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**Interactions between *Streptococcus oralis*, *Actinomyces*
oris, and *Candida albicans* in the development of
multispecies oral microbial biofilms on salivary pellicle**

Indira M.G. Cavalcanti^{1,2}, Altair A. Del Bel Cury¹, Howard F. Jenkinson² and Angela
H. Nobbs^{2*}

¹Department of Prosthodontics and Periodontology, Piracicaba Dental School - University of
Campinas, Limeira Avenue, 901, 13414-903 Piracicaba, SP, Brazil.

²School of Oral and Dental Sciences, University of Bristol, Lower Maudlin Street, Bristol,
BS1 2LY, United Kingdom.

Running header: Interkingdom biofilms on salivary pellicle

***Correspondence:** Dr Angela H. Nobbs, School of Oral and Dental Sciences, University of
Bristol, Lower Maudlin Street, Bristol BS1 2LY, United Kingdom Tel. +44-117-342-4424;
Fax. +44-117-342-4313; E-mail: angela.nobbs@bristol.ac.uk

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28 **SUMMARY**

29

30 The fungus *Candida albicans* is carried orally and causes a range of superficial infections
31 that may become systemic. Oral bacteria *Actinomyces oris* and *Streptococcus oralis* are
32 abundant in early dental plaque and on oral mucosa. The aims of this study were to
33 determine the mechanisms by which *S. oralis* and *A. oris* interact with each other and with *C.*
34 *albicans* in biofilm development. Spatial distribution of microorganisms was visualized by
35 confocal scanning laser microscopy of biofilms labelled by differential fluorescence or by
36 fluorescence in situ hybridization (FISH). *A. oris* and *S. oralis* formed robust dual-species
37 biofilms, or three-species biofilms with *C. albicans*. The bacterial components tended to
38 dominate the lower levels of the biofilms while *C. albicans* occupied the upper levels. Non-
39 fimbriated *A. oris* was compromised in biofilm formation in the absence or presence of
40 streptococci, but was incorporated into upper biofilm layers via binding to *C. albicans*. Biofilm
41 growth and hyphal filament production by *C. albicans* was enhanced by *S. oralis*. It is
42 suggested that the interkingdom biofilms are metabolically coordinated to house all three
43 components, and this study demonstrates that adhesive interactions between them
44 determine spatial distribution and biofilm architecture. The physical and chemical
45 communication processes occurring in these communities potentially augment *C. albicans*
46 persistence at multiple oral cavity sites.

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50 **Keywords:** human oral cavity; polymicrobial communities; colonization; FISH;
51 coaggregation

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54 INTRODUCTION

55 The pleiomorphic fungus *Candida albicans* colonizes the oral cavity, GI tract and genito-
 56 urinary tract where it can remain benign, or cause superficial infection, or invade body
 57 tissues to become systemic (Poulain, 2015). Although invasive disease can originate from
 58 oral cavity colonization in healthy subjects, it is much more likely to occur in individuals who
 59 are immunocompromised either as a result of HIV infection or medical procedures (surgery,
 60 radiotherapy, etc.) (Delaloye and Calandra, 2014; Martins *et al.*, 2014). A common oral
 61 condition involving *C. albicans* is denture stomatitis (Figueira *et al.*, 2007). Dentures from
 62 subjects with stomatitis carry surface biofilms containing networks of hyphal filaments
 63 characteristic of *C. albicans* (Douglas, 2003). The biofilms also contain bacterial components
 64 (Campos *et al.*, 2008; Salerno *et al.*, 2011) and there is evidence that oral streptococci can
 65 enhance growth of *C. albicans* biofilms (Xu *et al.*, 2014a) and augment fungal invasion of
 66 oral mucosa (Diaz *et al.*, 2012; Xu *et al.*, 2014b). The presence of *C. albicans* in sub-gingival
 67 plaque (Canabarro *et al.*, 2013) and in periodontal disease lesions (Urzúa *et al.*, 2008; Al
 68 Mubarak *et al.*, 2013) suggests that the fungus may possibly also have a role in the
 69 progression of periodontal disease. Furthermore, *C. albicans* has been found in supra-
 70 gingival plaque (Zijngel *et al.*, 2010) and in carious lesions (Marchant *et al.*, 2001; de
 71 Carvalho *et al.*, 2006), indicating that interactions with cariogenic bacteria such as
 72 *Streptococcus mutans* could potentially be significant in tooth decay (Koo and Bowen, 2014).

73 In order to colonize the salivary glycoprotein-coated hard or soft surfaces present
 74 within the oral cavity, *C. albicans* has acquired a range of adhesins that interact with salivary
 75 components (Cannon and Chaffin, 1999; Chaffin, 2008; Nobbs *et al.*, 2010; Demuyser *et al.*,
 76 2014). These include cell wall proteins (CWPs) Hwp1, Hwp2, Rbt1, Eap1, the Iff/Hyr family
 77 and the Als family (de Groot *et al.*, 2013). However, *C. albicans* is in competition for
 78 adhesion to salivary receptors with several hundred species of bacteria at any given oral
 79 cavity site (Jenkinson, 2011) and so interactions with bacteria that promote *C. albicans*
 80 colonization are important to define.

81 Mitis/sanguinis group streptococci and *Actinomyces* species (e.g. *oris*, *naeslundii*,
 82 *johnsonii*) are amongst the first oral bacteria to colonize a fresh salivary glycoprotein-coated
 83 surface (Nyvad and Kilian, 1987; Al-Ahmad *et al.*, 2009; Dige *et al.*, 2009). Oral streptococci
 84 express a wide range of salivary pellicle adhesins (Nobbs *et al.*, 2009; 2011), while
 85 *Actinomyces* type 1 fimbriae mediate interactions with salivary proline-rich proteins and
 86 statherin (Gibbons *et al.*, 1988; Clark *et al.*, 1989; Li *et al.*, 2001). *Actinomyces* type 2
 87 fimbriae on the other hand interact specifically with Gal β 1-3GalNAc found within the cell
 88 surface receptor polysaccharides (RPS) of mitis-group streptococci (Yoshida *et al.*, 2006b)
 89 and some salivary glycoproteins (Ruhl *et al.*, 2004). The type 2 fimbriae therefore mediate
 90 coaggregation with streptococci expressing RPS (Cisar *et al.*, 1983; Palmer *et al.*, 2003),
 91 and also appear to be involved in biofilm formation (Mishra *et al.*, 2010). In vitro studies have
 92 shown that mutualism in biofilm formation occurs between strains of *Streptococcus oralis*
 93 and *Actinomyces oris* (Palmer *et al.*, 2001). Interactions between these early colonizing
 94 bacteria and *C. albicans* could thus impact on the kinds of communities that develop upon
 95 oral cavity tissues and prostheses.

96 Several species of oral streptococci can form mixed-species biofilms with *C. albicans*
 97 (Xu *et al.*, 2014a). These biofilms are often more luxuriant than monospecies *C. albicans*
 98 biofilms and may contain a higher proportion of hyphal filaments (Dutton *et al.*, 2014; Sztajer
 99 *et al.*, 2014; Dutton *et al.*, 2015). While *S. oralis* is able to facilitate invasion of oral mucosal
 100 epithelium by *C. albicans* (Diaz *et al.*, 2012), concomitantly *S. oralis* growth is enhanced by
 101 the fungus. In addition, the pathogenic properties and invasive potential of streptococci
 102 bacteria can be augmented by *C. albicans* (Falsetta *et al.*, 2014; Xu *et al.*, 2014b). Much less
 103 is understood about how *Actinomyces* species interact with *C. albicans*, but coaggregation
 104 has been reported (Grimaudo *et al.*, 1996; Arzmi *et al.*, 2015), and there is evidence that *C.*
 105 *albicans* proliferation and biofilm development could be inhibited by *Actinomyces* species
 106 (Guo *et al.*, 2015).

107 Microbial synergy in biofilm community development involves primary colonizing
 108 species adhering to oral cavity surfaces and thus increasing the availability of receptors for

109 adhesion by secondary colonizers (Wright *et al.*, 2013). On the basis of studies showing
110 synergy between *S. oralis* and *C. albicans* in biofilm formation (Diaz *et al.*, 2012), and
111 synergy between *S. oralis* and *A. oris* in biofilm formation (Palmer *et al.*, 2001), we have
112 investigated if these interactions are maintained or disrupted in three-species biofilms. The
113 results here demonstrate that *C. albicans* assists incorporation of *S. oralis* and *A. oris* into
114 biofilms independently of the coaggregation interactions between the bacteria.

115 **METHODS**

116 **Microbial strains and growth conditions**

117 Bacterial strains utilized were as follows: *A. oris* T14V (wild type) and *A. oris* T14V Fim⁻
 118 (mutant lacking type 1 and type 2 fimbriae) (Cisar *et al.*, 1988); *S. oralis* 34 and *S. oralis* 34
 119 $\Delta wchA$ RPS⁻ (receptor polysaccharide negative isogenic mutant) (Yoshida *et al.*, 2006a).
 120 Bacteria were cultivated anaerobically at 37 °C on BHYN agar (per liter: 37 g Brain Heart
 121 Infusion, 5 g yeast extract, 5 g Bacto-Neopeptone, 15 g agar). Suspension cultures were
 122 grown in BHY medium (Brain Heart Infusion medium containing 5 g l⁻¹ yeast extract), in
 123 sealed bottles or tubes, and incubated stationary at 37 °C. *C. albicans* SC5314 was
 124 cultivated aerobically on Sabouraud Dextrose (SD) agar (LabM, Heywood, Leics. UK) at 37
 125 °C, and suspensions were grown in YMD medium (per liter: 20 g Oxoid Mycological
 126 Peptone, 10 g yeast extract, 20 g dextrose) in conical flasks at 37 °C with shaking (200
 127 r.p.m.) (Dutton *et al.* 2014). For preparation of cells for experiments, cultures were grown for
 128 16 h at 37 °C, centrifuged (5000 **g** for 7 min), the cell pellets were suspended and washed
 129 twice with YPT medium (1 x yeast nitrogen base, 20 mM NaH₂PO₄-H₃PO₄ buffer pH 7.0,
 130 0.1% tryptone) (Silverman *et al.*, 2010), and suspended in YPT medium at OD₆₀₀ 1.0.

131 **Bacterial coaggregation**

132 *A. oris* cells were fluorescently labelled with FITC (fluorescein isothiocyanate) as described
 133 by Dutton *et al.* (2014) and *S. oralis* cells were labelled with 1.3 mM TRITC
 134 (tetramethylrhodamine-5 (and 6) isothiocyanate) in 0.05 M Na₂CO₃ containing 0.15 M NaCl
 135 for 30 min at 20 °C in the dark with gentle shaking. Bacterial cells were washed three times
 136 with YPT and suspended at OD₆₀₀ 0.5 (2 x 10⁸ cells ml⁻¹) in YPT medium. Equal volumes of
 137 bacterial cell suspension were mixed in a glass tube, vortex mixed for 1 min and the tubes
 138 allowed to stand for 30 min at room temperature. Samples were taken onto glass
 139 microscope slides and visualized by fluorescence microscopy (Leica DMLB).

140 **Planktonic interactions of bacteria with *C. albicans***

141 *C. albicans* cell suspension at OD₆₀₀ 1.0 (approximately 1×10^7 cells ml⁻¹) (0.2 ml) was
 142 added to glass tubes containing 1.8 ml warm YPTG medium (YPT supplemented with 0.4%
 143 glucose) and incubated for 2 h at 37 °C with shaking (200 r.p.m.) to initiate hyphal filament
 144 formation. Fluorescently-labelled bacterial cell suspension (1 ml) was then added to *C.*
 145 *albicans* cell suspension (2 ml) and incubated for 1 h at 37 °C with gentle shaking. To each
 146 cell suspension was then added fluorescent brightener 28 (Calcofluor white, Sigma-Aldrich
 147 Co., St. Louis, MO) (1 µl of 0.2 mg ml⁻¹ stock solution) to fluoresce the *C. albicans*. Samples
 148 of suspension were applied to glass microscope slides and visualized by fluorescence
 149 microscopy (Leica DMLB) (Silverman *et al.* 2010).

150 **Salivary pellicle formation**

151 Collection of human saliva samples was approved by the National Research Ethics
 152 Committee South Central Oxford C (no. 08/H0606/87+5). Saliva was obtained from at least
 153 six healthy adult subjects, who provided written informed consent. The samples were
 154 pooled, treated with 0.25 M dithiothreitol on ice for 10 min, and centrifuged (8000 *g* for 10
 155 min). The supernatant was removed, diluted to 10% with sterile water, filter sterilized (0.45-
 156 µm pore-size membrane), and portions were stored at -20 °C. Salivary pellicle was formed
 157 on glass cover slips or in plastic culture dishes by incubating them with 10% saliva for 16 h
 158 at 4 °C.

159 **Biofilm development on cover slips**

160 Salivary glycoprotein-coated glass cover slips (19 mm diam.) were transferred to individual
 161 wells of a 12-well tissue culture plate containing 1.9 ml of YPTG medium. *C. albicans* cell
 162 suspension (0.1 ml) was added and incubated for 2 h at 37 °C, over which time fungal cells
 163 adhered to the substratum and began to form hyphal filaments. The unattached cells were
 164 then aspirated and, for monospecies biofilms, fresh medium (2 ml) was added and
 165 incubation continued. For dual species or three-species biofilms, the unattached fungal cells
 166 were aspirated, fresh medium (1 ml) was added together with *S. oralis* cell suspension in

YPTG (1 ml, $\sim 2 \times 10^8$ cells ml⁻¹) or *A. oris* cell suspension (1 ml, $\sim 2 \times 10^8$ cells ml⁻¹), or both added separately one immediately after the other, and incubated for 1 h at 37 °C. The bacterial cell suspensions were then removed, replaced with fresh YPTG medium (2 ml) and incubation was continued for up to 5 h. Coverslips were removed, rinsed gently with phosphate-buffered saline (PBS), dried, and stained with 0.5% crystal violet solution. The biofilms were then visualized by light microscopy (Leica DMLB). For biomass estimations the stain was released with 10% acetic acid and optical density at 595 nm (OD₅₉₅) was measured with a microtiter plate reader (Dutton *et al.*, 2014).

Biofilm development for confocal scanning laser microscopy (CSLM)

Biofilms (3 ml final volume) were grown in glass-bottom microwell dishes (35 mm diam., 14 mm microwell) (MatTek Corporation, Ashland, Mass.) exactly as described above. For visualizing *C. albicans* within biofilms, 2 µg Calcofluor ml⁻¹ was incorporated into the YPTG growth medium at the end of the experiment for 10 min to fluorescently label the fungal cells. Biofilms containing single species of bacteria (with or without *C. albicans*) were fluorescently labelled with FITC (10 µM, 20 min) at the end of the experiments. The dishes were washed twice with PBS and the biofilms were visualized by CSLM with a Leica SP5-AOBS confocal microscope attached to a Leica DM I6000 inverted epifluorescence microscope.

Dual-species or three-species biofilms were also subjected to fluorescence in situ hybridization (FISH) analysis by CSLM. Biofilms grown in glass-bottom microwell dishes were fixed with 4% paraformaldehyde (2 h), 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 16 rDNA-specific oligonucleotide probe for 150 min at 55 °C. The oligonucleotide probe sequences were 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 cover slips 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 upon the formamide concentration used during hybridization) for 15 min at 55 °C. After rinsing briefly in 0.9% NaCl the cover slips 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 one-way ANOVA followed by Tukey test respective to the biofilm combinations.

RESULTS

Planktonic interactions of *A. oris* and *S. oralis*

Coaggregation between various *A. oris* strains expressing type 2 fimbriae and *S. oralis* 34 has been well documented, and involves recognition of RPS on the surface of *S. oralis* by the type 2 fimbriae (Mishra *et al.*, 2010). To confirm that *A. oris* T14V, expressing type 1 or type 2 fimbriae (Cisar *et al.*, 1983), coaggregated with *S. oralis* 34, we performed dual-fluorescence coaggregation assays. The wild type strains formed a wide range of different sized coaggregates, some containing thousands of cells (Fig. 1A,B). An *A. oris* Fim⁻ strain lacking type 1 and type 2 fimbriae did not coaggregate with *S. oralis* (Fig. 1C), and an *S. oralis* RPS⁻ mutant did not interact with *A. oris* T14V or *A. oris* Fim⁻ (Fig. 1D,E).

Planktonic interactions of *A. oris*, *S. oralis* and *C. albicans*

A. oris T14V and *A. oris* Fim⁻ cells adhered to *C. albicans* hyphal filaments as single cells or clumps (Fig. 2A,B) and no specific regions of the filaments were targeted. Likewise, *S. oralis* 34 and *S. oralis* RPS⁻ cells interacted with hyphae (Fig. 2C,D). The RPS⁻ mutant strain formed shorter chains of cells than *S. oralis* wild type and more individual cells or cell pairs

adhered to the hyphal filaments. In three-species groupings, *A. oris* T14V and *S. oralis* 34 formed coaggregates as expected, and these coaggregates adhered to hyphae, in addition to single cell pairs or chains (Fig. 2E). Lack of fimbriae did not appear to affect the interaction of individual pairs or groups of *A. oris* Fim⁻ with *C. albicans* hyphae (Fig. 2F), or interaction of the accompanying *S. oralis* cells with hyphal filaments. *S. oralis* RPS⁻ cells also adhered to hyphal filaments (Fig. 2G) independently of *A. oris* T14V or Fim⁻ cells (Fig. 2H). A single hyphal filament was able to support adhesion of both actinomyces and streptococcal cells. Therefore the interactions of *A. oris* or *S. oralis* cells with *C. albicans* can occur independently of fimbriae or RPS, respectively, and there is seemingly no direct competition for binding sites on the candidal hyphae.

Biofilm temporal development

Development of monospecies biofilms or dual-species biofilms on salivary pellicle over 5-7 h was visualized by light microscopy. *C. albicans* cells that were adhered to the pellicle substratum had formed short hyphae at 2 h, and more extensive networks of hyphal filaments at 5 h and 7 h (Fig. 3A) as previously reported (Dutton et al., 2014). *A. oris* T14V cells formed a confluent biofilm by 5 h, but virtually no biofilm was formed by the *A. oris* Fim⁻ mutant (Fig. 3A), as reflected by biomass measurements (Fig. 4). When present in biofilms with *C. albicans*, *A. oris* T14V cells were relatively evenly distributed over the pellicle, as well as being associated with *C. albicans* hyphae (Fig. 3A). The *A. oris* Fim⁻ strain in this case formed a dual-species biofilm with *C. albicans* by adhering to the hyphal filaments. *S. oralis* 34 and RPS⁻ mutant both formed confluent monospecies biofilms by 5 h, but the RPS⁻ mutant biomass was significantly lower than strain 34 biomass (Fig. 4). In dual-species biofilms, the presence of the streptococci clearly stimulated formation of extensive networks of hyphal filaments (Fig. 3B). These results confirm that *A. oris* fimbriae and *S. oralis* RPS are not necessary for three-species biofilm formation with *C. albicans*, but that *A. oris* fimbriae are required for attachment to salivary pellicle in the absence of coaggregation partners.

Bacterial biofilm structure and architecture

Monospecies or dual-species bacterial biofilms developed on salivary pellicle were then analyzed by confocal scanning laser microscopy (CSLM). After 6 h, *A. oris* T14V produced a biofilm consisting of growth clusters (Fig. 5A), while the *A. oris* Fim⁻ mutant did not form a biofilm on pellicle (Fig. 5B), as previously noted (Fig. 3A). *S. oralis* 34 formed a dense biofilm of thickness ~15 µm that covered the pellicle surface uniformly (Fig. 5C), while the RPS⁻ mutant formed a more sparse and very much thinner biofilm (Fig. 5D). These images show detail not deduced from the light microscopy images of fixed and stained biofilms (Fig. 3). In dual-species bacterial biofilms the components were resolved by FISH. In *A. oris* T14V-*S. oralis* 34 biofilms, actinomyces and streptococci were interdigitated within a confluent biofilm (Fig. 5E). However, very little growth of the *A. oris* Fim⁻ mutant could be seen amongst *S. oralis* 34 (Fig. 5F) and biomass values of crystal violet-stained biofilms were significantly lower compared to dual species *A. oris* T14V-*S. oralis* 34 biofilms (Fig. 4). *A. oris* T14V formed luxuriant communities on the pellicle amongst the RPS⁻ streptococcal biofilm (Fig. 5G). There were no interspecies interactions occurring in these biofilms, in the absence of RPS, and the two strains coexisted side by side. In dual-species biofilms containing both mutants, no *A. oris* Fim⁻ cells were visible within the less dense biofilm of *S. oralis* RPS⁻ cells (Fig. 5H), and the biomass values of crystal violet-stained biofilms were significantly reduced compared to dual-species *A. oris* T14V-*S. oralis* 34 biofilms (Fig. 4).

Interkingdom biofilm architecture

In biofilms of the bacterial strains together with *C. albicans* the first thing to note is that, in the presence of *C. albicans*, all of the bacterial strains were able to form biofilms. *A. oris* T14V was well integrated with *C. albicans* with both organisms attached to the substratum (Fig. 6A), while *A. oris* Fim⁻ was also well established but not evidently bound to pellicle (Fig. 6B). Streptococcal strains covered the areas of pellicle not occupied by *C. albicans* to form basal layers (Fig. 6C,D), with the *C. albicans* hyphae emanating into the environment.

Biovolume measurements of these biofilms clearly show that *A. oris* Fim⁻ is incorporated well into biofilms with *C. albicans* (Fig. 7A). In addition, while *C. albicans* dominated the overall biovolume in dual-species biofilms, it was possible to detect increase in biovolume of *C. albicans* when *S. oralis* 34 cells were present (Fig. 7A).

In three-species biofilms, *C. albicans* and streptococci always predominated. *A. oris* T14V and *S. oralis* 34 colonized the pellicle with the upper surface layers of the biofilms almost completely consisting of *C. albicans* (Fig. 6E). This occurs presumably as a result of dispersed *C. albicans* cells recolonizing the biofilm by adhering to *S. oralis*. Biovolume measurements suggested that ~25% of the volume comprised streptococci while actinomyces comprised <10% of total biovolume (Fig. 7B). In biofilms containing *A. oris* Fim⁻, cells of the afimbrial mutant were associated with the *C. albicans* upper layers over a denser streptococcal basal layer (Fig. 6F). In biofilms containing the *S. oralis* RPS⁻ mutant, streptococci predominated within the basal layers together with clusters of *A. oris* T14V, similar to the effect observed in Fig. 5G, and there were fewer *C. albicans* (Fig. 6G).

Incorporation of *A. oris* T14V into *C. albicans*-*S. oralis* RPS⁻ mutant biofilms on the basis of biovolume measurements was ~10-fold reduced (Fig. 7B) compared with *C. albicans*-*S. oralis* 34 biofilms. In three-species biofilms of *S. oralis* RPS⁻, *A. oris* Fim⁻ and *C. albicans*, the *A. oris* Fim⁻ mutant was least well-incorporated of all the strains (Fig. 7B) and was associated only with the top layer of *C. albicans*, while the *S. oralis* RPS⁻ mutant formed a multi-layer biofilm on the pellicle substratum (Fig. 6H). Taken collectively the results suggest that *A. oris*, *S. oralis* and *C. albicans* are able to form robust polymicrobial biofilms. Furthermore, *C. albicans* can promote inclusion of bacteria into a biofilm even when the bacteria themselves do not physically coaggregate, or when they are impaired in attachment to salivary pellicle.

DISCUSSION

S. oralis is an early colonizer of the oral cavity in newborns (Pearce *et al.*, 1995) and of the salivary pellicle in dentate individuals (Diaz *et al.*, 2006). *Actinomyces* spp. are also found amongst the early colonizers of pellicle and are prominent components of the oral mucosal microbiota (Ellen, 1976). Therefore the presence of these bacterial species will almost always be an important consideration for *C. albicans* colonization of the oral cavity. A better understanding of these intermicrobial mechanisms and their capacity to influence *C. albicans* growth and persistence in the oral cavity may help to devise novel strategies to combat the wide range of infections caused by *C. albicans* in humans of all ages.

It is well documented that *S. oralis* 34 produces a type 1Gn RPS with the repeating structure $[-6\text{Gal}\beta 1 \rightarrow 6\text{GalNAc}\beta 1 \rightarrow 3\text{Gal}\alpha 1\text{-PO}_4^- \rightarrow 6\text{GalNAc}\alpha 1 \rightarrow 3\text{Rha}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow]_n$ (Yoshida *et al.*, 2006a), and that the GalNAc β 1-3Gal structure within the repeating unit is recognized by *A. oris* type 2 fimbriae (Cisar *et al.*, 1988). This mediates interbacterial adhesion, and salivary flow biofilms of *A. oris* and *S. oralis* were diminished with type 2 fimbriae negative or RPS⁻ mutants, respectively (Yoshida *et al.*, 2006b). However, in other studies utilizing salivary flow cell biofilms it was shown that *S. oralis* 34 or *A. oris* T14V were able only to form dual-species biofilms. Neither strain alone could form a biofilm under salivary flow, unlike the results presented here. Moreover *A. oris* and *S. oralis* were deficient in planktonic growth in saliva. Therefore nutrient limitations in these experiments were overcome by forming dual-species biofilms (Palmer *et al.*, 2001) and, under the conditions, interbacterial adhesion was essential for mutual metabolic benefit. By contrast, the experiments conducted in this current paper with a minimal growth medium under static conditions showed that RPS production by *S. oralis* was not essential for biofilm formation on salivary pellicle. However, biofilms of *S. oralis* RPS⁻ and *A. oris* T14V were comprised of patches of actinomyces and streptococci, as opposed to being interdigitated as within the *S. oralis*-*A. oris* wild-type biofilms. Thus, when nutritionally-sufficient conditions and available pellicle receptors are present, the ability to employ interbacterial adhesion is not absolutely necessary. On the other hand, afimbrial mutant strain *A. oris* Fim⁻ was unable to form a monospecies biofilm on salivary pellicle, and unable to form a mixed species biofilm with *S.*

oralis 34 or RPS⁻, consistent with the absence of both types of fimbriae. The *S. oralis* RPS⁻ biofilms were patchy and much thinner than wild-type, and so the RPS plays a role in cell-cell interactions and biofilm formation by *S. oralis* 34.

Interestingly, we did notice some interbacterial coaggregation of *A. oris* Fim⁻ and *S. oralis* 34 in the presence of *C. albicans* (Fig. 2). It is possible that there are additional coaggregation mechanisms between *A. oris* T14V and *S. oralis* 34 that are secondary to the fimbriae-RPS interaction. For example, we have recently shown that the *S. gordonii* SspB (antigen I/II-family) protein binds to a polysaccharide receptor present on *A. oris* T14V (Back *et al.*, 2015). *S. oralis* 34 also carries an antigen I/II protein-encoding gene, and so the *S. oralis* antigen I/II protein might act similarly. In addition, *A. oris* T14V carries cell-surface sialic acid (Jones *et al.*, 1986), which could be a target for other *S. oralis* adhesins such as serine-rich repeat proteins (Zhou and Wu, 2009).

Wild-type and mutant strains of *A. oris* or *S. oralis* were all able to bind *C. albicans* hyphal filaments. Fimbriae or RPS were clearly not requirements for interkingdom adhesion. Typically the bacteria, present as single cells, groups or coaggregates, bound to various sites along the *C. albicans* filaments. It is thought that *C. albicans* adhesins become clustered as nanodomains following their activation (Lipke *et al.*, 2012) and so these sites possibly represent adhesin clusters. The interaction of *S. gordonii* cells with *C. albicans* involves the streptococcal antigen I/II protein SspB binding to Als3 protein present on hyphal filaments (Silverman *et al.*, 2010). Again, since *S. oralis* 34 contains an antigen I/II family protein-encoding gene, it is possible that this protein drives the *S. oralis*-*C. albicans* interaction. Further work generating mutants and heterologous protein-expression strains would be required to confirm this. Als3 appears to be receptor for a range of different bacteria (Demuyser *et al.*, 2015) and could also be the target of *A. oris*. However, potential adhesins of *A. oris* aside from type 1 and type 2 fimbriae are currently unknown.

A. oris and *S. oralis* strains, wild type and mutants, were all able to form biofilms with *C. albicans*. The afimbriate mutant of *A. oris* was incorporated via interaction with *C. albicans*, since *A. oris* Fim⁻ cells were unable to adhere to pellicle (Fig. 5). The *S. oralis* RPS⁻

mutant formed thicker biofilms in the presence of *C. albicans* than in monospecies (Fig. 3 and Fig. 5). One explanation for this is that *C. albicans* directly provides additional nutrients or more conducive environmental conditions for the streptococci. Alternatively, it is possible that RPS is normally an additional nutrient source for *C. albicans* thus providing the fungus with a growth advantage. A wide range of hydrolases are produced by *C. albicans* in the presence or absence of streptococci (Polacheck *et al.*, 1987; Molloy *et al.*, 1994; Bramono *et al.*, 1995; Dutton *et al.*, 2015) and so the RPS could be broken down into sugar residues for uptake by the fungus.

In summary, *C. albicans* and *S. oralis* provide sites on pellicle for colonization by *A. oris*. In the absence of fimbriae production, *A. oris* is compromised in coaggregation with *S. oralis* (via type 2 fimbriae) and in adherence to salivary pellicle (via type 1 fimbriae), but is nevertheless incorporated into biofilms via binding to *C. albicans*. When *C. albicans* and *S. oralis* are mainly occupying pellicle binding sites, the *A. oris* cells tend to be found in the upper layers of the biofilm. Biofilm growth and hyphal filament production by *C. albicans* is enhanced by *S. oralis*. Under these conditions, the bacterial cells mainly occupy the pellicle binding sites and form a lower biofilm layer, while *C. albicans* forms the upper biofilm layers. We did not detect inhibition of *C. albicans* by *A. oris* in dual-species biofilms (Fig. 6) that has recently been shown for three *Actinomyces* species (Guo *et al.*, 2015). In fact there was growth synergy between *A. oris* and *C. albicans*, and between *S. oralis* and *C. albicans*, resulting in augmentation of total biofilm biomass in each case. We acknowledge that this present study utilized only three specific strains of microorganisms, but our results may be applicable generally to streptococci expressing RPS with Gal β 1-3GalNAc or GalNAc β 1-3Gal linkages that are recognized by *Actinomyces* type 2 fimbriae. There are many other coaggregation groupings of *Streptococcus* and *Actinomyces* (Kolenbrander *et al.*, 1983) that remain to be explored in the context of biofilm formation with *C. albicans*. The three-species biofilms that we describe here are thus able to become metabolically coordinated, with the adhesive interactions between the components determining their spatial distribution and overall architecture. Clearly *C. albicans* is well endowed with the ability to form a biofilm

directly on salivary pellicle, or to benefit from two early colonizing bacterial species forming a biofilm into which the fungus can be incorporated and thrive. Since *A. oris* and *S. oralis* are major colonizers of the buccal mucosa, they may also enhance *C. albicans* retention and growth at multiple oral cavity sites.

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The authors declare no conflict of interest

REFERENCES

- Al-Ahmad, A., Follo, M., Selzer, A.C., Hellwig, E., Hannig, M. and Hannig, C. (2009) Bacterial colonization of enamel in situ investigated using fluorescence in situ hybridization. *J Med Microbiol* **58**: 1359-1366.
- Al Mubarak, S., Robert, A.A., Baskaradoss, J.K. *et al.* (2013) The prevalence of oral *Candida* infections in periodontitis patients with type 2 diabetes mellitus. *J Infect Public Health* **6**: 296-301.
- Arzmi, M.H., Dashper, S., Catmull, D., Cirillo, N., Reynolds, E.C. and McCullough, M. (2015) Coaggregation of *Candida albicans*, *Actinomyces naeslundii* and *Streptococcus mutans* is *Candida albicans* strain dependent. *FEMS Yeast Res* **15**: fov038.
- Back, C.R., Douglas, S.K., Emerson, J.E., Nobbs, A.H. and Jenkinson, H.F. (2015) *Streptococcus gordonii* DL1 adhesin SspB V-region mediates coaggregation via receptor polysaccharide of *Actinomyces oris* T14V. *Mol Oral Microbiol* **30**: 411-424.
- Bamford, C.V., d'Mello, A., Nobbs, A.H., Dutton, L.C., Vickerman, M.M. and Jenkinson, H.F. (2009) *Streptococcus gordonii* modulates *Candida albicans* biofilm formation through intergeneric communication. *Infect Immun* **77**: 3696-3704.
- Bramono, K., Tsuboi, R. and Ogawa, H. (1995) A carbohydrate-degrading enzyme from *Candida albicans*: correlation between α -glucosidase activity and fungal growth. *Mycoses* **38**: 349-353.
- Campos, M.S., Marchini, L., Bernardes, L.A.S. *et al.* (2008) Biofilm microbial communities of denture stomatitis. *Oral Microbiol Immunol* **23**: 419-424.
- Canabarro, A., Valle, C., Farias, M.R., Santos, F.B., Lazera, M. and Wanke, B. (2013) Association of subgingival colonization of *Candida albicans* and other yeasts with severity of periodontitis. *J Periodontal Res* **48**: 428-432.
- Cannon, R.D. and Chaffin, W.L. (1999) Oral colonization by *Candida albicans*. *Crit Rev Oral Biol Med* **10**: 359-383.

- 432 Cavalcanti, Y.W., Morse, D.J., da Silva, W.J. *et al.* (2015) Virulence and pathogenicity of
 433 *Candida albicans* is enhanced in biofilms containing oral bacteria. *Biofouling* **31**: 27-38.
- 434 Chaffin, W.L. (2008) *Candida albicans* cell wall proteins. *Microbiol Mol Biol Rev* **72**: 495-544.
- 435 Cisar, J.O., Curl, S.H., Kolenbrander, P.E., Cisar, J.O. (1983) Specific absence of type 2
 436 fimbriae on a coaggregation-defective mutant of *Actinomyces viscosus* T14V. *Infect*
 437 *Immun* **40**: 759-765.
- 438 Cisar, J.O., Vatter, A.E., Clark, W.B. *et al.* (1988) Mutants of *Actinomyces viscosus* T14V
 439 lacking type 1, type 2, or both types of fimbriae. *Infect Immun* **56**: 2984-2989.
- 440 Clark, W.B., Beem, F.E., Nesbitt, W.E., Cisar, J.O., Tseng, C.C. and Levine, M.J. (1989)
 441 Pellicle receptors for *Actinomyces viscosus* type 1 fimbriae in vitro. *Infect Immun* **57**:
 442 3003-3008.
- 443 De Carvalho, F.G., Silva, D.S., Hebling, J., Spolidorio, L.C. and Spolidorio, D.M. (2006)
 444 Presence of mutans streptococci and *Candida* spp. in dental plaque/dentine of carious
 445 teeth and early childhood caries. *Arch Oral Biol* **51**: 1024-1028.
- 446 De Groot, P.W.J., Bader, O., de Boer, A.D., Weig, M. and Chauhan, N. (2013) Adhesins in
 447 human fungal pathogens: glue with plenty of stick. *Eukaryot Cell* **12**: 470-481.
- 448 Delaloye, J. and Calandra, T. (2014) Invasive candidiasis as a cause of sepsis in the
 449 critically ill patient. *Virulence* **5**: 161-169.
- 450 Demuyser, L., Jabra-Rizk, M.A. and Van Dijck, P. (2014) Microbial cell surface proteins and
 451 secreted metabolites involved in multispecies biofilms. *Pathog Dis* **70**: 219-230.
- 452 Diaz, P.I., Chalmers, N.I., Rickard, A.H. *et al.* (2006) Molecular characterization of subject-
 453 specific oral microflora during initial colonization of enamel. *Appl Environ Microbiol* **72**:
 454 2837-2848.
- 455 Diaz, P.I., Xie, Z., Sobue, T. *et al.* (2012) Synergistic interaction between *Candida albicans*
 456 and commensal oral streptococci in a novel in vitro mucosal model. *Infect Immun* **80**: 620-
 457 632.
- 458 Dige, I., Raarup, M.K., Nyengaard, J.R., Kilian, M. and Nyvad, B. (2009) *Actinomyces*
 459 *naeslundii* in initial dental biofilm formation. *Microbiology* **155**: 2116-2126.

- 460 Douglas, L.J. (2003) *Candida albicans* biofilms and their role in infection. *Trends Microbiol*
 461 **11**: 30-36.
- 462 Dutton, L.C., Nobbs, A.H., Jepson, K. *et al.* (2014) O-mannosylation in *Candida albicans*
 463 enables development of interkingdom biofilm communities. *mBio* **5**: e00911.
- 464 Dutton, L.C., Paszkiewicz, K.H., Silverman, R.J. *et al.* (2015) Transcriptional landscape of
 465 trans-kingdom communication between *Candida albicans* and *Streptococcus gordonii*.
 466 *Mol Oral Microbiol* **30**: doi: 10.1111/omi.12111 [Epub ahead of print].
- 467 Ellen, R.P. (1976) Establishment and distribution of *Actinomyces viscosus* and *Actinomyces*
 468 *naeslundii* in the human oral cavity. *Infect Immun* **14**: 1119-1124.
- 469 Falsetta, M.L., Klein, M.I., Colonne, P.M. *et al.* (2014) Symbiotic relationship between
 470 *Streptococcus mutans* and *Candida albicans* synergizes virulence of plaque biofilms in
 471 vivo. *Infect Immun* **82**: 1968-1981.
- 472 Figueira, M.H., Azul, A., Pinto, E. *et al.* (2007) Denture-related stomatitis: identification of
 473 aetiological and predisposing factors- a large cohort. *J Oral Rehabil* **34**:448-455.
- 474 Gibbons, R.J., Hay, D.I., Cisar, J.O. and Clark, W.B. (1988) Adsorbed salivary proline-rich
 475 protein 1 and statherin: receptors for type 1 fimbriae of *Actinomyces viscosus* T14V-J1 on
 476 apatitic surfaces. *Infect Immun* **56**: 2990-2993.
- 477 Grimaudo, N.J., Nesbitt, W.E. and Clark, W.B. (1996) Coaggregation of *Candida albicans*
 478 with oral *Actinomyces* species. *Oral Microbiol Immunol* **11**: 59-61.
- 479 Guo, Y., Wei, C., Liu, C. *et al.* (2015) Inhibitory effects of oral *Actinomyces* on the
 480 proliferation, virulence and biofilm formation of *Candida albicans*. *Arch Oral Biol* **60**: 1368-
 481 1374.
- 482 Jack, A.A., Daniels, D.E., Jepson, M.A. *et al.* (2015) *Streptococcus gordonii* comCDE
 483 (competence) operon modulates biofilm formation with *Candida albicans*. *Microbiology*
 484 **161**: 411-421.
- 485 Jenkinson, H.H. (2011) Beyond the oral microbiome. *Environ Microbiol* **13**: 3077-3087.
- 486 Jenkinson, H.F., Lala, H.C. and Shepherd, M.G. (1990) Coaggregation of *Streptococcus*
 487 *sanguis* and other streptococci with *Candida albicans*. *Infect Immun* **58**: 1429-1436.

- 488 Jones, A.H., Lee, C.C., Moncla, B.J., Robinovitch, M.R. and Birdsell, D.C. (1986) Surface
489 localization of sialic acid on *Actinomyces viscosus*. *J Gen Microbiol* **132**: 3381-3391.
- 490 Kolenbrander, P.E., Inouye, Y. and Holdeman, L.V. (1983) New *Actinomyces* and
491 *Streptococcus* coaggregation groups among human oral isolates from the same site.
492 *Infect Immun* **41**: 501-506.
- 493 Koo, H. and Bowen, W.H. (2014) *Candida albicans* and *Streptococcus mutans*: a potential
494 synergistic alliance to cause virulent tooth decay in children. *Future Microbiol* **9**: 1295-
495 1297.
- 496 Li, T., Khah, M.K., Slavnic, S., Johansson, I. and Strömberg, N. (2001) Different type1
497 fimbrial genes and tropisms of commensal and potentially pathogenic *Actinomyces* spp.
498 with different salivary acidic proline-rich protein and statherin ligand specificities. *Infect*
499 *Immun* **69**: 7224-7233.
- 500 Lipke, P.N., Garcia, M.C., Alsteens, D., Ramsook, C.B., Klotz, S.A. and Dufrêne, Y.F. (2012)
501 Strengthening relationships: amyloids create adhesion nanodomains in yeasts. *Trends*
502 *Microbiol* **20**: 59-65.
- 503 Marchant, S., Brailsford, S.R., Twomey, A.C., Roberts, G.J. and Beighton, D. (2001) The
504 predominant microflora of nursing caries lesions. *Caries Res* **35**: 397-406.
- 505 Martins, N., Ferreira, I.C., Barros, L., Silva, S. and Henriques, M. (2014) Candidiasis:
506 predisposing factors, prevention, diagnosis and alternative treatment. *Mycopathologia*
507 **177**: 223-240.
- 508 Mishra, A., Wu, C., Yang, J., Cisar, J.O., Das, A. and Ton-That, H. (2010) The *Actinomyces*
509 *oris* type 2 fimbrial shaft FimA mediates co-aggregation with oral streptococci,
510 adherences to red blood cells and biofilm development. *Mol Microbiol* **77**: 841-854.
- 511 Molloy, C., Cannon, R.D., Sullivan, P.A. and Shepherd, M.G. (1994) Purification and
512 characterization of two forms of *N*-acetylglucosaminidase from *Candida albicans* showing
513 widely different outer chain glycosylation. *Microbiology* **140**: 1543-1553.
- 514 Nobbs, A.H., Jenkinson, H.F. and Jakubovics, N.S. (2011) Stick to your gums: mechanisms
515 of oral microbial adherence. *J Dent Res* **90**: 1271-1278.

- 516 Nobbs, A.H., Lamont, R.J. and Jenkinson, H.F. (2009) *Streptococcus* adherence and
517 colonization. *Microbiol Mol Biol Rev* **73**: 407-450.
- 518 Nobbs, A.H., Vickerman, M.M. and Jenkinson, H.F. (2010) Heterologous expression of
519 *Candida albicans* cell wall-associated adhesins in *Saccharomyces cerevisiae* reveals
520 differential binding specificities in adherence and biofilm formation and in binding oral
521 *Streptococcus gordonii*. *Eukaryot Cell* **9**: 1622-1634.
- 522 Nyvad, B. and Kilian, M. (1987) Microbiology of the early colonization of human enamel and
523 root surfaces in vivo. *Scand J Dent Res* **96**: 369-80.
- 524 Palmer, R.J. Jr, Gordon, S.M., Cisar, J.O. and Kolenbrander, P.E. (2003) Coaggregation-
525 mediated interactions of streptococci and actinomyces detected in initial human dental
526 plaque. *J Bacteriol* **185**: 3400-3409.
- 527 Palmer, R.J. Jr, Kazmerzak, K., Hansen, M.C. *et al.* (2001) Mutualism versus independence:
528 strategies of mixed-species oral biofilms in vitro using saliva as the sole nitrogen source.
529 *Infect Immun* **69**: 5794-5804.
- 530 Pearce, C., Bowden, G.H., Evans, M. *et al.* (1995) Identification of pioneer viridans
531 streptococci in the oral cavity of human neonates. *J Med Microbiol* **42**: 67-72.
- 532 Polacheck, I., Melamed, M., Bercovier, H. and Salkin, I.F. (1987) β -Glucosidase in *Candida*
533 *albicans* and its application in yeast identification. *J Clin Microbiol* **25**: 907-910.
- 534 Poulain, C. (2015) *Candida albicans*, plasticity and pathogenesis. *Crit Rev Microbiol* **41**: 208-
535 217.
- 536 Ruhl, S., Sandberg, A.L. and Cisar, J.O. (2004) Salivary receptors for the proline-rich
537 protein-binding and lectin-like adhesins of oral actinomyces and streptococci. *J Dent Res*
538 **83**: 505-510.
- 539 Salerno, C., Pascale, M., Contaldo, M. *et al.* (2011) *Candida*-associated denture stomatitis.
540 *Med Oral Patol Oral Cir Bucal* **16**: e139-143.
- 541 Silverman, R.J., Nobbs, A.H., Vickerman, M.M. *et al.* (2010) Interaction of *Candida albicans*
542 cell wall Als3 protein with *Streptococcus gordonii* SspB adhesin promotes development of
543 mixed-species communities. *Infect Immun* **78**: 4644-4652.

- 544 Sztajer, H., Szafranski, S.P., Tomasch, J. *et al.* (2014) Cross-feeding and interkingdom
 545 communication in dual-species biofilms of *Streptococcus mutans* and *Candida albicans*.
 546 *ISME J* **8**: 2256-2271.
- 547 Thurnheer, T., Gmür, R. and Guggenheim, B. (2004) Multiplex FISH analysis of a six-
 548 species bacterial biofilm. *J Microbiol Methods* **56**: 37-47.
- 549 Urzúa, B., Hermosilla, G., Gamonal, J. *et al.* (2008) Yeast diversity in the oral microbiota of
 550 subjects with periodontitis: *Candida albicans* and *Candida dubliniensis* colonize the
 551 periodontal pockets. *Med Mycol* **46**: 783-793.
- 552 Wright, C.J., Burns, L.H., Jack, A.A. *et al.* (2013) Microbial interactions in building of
 553 communities. *Mol Oral Microbiol* **28**: 83-101.
- 554 Xu, H., Jenkinson, H.F. and Dongari-Bagtzoglou, A. (2014a) Innocent until proven guilty:
 555 mechanisms and roles of *Streptococcus-Candida* interactions in oral health and disease.
 556 *Mol Oral Microbiol* **29**: 99-116.
- 557 Xu, H., Sobue, T., Thompson, A. *et al.* (2014b) Streptococcal co-infection augments *Candida*
 558 pathogenicity by amplifying the mucosal inflammatory response. *Cell Microbiol* **16**: 214-
 559 231.
- 560 Yoshida, Y., Ganguly, S., Bush, C.A. and Cisar, J.O. (2006a) Molecular basis of L-rhamnose
 561 branch formation in streptococcal coaggregation receptor polypeptides. *J Bacteriol* **188**:
 562 4125-4130.
- 563 Yoshida, Y., Palmer, R.J., Yang, J., Kolenbrander, R.E. and Cisar, J.O. (2006b)
 564 Streptococcal receptor polysaccharides: recognition molecules for oral biofilm formation.
 565 *BMC Oral Health* **6** (Suppl 1): S12.
- 566 Zhou, M. and Wu, H. (2009) Glycosylation and biogenesis of a family of serine-rich bacterial
 567 adhesins. *Microbiology* **155**: 317-327.
- 568 Zijnge, V., van Leeuwen, M.B., Degener, J.E. *et al.* (2010) Oral biofilm architecture on natural
 569 teeth. *PLoS One* **5**: e9321.
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572

573 **Figure legends**

574 **Figure 1** Fluorescence micrographs of planktonic interactions between *S. oralis* and *A. oris*
 575 wild types and mutant strains. Streptococci were labelled with TRITC (red) and actinomyces
 576 with FITC (green), vortex-mixed and incubated for 1 h as described in Material and Methods.
 577 Very large aggregates of *S. oralis* 34 and *A. oris* T14V developed containing thousands of
 578 mixed species cells (Panel A) and smaller aggregates were composed of similar numbers of
 579 the two cell types (Panel B). There was no coaggregation observed between *S. oralis* 34 and
 580 *A. oris* Fim⁻ (Panel C), *S. oralis* 34 RPS⁻ and *A. oris* T14V (Panel D), or *S. oralis* RPS⁻ and *A.*
 581 *oris* Fim⁻ (Panel E). Scale bars = 20 µm.

582 **Figure 2** Fluorescence micrographs of *A. oris* and *S. oralis* wild types or mutant strains with
 583 *C. albicans*. In Panels A-D, bacteria have been labelled with FITC (green) and *C. albicans*
 584 with Calcofluor (blue). *A. oris* T14V (Panel A) and *A. oris* Fim⁻ (Panel B) bound to *C. albicans*
 585 as single cells (arrow a) or clumps (arrow b) to hyphae at no specific site. Likewise, *S. oralis*
 586 34 cells were often in chains (arrow c) that interacted with hyphae (Panel C), as did cell pairs
 587 of *S. oralis* RPS⁻ (Panel D). The RPS⁻ mutant strain formed shorter chains of cells than wild
 588 type and more individual cells or cell pairs adhered to the hyphal filaments (arrow d). In
 589 three-species experiments (Panels E-H), *S. oralis* was labelled with TRITC (red), *A. oris* with
 590 FITC (green) and *C. albicans* with Calcofluor (blue). Wild type strains of *A. oris* and *S. oralis*
 591 formed coaggregates (Panel E) that adhered to hyphae (arrow e), in addition to single cell
 592 pairs or chains (arrow a). Lack of fimbriae did not appear to affect the interaction of individual
 593 pairs or groups of *A. oris* (arrow a) or *S. oralis* (arrow c) with hyphal filaments (Panel F).
 594 Neither did lack of RPS in *S. oralis* (Panel G) appear to affect streptococcal interactions with
 595 hyphal filaments (arrow a), but interestingly there were a few coaggregates with *A. oris*
 596 observed in these experiments (arrow f). Cells of *S. oralis* RPS⁻ (arrow a) and *A. oris* Fim⁻
 597 (arrow b) were both able to interact with hyphae (Panel H) but as expected there was no
 598 bacterial coaggregation observed. Scale bars = 40 µm.

599 **Figure 3** Light micrographs of dual-species biofilms stained with crystal violet. In biofilms
 600 containing *C. albicans*, fungal cells were incubated at 37 °C with the pellicle surface for 2 h,
 601 removed and fresh medium was added, with or without bacteria, for 1 h. The medium was
 602 then removed, replaced by fresh medium, and biofilms were incubated for up to 7 h. For
 603 monospecies bacterial biofilms, bacterial cells were incubated with pellicle for 1 h, removed
 604 and replaced with fresh medium, and then incubated for up to 5 h. Panel A, *C. albicans* and
 605 *A. oris*. Note the following: there was virtually no biofilm formed by *A. oris* Fim⁻ after 5 h; the
 606 presence of *C. albicans* reduced the biofilm coverage by *A. oris* T14V at 7 h; conversely, *C.*
 607 *albicans* provided receptors for biofilm retention of *A. oris* Fim⁻ (inset bottom right hand panel
 608 shows coaggregates of *C. albicans* and *A. oris* Fim⁻). Panel B, *C. albicans* and *S. oralis*.
 609 Note the following: there was similar overall coverage of pellicle by *S. oralis* 34 and *S. oralis*
 610 RPS⁻ at 5 h, but strain 34 formed more clusters; hyphal filament formation by *C. albicans* was
 611 greatly enhanced by *S. oralis* 34 and *S. oralis* RPS⁻ at 7 h. Scale bars = 50 µm.

612 **Figure 4** Biomass values for monospecies or dual-species biofilms grown on salivary pellicle
 613 for 6 h at 37 °C. Biofilms were stained with crystal violet which was then released with 10%
 614 acetic acid for biomass quantification (OD₅₉₅). Error bars are ± SD for triplicates over two
 615 independent experiments (n = 2).

616 **Figure 5** CSLM images of monospecies or dual-species biofilms formed on salivary pellicle
 617 after 6 h at 37 °C. Biofilm images are presented in 3D (xyz) and side (xz) views. Panels A-D,
 618 bacteria were stained with FITC (green). Panels: A, *A. oris* T14V; B, *A. oris* Fim⁻; C, *S. oralis*
 619 34; D, *S. oralis* RPS⁻. *A. oris* T14V produced a biofilm consisting of clusters of growth (A)
 620 while the *A. oris* Fim⁻ mutant did not form a biofilm on pellicle (B). Note in (C) that the pellicle
 621 surface is covered uniformly by *S. oralis* 34, while the RPS⁻ mutant (D) showed a patchy
 622 distribution and a very much thinner biofilm. Panels E-H, dual-species biofilms of *A. oris*
 623 (green) and *S. oralis* (red) detected by FISH. Panels: E, *A. oris* T14V + *S. oralis* 34; F, *A.*
 624 *oris* Fim⁻ + *S. oralis* 34; G, *A. oris* T14V + *S. oralis* RPS⁻; H, *A. oris* Fim⁻ + *S. oralis* RPS⁻. In
 625 (E) the actinomyces and streptococci are interdigitated within a confluent biofilm, while in (F)

few *A. oris* Fim⁻ cells are visible. In (G), *A. oris* T14V grows luxuriantly in communities amongst the RPS⁻ streptococci, while in (H) no *A. oris* Fim⁻ cells are visible within the sparser biofilm of *S. oralis* RPS⁻ mutant. Scale bars = 50 μ m.

Figure 6 CLSM images of dual- or three-species biofilms of *A. oris* and *S. oralis* wild type and mutant strains with *C. albicans*. Biofilms were grown in YPTG medium as described in Materials and Methods for 6 h at 37 °C. All biofilms contained *C. albicans*. In Panels A-D, bacteria are stained green, *C. albicans* in red. Panels: A, *A. oris* T14V; B, *A. oris* Fim⁻; C, *S. oralis* 34; D, *S. oralis* RPS⁻; Note the distribution of *C. albicans* and *A. oris* within the layers of the biofilms (A), and in (B) where *A. oris* Fim⁻ is clearly incorporated, the predominance of *C. albicans* within the layer in contact with pellicle. Two distinct cellular layers were seen in combinations of *Candida* with streptococci (C and D). Panels E-H, three-species biofilms of *S. oralis* (red), *A. oris* (green) and *C. albicans* (blue) detected by FISH. Panels: E, *A. oris* T14V + *S. oralis* 34; F, *A. oris* Fim⁻ + *S. oralis* 34; G, *A. oris* T14V + *S. oralis* RPS⁻; H, *A. oris* Fim⁻ + *S. oralis* RPS⁻. Note that the wild type strains of bacteria colonized the pellicle together, with hyphal filaments of *C. albicans* predominantly in the upper layers (E). *A. oris* Fim⁻ cells were only barely visible in (F) while cells of *A. oris* T14V colonized the pellicle substratum in (G). *C. albicans* enabled the *A. oris* Fim⁻ strain to become incorporated into the biofilm with *S. oralis* RPS⁻. Scale bars = 50 μ m.

Figure 7 Biovolume measurements of individual components of monospecies or dual-species biofilms (panel A) and a three-species biofilm (Panel B), as shown in Figures 5 and 6. Since these are biovolume measurements from the individual biofilms there are no error bars. 3D Micrograph of *C. albicans* SC5314 monospecies biofilm is not reproduced in this paper as has been published elsewhere (Dutton *et al.*, 2014). Fluorescence was visualized by CSLM and quantified using Imaris® Bitplane software. Ca, *C. albicans*; Ao, *A. oris*; So, *S. oralis*.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7