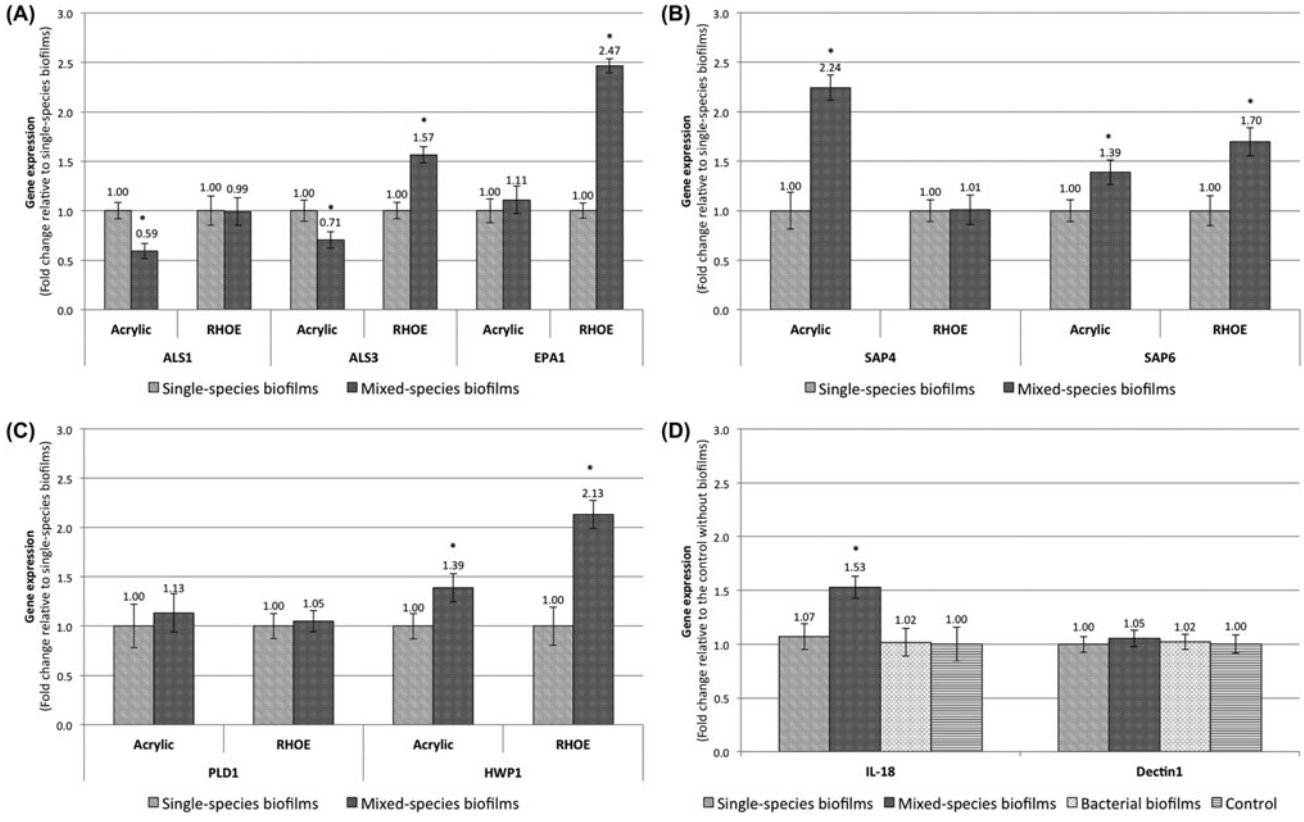

Figure 4. Relative gene expression in single and mixed-species biofilms on acrylic (n = 12), or in biofilms infecting RHOE (n = 6) of (A) *C. albicans* adhesins (ALS1, ALS3 and EPA1); (B) *C. albicans* secreted aspartyl proteinases (SAP4 and SAP6); and (C) *C. albicans* phospholipase D (PLD1) and hyphal wall protein (HWP1). (D) Relative gene expression of human interleukin 18 (IL-18) and of a human epithelial cell receptor for *C. albicans* (Dectin-1), in RHOE samples (n = 6) infected with single-*Candida* biofilm, mixed-species biofilm, bacterial biofilm, or control (without infection). *C. albicans* target genes were normalised using the *C. albicans* housekeeping gene (ACT1). Human target genes were normalised using a human housekeeping gene (β-actin). Analysis of quantitative RT-PCR was made by the $^{\Delta\Delta}$Ct method. Single-species biofilms were used as reference samples for expression *C. albicans* genes. Control RHOE samples (uninfected) were used as reference samples for expression of human genes. Bars show averages and SDs within each group. *Statistically different gene expression relative to controls (single-species biofilms – for analysis of *Candida* genes expression; uninfected samples – for analysis of human genes expression).

Discussion

Candida–bacteria interaction will invariably occur when associated together in oral biofilms. Mutualistic interactions between *C. albicans* and streptococci have previously been reported (Diaz, Xie, et al. 2012; Diaz, Strausbaugh, et al. 2014; Ricker et al. 2014; Xu et al. 2014) and were confirmed in the present study. Other studies have shown antagonistic interactions between *C. albicans* and other bacteria, such as *Lactobacillus* (Juárez Tomás et al. 2011) and *Pseudomonas aeruginosa* (Purschke et al. 2012; Méar et al. 2013). Such antagonism could occur due to bacterial-derived and quorum sensing regulated molecules such as pyocyanin and hydrogen peroxide (Hogan et al. 2004; Morales & Hogan 2010; Tian et al. 2013). To date, there have been limited studies examining the role oral bacteria play in directly influencing *C. albicans* virulence.

The aim of the present study was to compare *C. albicans* virulence attributes in biofilms on acrylic containing *C. albicans*-only, or biofilms also including selected oral bacteria. *Streptococcus* and *Actinomyces* species were the selected bacteria, as these are known primary colonisers of the oral cavity and are present in denture biofilms (Pereira-Cenci et al. 2010; Teles et al. 2012). In addition to examining biofilms on acrylic, the pathogenicity of these biofilms was compared by interfacing them with the surface of RHOE.

Importantly, the results show that *C. albicans* hyphal proliferation and expression of several putative candidal virulence genes significantly increased in the presence of bacteria. Associated with this, was enhanced RHOE damage and tissue invasion by *C. albicans* hyphae in mixed species biofilm infections, along with increased expression of the IL-18 gene (encoding a pro-inflammatory cytokine).

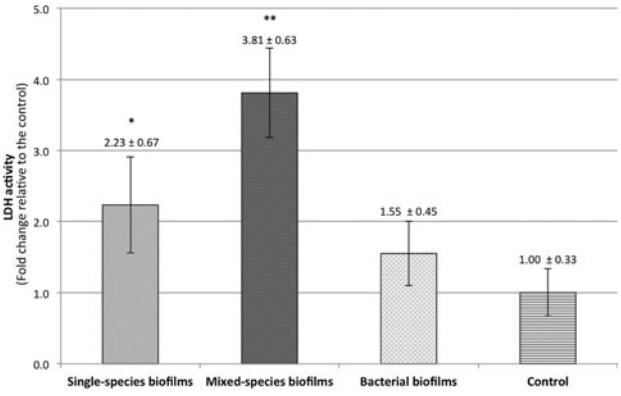

Figure 5. Relative LDH activity measured in the culture medium supernatant of RHOE tissues (n = 6, for each group) after 12 h incubation of samples infected with single-species *Candida* biofilm, mixed-species biofilm, bacteria only biofilm, or control (devoid of infection). Basal culture medium was used to 'blank' samples and the fold change calculations were based on a control group. Bars show averages and SDs within each group. *Statistically higher LDH activity relative to control and to bacterial biofilm. **Statistically higher LDH activity relative to control, single-species (*C. albicans*) and bacterial biofilms.

Enhanced *C. albicans* hyphal development in biofilms with bacteria and subsequent significant tissue invasion in RHOE could arise from direct interaction between bacteria and *C. albicans*. Indeed, it is known that streptococci interact with the cell-wall proteins of *Candida* (Bamford et al. 2009; Diaz, Xie, et al. 2012; Ricker et al. 2014) and inhibit farnesol-mediated hyphal suppression (Morales & Hogan 2010; Gow et al. 2011). Furthermore, bacterial metabolites (Purschke et al. 2012; Ricker et al. 2014) may influence biochemical pathways involved in candidal and hyphal transition. Some secreted bacterial quorum sensing molecules (eg competence-stimulating peptide secreted by *S. mutans*, and autoinducer-2 secreted by *Aggregatibacter actinomycetemcomitans*) have been reported to inhibit hyphal formation (Jarosz et al. 2009; Bachtiar et al. 2014).

Furthermore, it is likely that bacteria in mixed species biofilms affect the local environment by changing parameters such as nutrient or carbon dioxide levels, which are factors known to be involved in *C. albicans* hyphal transition and virulence (Morales & Hogan 2010; Gow et al. 2011; Lu et al. 2013; Buu & Chen 2014). Environmental pH can influence yeast–hyphal transition (Bensen et al. 2004; Gow et al. 2011) and co-cultivation of *C. albicans* with streptococci would lower the environmental pH, possibly inactivating the *C. albicans* transcription factor (Rim101 pathway) involved in hyphal morphogenesis (Bensen et al. 2004; Gow et al. 2011). Excessive environmental changes would, however, have been partially mitigated through daily medium replacement.

Hyphal growth of *C. albicans* is generally regarded as more pathogenic than yeast (Dalle et al. 2010; Martin et al. 2011; Kuo et al. 2013), with the former able to migrate and invade tissues (Gow et al. 2011; Diaz, Xie, et al. 2012). Perhaps unsurprisingly, associated with increased hyphal development was the concurrent up-regulation of HWP1, along with SAP4 and SAP6, which are genes implicated in *C. albicans* hyphal development (Jayatilake et al. 2006; Nailis et al. 2010; Zhu & Filler 2010; Martin et al. 2011; Alves et al. 2014).

With regards to expression of *C. albicans* putative virulence genes, only ALS1 and ALS3 were up-regulated in *C. albicans*-only biofilms on acrylic, and these are associated with substratum adhesion (Hoyer 2001; Zhu & Filler 2010; Méar et al. 2013). Interestingly, similar down-regulation has previously been demonstrated in mixed species biofilms on abiotic surfaces (Park et al. 2014). ASL1 is associated with biofilm maturation (Nailis et al. 2010; Zhu & Filler 2010) and its reduced expression caused by bacteria has been linked to altered nutrient availability, with associated slower biofilm development (Buu & Chen 2014; Park et al. 2014). ALS3 is a hyphal-specific cell surface protein and receptor for the streptococcal adhesins SspA and SspB (Bamford et al. 2009; Diaz, Xie, et al. 2012; Diaz, Strausbaugh, et al. 2014). The fact that higher hyphal proportions were present in mixed species acrylic biofilms would have presumably led to elevated ALS3 expression. However, this was not seen. It might be that the hyphal development arose prior to ALS3 being down-regulated due to bacterial factors.

Phospholipase D expression by *C. albicans* was not affected by bacteria and might indicate constitutive expression, corroborating previous findings (Nailis et al. 2010; Alves et al. 2014).

Other *C. albicans* putative virulence genes, related to hyphal proliferation (HWP1) and secretion of aspartyl proteinases (SAP4 and SAP6) were up-regulated in mixed species biofilms on acrylic. HWP1 is related to hyphal proliferation and substratum adhesion (Nailis et al. 2010; Zhu & Filler 2010; Martin et al. 2011; Alves et al. 2014), whilst SAP4 and SAP6 are associated with yeast–hyphal transition and tissue invasion (Naglik et al. 2003; Jayatilake et al. 2006; Naglik, Moyes, et al. 2008; Dalle et al. 2010; Martin et al. 2011). Given the changes to the *C. albicans* phenotype in the presence of bacteria in biofilms on acrylic, it was hypothesised that an increased pathogenic effect would be apparent when these biofilms were overlaid on RHOE. In these studies, the presence of bacteria led to enhanced expression of putative *Candida* virulence genes related to cell wall adhesins (ALS3 and EPA1), secreted aspartyl proteinases (SAP6) and hyphal proliferation (HWP1). Unsurprisingly, enhanced tissue invasion by *C. albicans* in the mixed species biofilms was associated with significantly

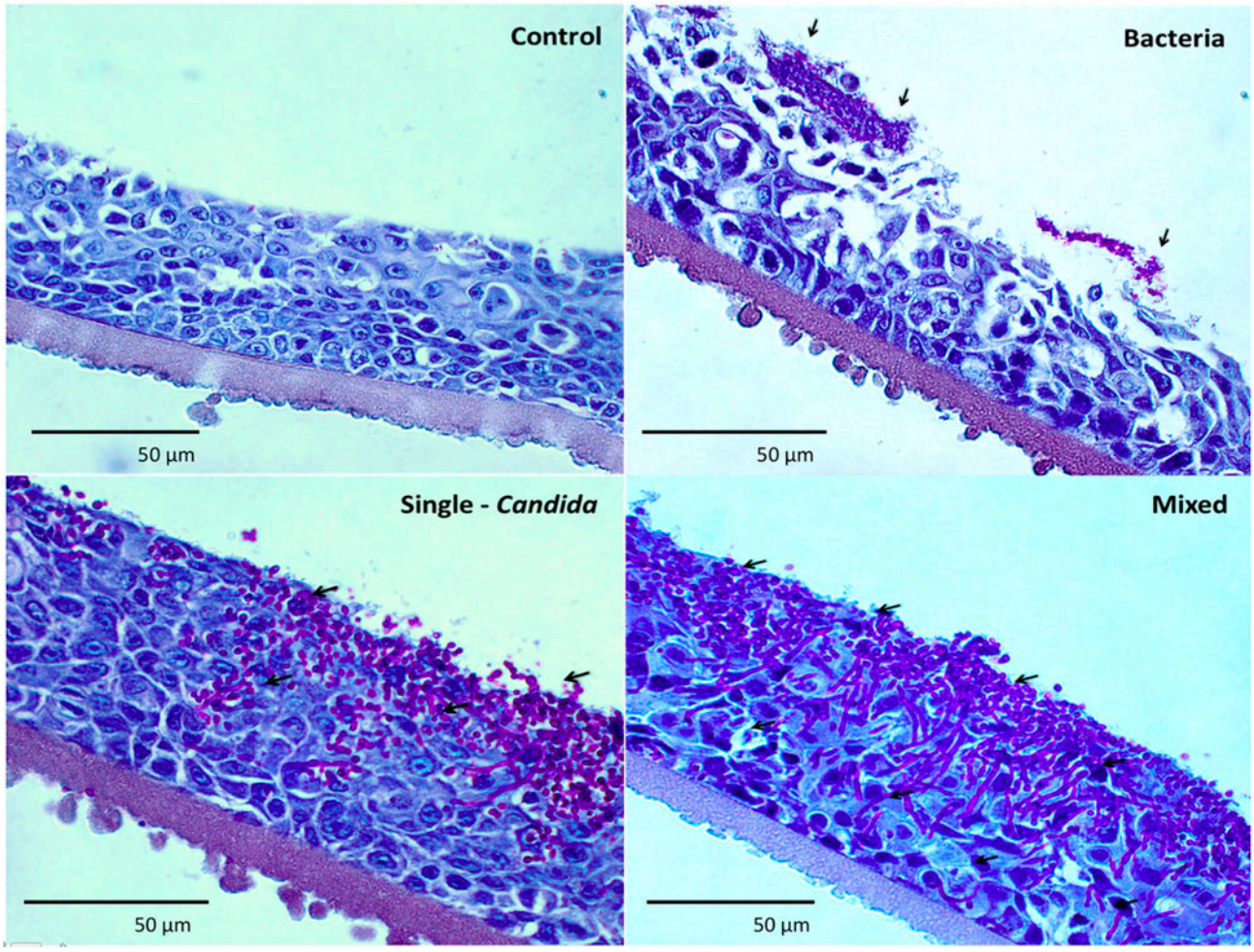

Figure 6. Representative light microscope images of fixed RHOE (n = 6, for each group) stained with PAS. Groups were comprised of healthy RHOE (control – upper left), and RHOE infected with bacterial biofilm (upper right); single-species *C. albicans* biofilm (lower left); or with mixed-species biofilm (lower right). Arrows show biofilms infecting tissues and invading the epithelia. Hyphae were seen in both single-species (*C. albicans*) and mixed-species biofilms but higher proportions of *C. albicans* hyphae and invasion were detected in mixed-species biofilms.

higher tissue damage, as determined by the LDH assay. Also, the increased proportion of *C. albicans* hyphae in mixed infections may have been responsible for the enhanced RHOE immune response seen by up-regulation of the gene encoding human interleukin IL-18 (Tardif et al. 2004; Martin et al. 2011; Kuo et al. 2013; Lowman et al. 2014).

Enhanced expression of ALS3 and EPA1 in mixed species biofilms infecting RHOE indicate that *C. albicans* adhesion to epithelial cells might also be increased in the presence of bacteria. The EPA1 gene translates a specific adhesin to epithelial cells (Kuhn & Vyas 2012), whilst ALS3 adhesin binds to the specific host cell receptors E-cadherin and N-cadherin (Zhu & Filler 2010; Liu & Filler 2011). Interestingly, the RHOE appeared to negate the previously observed lower ALS3 expression in mixed biofilms and two factors might explain this. Firstly, invasion of hyphae into the RHOE might protect against inhibitory effects of the bacteria, and secondly, the RHOE might provide additional nutrients to limit bacterial impact.

The mechanism by which the bacterial component of the biofilm induced the observed changes in *C. albicans* remains unclear. The effect could be due to several factors, eg alteration of environmental parameters including nutrient limitation, hypoxia, or specific effects such as inhibition of the farnesol pathway of hyphal suppression (Morales & Hogan 2010; Peleg et al. 2010; Gow et al. 2011; Lu et al. 2013; Buu & Chen 2014). Since many studies have shown antagonism of *C. albicans* by other bacteria (Hogan et al. 2004; Tian et al. 2013), studies assessing specific probiotics and quorum sensing molecules to manage candidal infection might also be of future value in this regard.

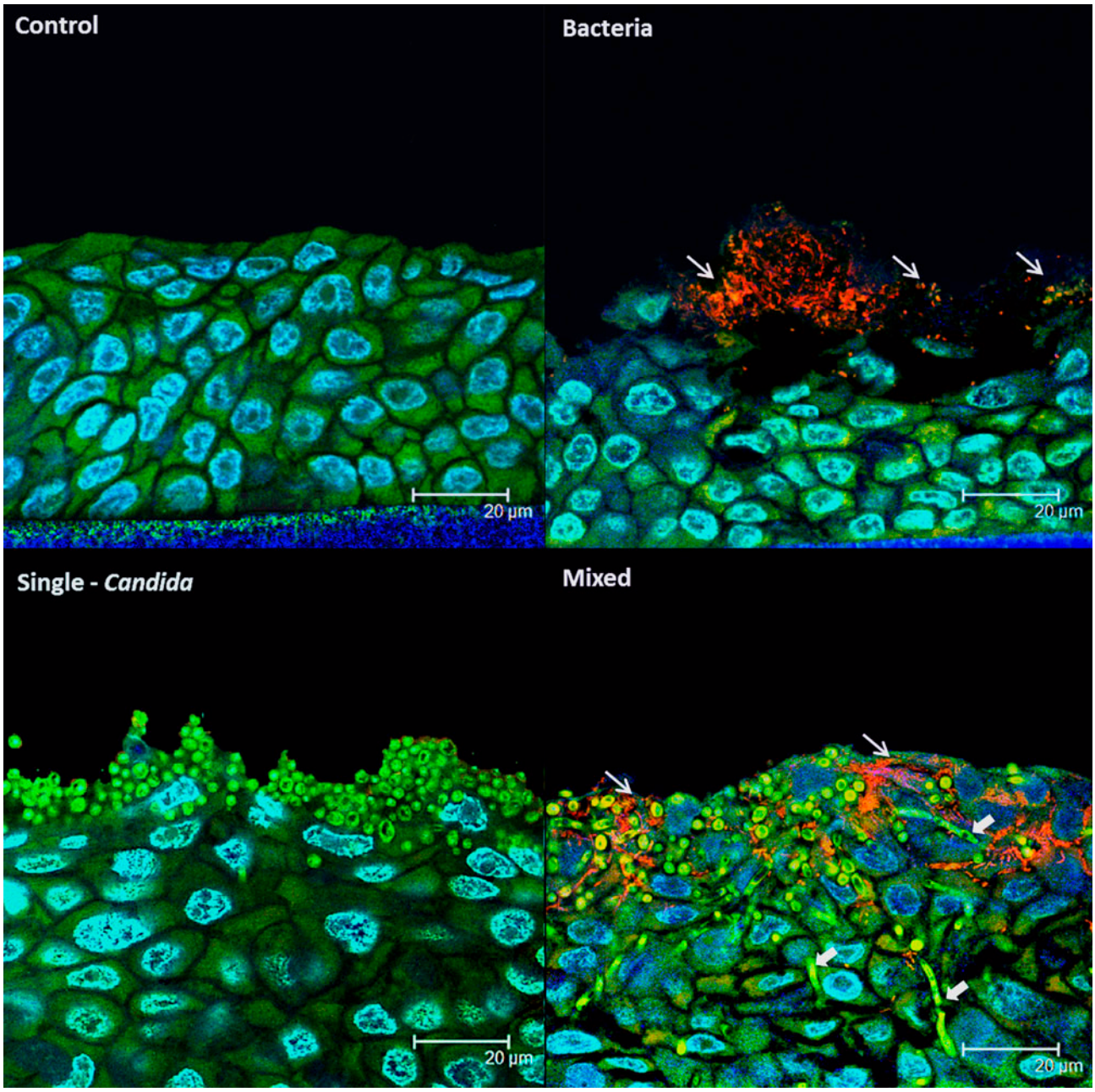

Figure 7. Representative CLSM images of fixed RHOE (n = 6, for each group) stained with fluorescent probes, after FISH. Groups comprised of healthy RHOE (control – upper left), and RHOE infected with bacterial biofilm (upper right); single-species *C. albicans* (lower left); or with mixed-species biofilm (lower right). Light arrows show bacterial species detected by a universal PNA probe (seen in red) in both bacterial and mixed-species biofilms. Bold arrows show hyphae and *C. albicans* invading the epithelium. A higher proportion of hyphae and invasion were detected in mixed-species biofilms. Epithelial nuclei were stained blue by Hoechst staining and epithelial cells were detected using pan-cytokeratin immunofluorescence. *Candida* was stained green by a PNA probe (YTL kit) and the cell wall was stained red by concanavalin-A conjugated lectin (Alexa Fluor® 594). Bacterial cells stained red following hybridisation with a bacterium-specific PNA probe.

This study found that an important factor in biofilm pathogenicity was the influence of the oral bacterial component on *C. albicans*. It may well be the case that different bacterial components modulate *C. albicans* pathogenicity *in vivo*, either by enhancing or reducing it. Since most attention in managing denture stomatitis has previously been directed towards the colonising *Candida*, based on these findings, further consideration should be given to the oral bacterial component of denture biofilms.

Conclusion

The presence of specific bacteria promotes proliferation of *C. albicans* hyphae and leads to enhanced tissue invasion and damage in mixed species biofilms. Higher virulence and pathogenicity of *Candida* biofilms was associated with up-regulation of virulence genes related to cell adhesion, hyphal proliferation and secreted aspartyl proteinases. These results are important, as in oral candidosis involving denture biofilms, progression and management of the infection may in part be dependent on the bacterial component present, an aspect that is frequently not a primary focus during clinical management.

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Conflict of interest disclosure statement

No potential conflict of interest was reported by the author(s).

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