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Interkingdom cooperation between *Candida albicans*, *Streptococcus oralis* and *Actinomyces oris* modulates early biofilm development on denture material

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Running header: Interkingdom biofilms on denture acrylic

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ABSTRACT

Candida-associated stomatitis affects up to 60% of denture wearers, and *Candida albicans* remains the most commonly isolated fungal species. The oral bacteria *Actinomyces oris* and *Streptococcus oralis* are abundant in early dental plaque. The aims of this study were to determine the effects of *S. oralis* and *A. oris* on the development of *C. albicans* biofilms on denture material. Resin discs were coated with saliva and at early (1.5 h) or later (24 h) stages of biofilm development, cell numbers of each species were determined. Spatial distribution of microorganisms was visualized by confocal scanning laser microscopy of biofilms labelled by differential fluorescence or by fluorescence in situ hybridization (FISH). Interkingdom interactions underpinning biofilm development were also evaluated planktonically utilizing fluorescence microscopy. Synergistic interactions between all three species occurred within biofilms and planktonically. Bacterial cells coaggregated with each other and adhered singly or in coaggregates to *C. albicans* hyphal filaments. *S. oralis* appeared to enhance hyphal filament production and *C. albicans* biovolume was increased two-fold. Concomitantly, cell numbers of *S. oralis* and *A. oris* were enhanced by *C. albicans*. Thus cooperative physical and metabolic processes occurring between these three microbial species intensify pathogenic plaque communities on denture surfaces.

Keywords: human oral cavity; microbial communities; stomatitis; FISH; coaggregation

Text for graphical abstract:

Interactive processes occurring between *Streptococcus oralis, Actinomyces oris*, and *Candida albicans* intensify pathogenic plaque communities on denture surfaces

INTRODUCTION

Denture stomatitis is a common disease, often recurring, and is seen in up to 60% of otherwise healthy denture wearers (Arendorf and Walker, 1987; Figueira *et al.* 2007; Geerts *et al.* 2008). It is characterized by inflamed mucosa, particularly under an upper denture (palatal mucosa), causing a burning sensation, soreness and bad taste (Ramage *et al.* 2004). Denture samples from subjects with denture stomatitis generally carry surface biofilms containing networks of hyphal filaments characteristic of *Candida albicans* (Douglas, 2003). The fungi have a high propensity for adhesion to oral acrylic dentures (Pereira-Cenci *et al.* 2007), and this is affected by the surface roughness of the denture resin (Verran and Maryan, 1997; Jackson *et al.* 2014; Mayahara *et al.* 2014) and by the type of dietary carbohydrates (Santana *et al.* 2013).

While *C. albicans* and other *Candida* species that can co-exist with *C. albicans* (Coco *et al.* 2008) have been a focus in treatment strategies for oral stomatitis (Redding *et al.*, 2009; Salerno *et al.* 2011), studies have underlined that the plaque accumulated on dentures during stomatitis contains both bacterial and fungal components (Campos *et al.* 2008; Salerno *et al.* 2011). This is potentially significant because it has been shown that oral bacteria, such as streptococci, can form mixed-species biofilms with *C. albicans* (Xu *et al.* 2014a) that are more luxurious and contain more hyphal filaments than monospecies biofilms (Dutton *et al.* 2014; Sztajer *et al.* 2014; Dutton *et al.* 2015). *Streptococcus oralis* appears to be able to facilitate invasion of oral mucosa by *C. albicans* (Diaz *et al.* 2012), while growth of *S. oralis* itself is promoted by the fungus. In addition, the pathogenic properties and invasive potential of the bacteria can be elevated by the presence of *C. albicans* (Falsetta *et al.* 2014; Xu *et al.* 2014b). Less is known about how other bacterial colonizers of hard surfaces, for example species of *Actinomyces* or *Veillonella*, interact with *C. albicans* and influence biofilm development.

Mitis/sanguinis group streptococci and *Actinomyces* species (e.g. *oris*, *naeslundii*, *johnsonii*) are amongst the first oral bacteria to colonize a fresh salivary glycoprotein-surface (Nyvad and Kilian, 1987; Al-Ahmad *et al.* 2009; Dige *et al.* 2009). In vitro studies have indicated that mutualism in biofilm formation occurs between strains of *S. oralis* and *Actinomyces oris* (formerly *A. naeslundii)*. Neither organism alone was able to form a salivary-flow biofilm, but a robust dual-species biofilm could be formed owing to nutritional cross-feeding (Palmer *et al.* 2001). Interactions between these species and *C. albicans* in triadic conditions has not before been studied, but would be significant in respect of early colonization of denture materials. The concept of synergy in biofilm community development rests on the notion that a primary species

preferentially adheres to the salivary pellicle-coated oral cavity surface and by doing so, provides additional sites for adhesion of secondary colonizers (Wright *et al.* 2013). Based upon this notion, and upon previous work showing synergistic interactions between streptococci and *C. albicans* (Bamford *et al.* 2009; Jack *et al.* 2015), it was hypothesized that colonization of salivary glycoprotein pellicle-coated denture material by *C. albicans* might be modulated when both streptococci and actinomyces are present. The results demonstrate that there is synergy in triadic-species biofilm formation, and that the increased presence of *C. albicans* enhances the development of bacterial plaque. This suggests that antibacterial approaches should be adopted in addition to antifungal strategies to more effectively cleanse dentures of biofilms and control stomatitis.

MATERIALS AND METHODS

Microbial strains and growth conditions

A. oris T14V and *S. oralis* 34 were cultivated anaerobically at 37 °C on BHYN agar (per liter: 37 g Brain Heart Infusion, 5 g yeast extract, 5 g Bacto-Neopeptone and 15 g agar). Suspension cultures were grown in BHY medium (Brain Heart Infusion medium containing 5 g L^{-1} yeast extract), in sealed bottles or tubes, and incubated stationary at 37 °C. *C. albicans* SC5314 was cultivated aerobically on Sabouraud Dextrose (SD) agar (LabM, Heywood, Leics. UK) at 37 °C, and suspensions were grown in YMD medium (per liter: 20 g Oxoid Mycological Peptone, 10 g yeast extract, 20 g dextrose) in conical flasks at 37 °C with shaking (200 r.p.m.) (Dutton *et al.* 2014). For preparation of cells for experiments, cultures were grown for 16 h at 37 °C, centrifuged (5000 ***g*** for 7 min), the cell pellets were suspended and washed twice with YPT medium (1 x yeast nitrogen base, 20 mM NaH_2PO_4-H_3PO_4 buffer pH 7.0, 0.1% tryptone) (Silverman *et al.*, 2010), and suspended in YPT medium at OD_{600} 1.0.

Planktonic interaction assay

C. albicans cell suspension at OD_{600} 1.0 (approximately 1 x 10^7 cells mL^{-1}) (0.2 mL) was added to glass tubes containing 1.8 mL warm YPTG medium (YPT supplemented with 0.4% glucose) and incubated for 2 h at 37 °C with shaking (200 r.p.m.) to initiate hyphal filament formation. During this period, *A. oris* cells were fluorescently labelled with FITC (fluorescein isothiocyanate) as described by Dutton *et al.* (2014) and *S. oralis* cells were labelled with 1.3 mM TRITC (tetramethylrhodamine-5 (and 6) isothiocyanate) in 0.05 M Na_2CO_3 containing 0.15 M NaCl for 30 min at 20 ^oC in the

dark with gentle shaking. Bacterial cells were washed three times with YPT and suspended at OD_{600} 0.5 (2 x 10^8 cells mL^{-1}) in YPT medium. Fluorescently-labelled bacterial cell suspension (1 ml) was added to *C. albicans* cell suspension (2 ml) and incubated for 1 h at 37 °C with gentle shaking. To each cell suspension was then added fluorescent brightener 28 (Calcofluor white, Sigma-Aldrich Co., St. Louis, MO) (1 µL of 0.2 mg mL^{-1} stock solution) to fluoresce the *C. albicans*. Samples of suspension were applied to glass microscope slides and visualized by fluorescence microscopy (Leica DMLB) (Silverman *et al.* 2010).

PMMA disc preparation

Discs were fabricated using poly(methyl methacrylate) (PMMA) resin (QC-20 PMMA, Dentsply International, York, PA) and polymerized in a hot water bath according to the manufacturer's instructions within a metal device (10 mm diameter x 2 mm thick). The discs were ground progressively with smoother aluminum oxide papers, grids 320, 400 and 600, in a horizontal polisher (Arotec APL-4, São Paulo, SP, Brazil) and then standardized for surface roughness by profilometry (Verran and Maryan, 1997) to R_a = 0.31 ± 0.02 (R_a value is the arithmetical average of all departures of the surface profile through the mean sample length). They were cleaned in an ultrasonic bath (Thornton T 740, Thornton-Inpec Eletronica LTDA, Vinhedo, SP, Brazil) for 2 x 10 min to remove surface contaminants, disinfected with 70% ethanol, washed with sterile distilled water and allowed to dry under aseptic conditions.

Salivary pellicle formation

Collection of human saliva samples was approved by the National Research Ethics Committee South Central Oxford C (no. 08/H0606/87+5). Saliva was collected from at least 6 adult subjects, who provided written informed consent. The samples were pooled, treated with 0.25 M dithiothreitol on ice for 10 min, and centrifuged (8000 ***g*** for 10 min). The supernatant was removed, diluted to 10% with sterile water, filter sterilized (0.45-µm pore-size membrane), and stored at -20°C. Salivary pellicle was formed on the resin discs by incubating them in 10% sterile saliva for 2 h at 37 °C.

Biofilm development

Saliva-coated PMMA discs were added to individual wells of a 24-well plate each containing 1.8 mL YPTG medium at 37 ^oC. Microbial cell suspensions in YPT medium, prepared as described above, were then added (75 µL) together with 150 µL, 75 µL or 0 µL YPTG medium as appropriate (total 225 µL) to generate mono-, dual- or triadic-species biofilms respectively in triplicate. After 1.5 h a number of the resin discs were

removed, rinsed, and analysed for biofilm formation. For the remaining discs, unattached cell suspension was aspirated from the wells and replaced with 2 mL fresh YPTG medium. The plates were incubated at 37 ºC for 24 h with gentle agitation as previously described (Silverman *et al.* 2010; Cavalcanti *et al.* 2014).

Measurement of viable cell numbers

After 1.5 h incubation, or after 24 h, the PMMA discs were removed from the culture wells, rinsed gently with sterile PBS (50 mM Na_2HPO_4-KH_2PO_4, 0.15 M NaCl, pH 7.2), added to 3 mL PBS in a polypropylene tube, and sonicated at 7 W for 30 s in a sonication bath to disrupt the biofilms. Portions (200 µL) of the homogenized suspensions were removed, serially 10-fold diluted and 20 µL aliquots were applied to BHYN agar plates containing 50 µg nystatin mL^{-1} (to inhibit growth of *C. albicans*) for estimating numbers of bacterial colony-forming units (CFU), or onto SD agar for estimating CFU of *C. albicans*. The plates were incubated anaerobically at 37 ^{o}C for 24 h (BHYN agar) or aerobically at 37 ^{o}C for 16 h (SD agar), and colony counts were subsequently converted to CFU mL^{-1}. It was possible to differentiate between *S. oralis* and *A. oris* on the basis of colony size and colour. Control experiments determined that the sonication treatment was sufficient to remove >90% cells from the surfaces of the discs, and at the same time was sufficiently gentle to result in <5% loss in cell viability.

Fluorescence microscopy and confocal scanning laser microscopy (CSLM)

For visualizing *C. albicans* monospecies biofilms, 2 µg Calcofluor mL^{-1} was incorporated into the YPTG growth medium to fluorescently label the fungal cells. Monospecies biofilms of bacteria were fluorescently labelled with FITC (10 µM, 20 min) at the end of the experiment. The resin discs were gently washed twice with PBS and the biofilms were visualized by fluorescence microscopy (Leica DMLB) or by CSLM with a Leica SP5-AOBS confocal microscope attached to a Leica DM I6000 inverted epifluorescence microscope.

Dual-species or triadic-species biofilms were also subjected to fluorescence in situ hybridization (FISH) analysis by CSLM. Biofilms were fixed in 4% paraformaldehyde (2 h), permeabilized using lysozyme for 30 min at 37 °C, washed twice in PBS and then incubated in hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl pH 8.0, 0.01% SDS, and 10-30% formamide depending upon the probe) containing 5 µg mL^{-1} fluorescently-labelled 16S rRNA-specific oligonucleotide probe for 150 min at 55 °C. The oligonucleotide probe sequences were as described by Thurnheer *et al.* (2004) and were labelled (Eurofins Genomic Services Ltd., Wolverhampton, UK) as follows: *S. oralis* probe MIT447_488 (Alexa 488), *A. oris* probe ANA103_647 (Alexa

647) and *C. albicans* EUK516_555 (Alexa 555). Following hybridization the discs were incubated in washing buffer (20 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.01% SDS, and between 159 and 636 mM NaCl depending on the formamide concentration used during hybridization) for 15 min at 55 °C. After rinsing briefly in 0.9% NaCl the discs were inverted onto a glass slide to be visualized by CSLM. Volocity® software was utilized to prepare three-dimensional (3D) images and Imaris® v7.5 software (Bitplane AG, Zurich, Switzerland) was used to calculate biovolumes (μm^3).

Statistical analysis

Data were processed by Prism 6 software at a confidence level of 95% using the two-way ANOVA followed by Tukey test for biovolumes or for total biofilm counts, respective to the time points or the biofilm combinations.

RESULTS

Planktonic interactions between bacteria and fungi

Approximately 60% of *C. albicans* cells formed hyphal filaments after 2 h planktonic incubation at 37 ^{o}C in YPTG medium. *S. oralis* (labelled with TRITC) adhered principally to hyphal filaments, with small chains of streptococci aligned along the hyphae (Fig. 1A), or adhered in patches as single cells or doublets (Fig. 1A). The actinomyces cell pairs showed characteristic whiplash morphology and small aggregates were visible freely and in association with hyphae (Fig. 1B). In triadic associations, the actinomyces and streptococci formed large coaggregates and these adhered principally to the fungal filaments (Fig. 1). Coaggregates of *S. oralis* and *A. oris* were also seen independently of candidal cells (Fig. 1C). Therefore coaggregation between these three microorganisms occurred pairwise as well as cooperatively.

Early biofilm formation on resin discs

To assess the capacity for the three microbial species, or combinations thereof, to bind saliva-coated resin, microbial cells were incubated with resin discs for 1.5 h. Non-adherent cells were then removed, and levels of attachment visualized by fluorescence microscopy following staining of the discs with FITC. Because of the uneven surfaces of the resin discs, it was not possible to capture the entire population of attached cells in a single plane of focus. Nonetheless, all three species could be seen to attach to saliva-coated resin, both as individual cells or as aggregates (Fig. 2, upper panels). Overall coverage was uniform, although the natural grooves of the resin surface

seemed to promote microbial deposition, especially of bacteria, compared to smoother regions of the resin.

For dual-species or triadic samples, *S. oralis* or *A. oris* could be seen interacting both directly with the salivary pellicle and with candidal cells (Fig. 2, lower panels). Patterns of association were similar overall to those seen under planktonic conditions (compare Figs. 1 and 2). For *C. albicans* cells incubated with resin either alone or in combination with bacteria, a mixture of blastospores and hyphae was evident attached to the surface. In dual-species biofilms of *C. albicans* and *A. oris*, the *A. oris* cells tended to associate with the *C. albicans* as opposed to adhering to the pellicle (Fig. 2). This suggested that the pellicle receptors for *A. oris* might be modified by the presence of *C. albicans*, or that *A. oris* bound preferentially to *C. albicans* rather than to the coated resin. In triadic-species biofilms, the *C. albicans* cells forming hyphal filaments were distributed more evenly over the surface, while notably the bacteria formed clusters associated with the *C. albicans* hyphae (Fig. 2). Although not quantified directly in these biofilms, a higher proportion of hyphal filaments was present in dual- or triadic-species samples compared to monospecies *C. albicans* samples (Fig. 2).

At 1.5 h the viable counts of bacteria recovered from the biofilms were similar (Table 1), while the *C. albicans* CFU were ten-fold less than bacterial cell numbers. However, C. *albicans* cells are approximately ten-fold larger in size/volume than the bacterial cells. The CFU of *C. albicans* were reduced by about 40% in the presence of *A. oris* and about 20% by *S. oralis* (Table 2). By contrast, there were no significant differences ($P \geq 0.05$) in CFU of streptococci or actinomyces in the presence of *C. albicans* (Table 2). The numbers of *C. albicans* CFU were also reduced in the presence of *S. oralis* and *A. oris* together.

To overcome the difficulties of visualizing the roughened surface of the resin discs by fluorescence microscopy, monospecies biofilm formation was also investigated by CSLM. Microbial cells were incubated with saliva-coated resin discs for 1.5 h. Non-adherent cells were then removed, and biofilms were visualized by fluorescent labelling with FITC (*S. oralis*, *A. oris*) or Calcofluor (*C. albicans*). At 1.5 h all three species exhibited relatively uniform coverage of the resin surface, with areas of elevated cell density in the resin grooves, particularly for *S. oralis* (Fig. 3). Overall levels of coverage were comparable between *S. oralis* and *A. oris*, while *C. albicans* cells had a more sparse distribution. All three species formed distinct microcolonies.

Later (24 h) biofilms on resin discs

To investigate biofilm formation by these microorganisms in more detail, later stage (24 h) biofilms on resin discs were also examined. Using CSLM, no obvious changes were

seen in *S. oralis* biomass levels from 1.5 h to 24 h (Fig. 3). By contrast, biofilms formed by *A. oris* or *C. albicans* increased in density and depth over the 24 h period to form a thick 'mat' on the resin (Fig. 3). Additionally, for *C. albicans*, a distinct transition from predominately blastospores and short hyphal filaments to long hyphal filaments was seen from 1.5 h to 24 h (Fig. 3).

At 24 h the viable counts (CFU) of each of the monospecies biofilms had increased, with *A. oris* showing a 3.6-fold increase and *C. albicans* a 30-fold increase (Table 1) compared to 1.5 h CFU levels. However, in the dual- or triadic-species biofilms there were notable interactive effects of the organisms. In the presence of *C. albicans,* the CFU of *S. oralis* and *A. oris* increased between 1.5 h and 24 h by 14-fold and 19-fold respectively, while *C. albicans* exhibited up to 38-fold increased CFU (Table 2). In the triadic-species biofilm total bacterial CFU increased 20-fold over the 1.5 h to 24 h time period. Therefore, based upon these CFU data, biofilm formation by *S. oralis* and *A. oris* was highly augmented in the presence of *C. albicans*. It should be noted that for *C. albicans*, CFU are not a meaningful indicator of biomass because multicellular hyphal filaments were the major morphological components of *C. albicans* 24 h biofilms. We were unable to measure total biomass by conventional staining methods because the resin adsorbed the stains.

In the experiments so far described the bacteria and fungi were added simultaneously to the resin discs. To determine if the order of deposition had any effect upon the biofilms formed, we first incubated the resin discs with bacteria for 1.5 h, then added *C. albicans* and measured CFU at 3 h or 24 h. A similar pattern of results was obtained (Supplemental Table S1) to those when the microorganisms were inoculated together at the same time (Table 2). *S. oralis* and *A. oris* CFU increased 8-fold and 12-fold over the time period, while *C. albicans* CFU increased up to 15-fold. The bacterial components of the biofilm were again augmented, especially bacterial CFU present within the triadic-species biofilms (Table S1).

Microbial composition of 24 h biofilms determined by FISH

To visualize relative proportions of microbes present in dual- or triadic-species biofilms, 1.5 h or 24 h biofilms were grown on saliva-coated resin, as above, and the presence of each microbial species detected using FISH. For all combinations the microorganisms formed fully integrated biofilms, interacting with one another and with salivary pellicle (Fig. 4). At 1.5 h, both bacterial species were in close contact with the pellicle, and this was particularly evident for *A. oris*. (Fig. 4). Initial deposition of *C. albicans* appeared to be enhanced by *S. oralis* (compare Fig. 4 and Fig. 3) but was unaffected by *A. oris*. This appears to contradict the corresponding CFU data (above),

but there are limitations in CFU determinations for *C. albicans* undergoing filamentation (see Discussion). At 24 h, *S. oralis* cells were integrated from the base to the surface of the dual-species biofilm, while *A. oris* cells were particularly associated with the upper biofilm layers (Fig. 4). These biofilms were composed mainly of hyphal filaments with integrated patches of bacteria. The triadic-species biofilms were also composed mainly of hyphal filaments, with integrated groups of *S. oralis* and fewer *A. oris* (Fig. 4).The biovolume data at 24 h demonstrated that the presence of *S. oralis* enhanced *C. albicans* biofilm development (biovolume) two-fold (Fig. 5), compared with *C. albicans* alone. *A. oris* had a lesser stimulatory effect on *C. albicans* (Fig. 5). *C. albicans* biovolume was also enhanced in the triadic-species biofilms (Fig. 5), but not as much as with *S. oralis* alone. In conclusion, *S. oralis* and *A. oris* individually augment biofilm formation by *C. albicans*, but *S. oralis* appears to out-compete *A. oris* in the presence of *C. albicans*.

DISCUSSION

The understanding of multispecies biofilm partnerships and their impact on human health has grown over recent years. Further investigation is required to take into account the potential for a wide spectrum of different microbial communities to develop in the human host and affect health. The interkingdom interactions of *C. albicans* and bacteria in the context of human disease have revived attention since one of the first descriptions of coaggregation between Gram-positive cocci and *Candida* (Jenkinson *et al*. 1990). Some bacterial species produce molecules that kill or inhibit *C. albicans* (Morales *et al.* 2010; 2013), while others promote growth of the fungus (Xu *et al.* 2014a). On the other side, the pathogenic properties of some bacteria may be enhanced (Schlecht *et al.* 2015) or suppressed (Lopez-Medina *et al.* 2015) in the presence of *C. albicans*. Dual-species biofilms of *Streptococcus gordonii* and *C. albicans* have at least two-fold increased biomass over and above the biomass sum of the monospecies biofilms, showing synergy in growth of these species (Bamford *et al.* 2009; Dutton *et al.* 2014). Moreover, dual-species communities of *S. oralis* and *C. albicans* show pathogenic synergy, and induce an exaggerated inflammatory response in the host, over and above the inflammatory response levels of the individual species (Xu *et al.* 2014b). This latter observation is clearly relevant to the condition of denture stomatitis, the inflammatory nature of which might therefore be exacerbated by the presence of specific bacterial species in association with *C. albicans* in denture biofilms (Cavalcanti *et al.* 2015).

In the current article we investigated whether the interactions of two early-colonizing bacterial species affected the manner in which *C. albicans* could be incorporated into the biofilm and colonize denture acrylic surface. We found that both species of bacteria interacted with *Candida* hyphae under planktonic conditions. There was no evidence that bacteria bound specific regions of the filaments, for example the septal domain, suggesting that receptors for adhesion were distributed all along the length. Bacterial cells attached to hyphal filaments individually or in small chains, and aggregates were visible. *A. oris* and *S. oralis* were mixed simultaneously with *C. albicans* in the triadic-species combinations, so the patterns of coaggregation indicated that there were sufficient binding sites on *C. albicans* available for both bacterial species. Moreover, the hyphae were able to support larger coaggegates of *S. oralis* and *A. oris*.

In dual- and triadic-species biofilms, *C. albicans* hyphal filaments appeared to be incorporated throughout the biofilm layers. *S. oralis* cells formed a basal layer upon the salivary pellicle, in dual-species biofilms, colonizing areas not occupied by *C. albicans*. This is similar to the stratified appearance of *S. oralis-C. albicans* biofilms described recently by Bertolini *et al.* (2015). Streptococci could also be seen in patches at all levels within the 24 h biofilm. *A. oris* on the other hand did not effectively colonize the pellicle in 24 h dual-species biofilms with *C. albicans*, and instead was mainly associated with the hyphal filaments. The dual-species biofilms exhibited synergistic behaviours and bacterial CFU were higher in the presence of *C. albicans* than in the absence. *A. oris* CFU were increased 3.7-fold, and *S. oralis* CFU were increased 8.8-fold, in the presence of *C. albicans* compared to CFU within the respective monospecies biofilms. Measurements of *C. albicans* CFU in biofilms after ~2 h incubation have limitations as more hyphal filaments are formed. The limitations are clearly evident in that *C. albicans* biovolume within the triadic-species biofilms increased nearly two-fold in the presence of bacteria, while bacteria themselves comprised <10% of the total biovolume. Interestingly, incorporation of *A. oris* into the *C. albicans-S. oralis* biofilm dampened in part the augmenting effect of *S. oralis* on *C. albicans.* This indicates that *A. oris* may have some growth inhibitory effect on *C. albicans* in the presence of *S. oralis*. However, we did not observe direct inhibition of *C. albicans* proliferation by *A. oris*, as has been reported recently (Guo *et al.* 2015). This might be because we are using different strains of *C. albicans* and *A. oris*, as it is known that coaggregation between *C. albicans*, actinomyces and streptococci is strain-dependent (Arzmi *et al.* 2015).

The biofilm model described could be valuable in evaluating means of controlling oral candidosis, or growth of denture biofilms, because it contains two early-

colonizing species of bacteria as well as *C. albicans*. In vivo, *C. albicans* forms biofilms in association with bacteria, and colonization of a surface may require the ability to successfully compete with or live alongside the early colonizers. Our results indicate that these two early colonizing strains of bacteria are promoted by the presence of *C. albicans*, with the fungus providing an alternative substratum for *A. oris*. Concomitantly we see greater levels of *C. albicans* biofilm development occurring in the presence of bacteria, especially with *S. oralis*, compared to *C. albicans* monospecies biofilm. Further work is underway to determine the kinds of signals, nutritional or otherwise, that are produced by the microorganisms in order to mediate these synergistic interactions. This knowledge would be important for impact on clinical intervention strategies or antimicrobial therapies to control these polymicrobial infections.

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Table 1. Colony-forming units (CFU) for monospecies biofilms of *S. oralis*, *A. oris* or *C. albicans* formed on resin discs at 1.5 h or 24 h

Biofilm (h)	CFU per biofilm (x 10^6)		
	S. oralis	*A. oris*	*C. albicans*
1.5	1.0 ± 0.3	1.0 ± 0.01	0.10 ± 0.003
24	1.3 ± 0.7	3.6 ± 1.6*	3.0 ± 0.8*

n = 2, mean ± SD x 10^6, * $P < 0.05$ versus 1.5 h

Table 2. Colony forming units (CFU x 10^6) per disc of *C. albicans*, *S. oralis* and/or *A. oris* in dual- or triadic-species biofilms formed on resin discs at 1.5 h or 24 h

Biofilm (h)	*C. albicans* + *S. oralis*		*C. albicans* + *A. oris*		*C. albicans* + *S. oralis* + *A. oris*	
	S. oralis	*C. albicans*	*A. oris*	*C. albicans*	Bacteria	*C. albicans*
1.5	0.8 ± 0.2	0.08 ± 0.02	0.7 ± 0.04	0.06 ± 0.02	0.7 ± 0.07	0.08 ± 0.02
24	11.5 ± 6.9*	2.6 ± 0.7*	13.4 ± 4.9*	2.3 ± 0.8*	14.4 ± 3.5*	2.2 ± 0.7*

n = 2, mean ± SD x 10^6, * $P < 0.05$ versus 1.5 h

FIGURES

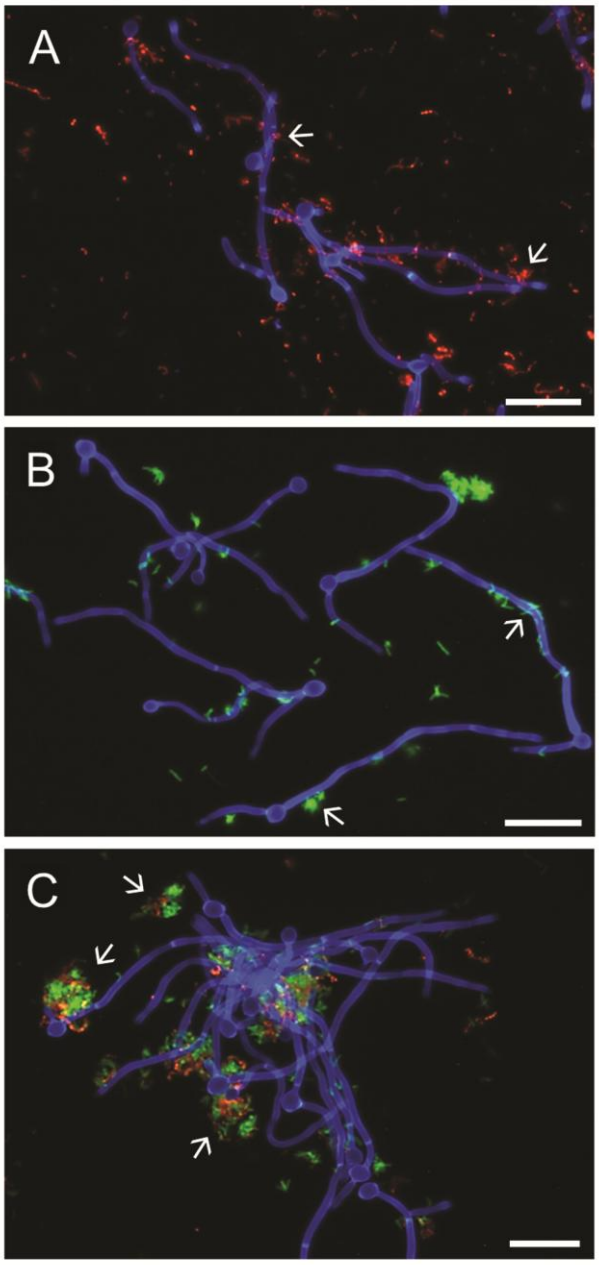

Figure 1. Fluorescence micrographs of planktonic interactions between *C. albicans* hypha-forming cells and *S. oralis* or *A. oris*. *C. albicans* cells were induced to form hyphae, as described in Materials and Methods, and then incubated with TRITC-labelled (red) *S. oralis* (panel A), FITC-labelled (green) *A. oris* (panel B), or both bacterial species (panel C). Arrows in panel A show clusters of streptococci associated

with hyphal filaments, while arrows in panel B show similar accumulations of actinomyces. In panel C, arrows indicate coaggregated clumps of bacteria adhered to hyphal filaments. Scale bars 50 µm.

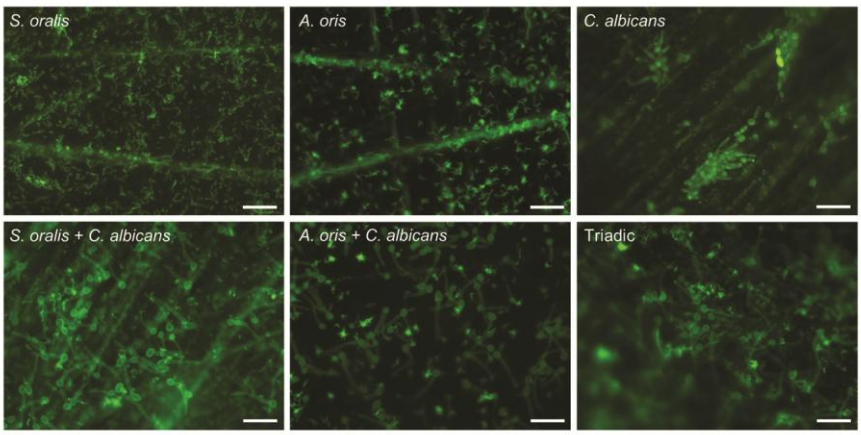

Figure 2. Fluorescence micrographs of *S. oralis*, *A. oris* or *C. albicans* adhered to salivary pellicle-coated denture acrylic resin. Cells were incubated with resin discs for 1.5 h and stained with FITC, as described in Materials and Methods. Scale bars 50 µm.

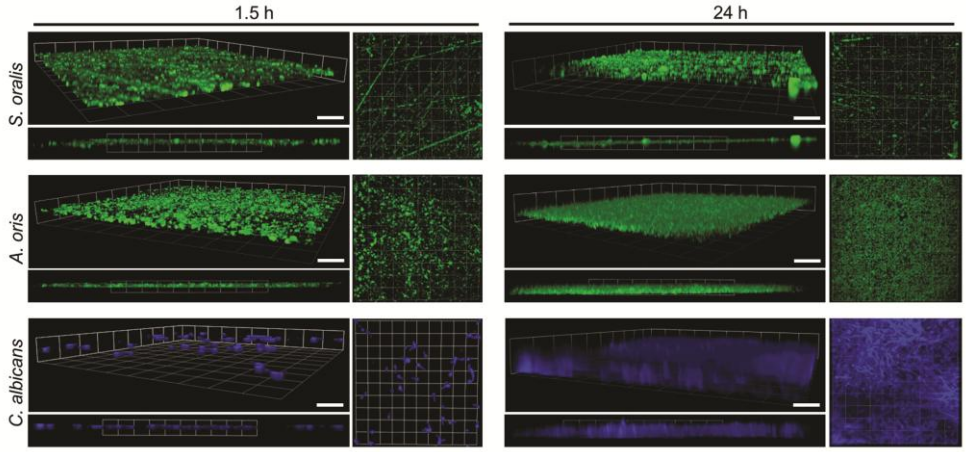

Figure 3. CSLM images of mono-species biofilms formed on salivary pellicle-coated denture acrylic resin after 1.5 h or 24 h incubation. Bacteria were stained with FITC while *C. albicans* was stained with Calcofluor, as described in Materials and Methods. Each biofilm is presented in 3D (xyz), side view (xz), and top down view (xy). Note that *S. oralis* formed an adhesive early biofilm that was incapable of growing over time, while the *A. oris* and *C. albicans* biofilms continued to develop over the time period. Scale bars 50 µm.

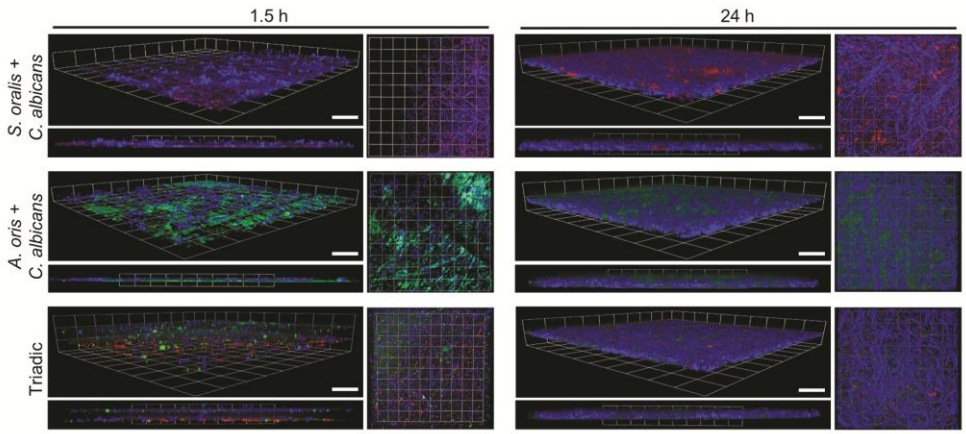

Figure 4. CSLM images of dual-species or triadic biofilms formed on salivary pellicle-coated denture acrylic resin after 1.5 h or 24 h incubation. Microbial cells were labelled by FISH probes for *S. oralis* (red), *A. oris* (green) or *C. albicans* (blue). At 24 h, *S. oralis* cells were integrated from the base to the surface of the dual-species biofilm, while *A. oris* cells were mainly associated with the upper biofilm layers. Scale bars 50 µm.

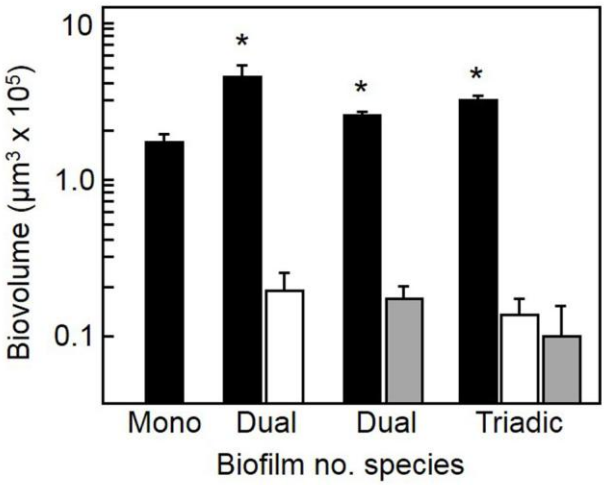

Figure 5. Biovolume data for individual species within 24 h biofilms. Fluorescence volumes were calculated for blue *C. albicans* (filled columns), red *S. oralis* (open columns) and green *A. oris* (grey shade columns) following CSLM imaging using Imaris® v7.5 software. Monospecies *C. albicans* biovolume = 2.29 ± 0.4 x 10^5 µm^3. Error bars are ± SD (n=2). *Significant increase (*P* <0.05) compared to monospecies biofilm.