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1 **Interactions between *Streptococcus oralis*, *Actinomyces***

2 ***oris*, and *Candida albicans* in the development of**

3 **multispecies oral microbial biofilms on salivary pellicle**

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16 Running header: Interkingdom biofilms on salivary pellicle

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SUMMARY

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

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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 (Zijnge *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 Galβ1-3GalNAc 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 Δ*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 NaH_2PO_4-H_3PO_4 buffer pH 7.0,

130 0.1% tryptone) (Silverman *et al.*, 2010), and suspended in YPT medium at OD_{600} 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 Na_2CO_3 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 OD_{600} 0.5 (2 x 10^8 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 OD_{600} 1.0 (approximately 1 x 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

167 YPTG (1 ml, ~2 x 10^8 cells ml^{-1}) or *A. oris* cell suspension (1 ml, ~2 x 10^8 cells ml^{-1}), or both

168 added separately one immediately after the other, and incubated for 1 h at 37 °C. The

169 bacterial cell suspensions were then removed, replaced with fresh YPTG medium (2 ml) and

170 incubation was continued for up to 5 h. Coverslips were removed, rinsed gently with

171 phosphate-buffered saline (PBS), dried, and stained with 0.5% crystal violet solution. The

172 biofilms were then visualized by light microscopy (Leica DMLB). For biomass estimations the

173 stain was released with 10% acetic acid and optical density at 595 nm (OD_{595}) was

174 measured with a microtiter plate reader (Dutton *et al.*, 2014).

175 **Biofilm development for confocal scanning laser microscopy (CSLM)**

176 Biofilms (3 ml final volume) were grown in glass-bottom microwell dishes (35 mm diam., 14

177 mm microwell) (MatTek Corporation, Ashland, Mass.) exactly as described above. For

178 visualizing *C. albicans* within biofilms, 2 µg Calcofluor ml^{-1} was incorporated into the YPTG

179 growth medium at the end of the experiment for 10 min to fluorescently label the fungal cells.

180 Biofilms containing single species of bacteria (with or without *C. albicans*) were fluorescently

181 labelled with FITC (10 µM, 20 min) at the end of the experiments. The dishes were washed

182 twice with PBS and the biofilms were visualized by CSLM with a Leica SP5-AOBS confocal

183 microscope attached to a Leica DM I6000 inverted epifluorescence microscope.

184 Dual-species or three-species biofilms were also subjected to fluorescence in situ

185 hybridization (FISH) analysis by CSLM. Biofilms grown in glass-bottom microwell dishes

186 were fixed with 4% paraformaldehyde (2 h), washed twice in PBS and then incubated in

187 hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl pH 8.0, 0.01% SDS, and 10-30%

188 formamide depending upon the probe) containing 5 µg ml^{-1} fluorescently-labelled 16 rDNA-

189 specific oligonucleotide probe for 150 min at 55 °C. The oligonucleotide probe sequences

190 were described by Thurnheer *et al.* (2004) and were labelled (Eurofins Genomic Services

191 Ltd., Wolverhampton, UK) as follows: *S. oralis* probe MIT447_488 (Alexa 488), *A. oris* probe

192 ANA103_647 (Alexa 647) and *C. albicans* EUK516_555 (Alexa 555). Following hybridization

193 the cover slips were incubated in washing buffer (20 mM Tris-HCl pH 7.5, 5 mM EDTA,

194   0.01% SDS, and between 159 and 636 mM NaCl depending upon the formamide

195   concentration used during hybridization) for 15 min at 55 °C. After rinsing briefly in 0.9%

196   NaCl the cover slips were inverted onto a glass slide to be visualized by CSLM. Volocity®

197   software was utilized to prepare three-dimensional (3D) images and Imaris® v7.5 software

198   (Bitplane AG, Zurich, Switzerland) was used to calculate biovolumes (μm^3).

199   **Statistical analysis**

200   Data were processed by Prism 6 software at a confidence level of 95% using one-way

201   ANOVA followed by Tukey test respective to the biofilm combinations.

202

203

204   **RESULTS**

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

206   Coaggregation between various *A. oris* strains expressing type 2 fimbriae and *S. oralis* 34

207   has been well documented, and involves recognition of RPS on the surface of *S. oralis* by

208   the type 2 fimbriae (Mishra *et al*., 2010). To confirm that *A. oris* T14V, expressing type 1 or

209   type 2 fimbriae (Cisar *et al*., 1983), coaggregated with *S. oralis* 34, we performed dual-

210   fluorescence coaggregation assays. The wild type strains formed a wide range of different

211   sized coaggregates, some containing thousands of cells (Fig. 1A,B). An *A. oris* Fim⁻ strain

212   lacking type 1 and type 2 fimbriae did not coaggregate with *S. oralis* (Fig. 1C), and an *S.*

213   *oralis* RPS⁻ mutant did not interact with *A. oris* T14V or *A. oris* Fim⁻ (Fig. 1D,E).

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

215   *A. oris* T14V and *A. oris* Fim⁻ cells adhered to *C. albicans* hyphal filaments as single cells or

216   clumps (Fig. 2A,B) and no specific regions of the filaments were targeted. Likewise, *S. oralis*

217   34 and *S. oralis* RPS⁻ cells interacted with hyphae (Fig. 2C,D). The RPS⁻ mutant strain

218   formed shorter chains of cells than *S. oralis* wild type and more individual cells or cell pairs

219   adhered to the hyphal filaments. In three-species groupings, *A. oris* T14V and *S. oralis* 34

220   formed coaggregates as expected, and these coaggregates adhered to hyphae, in addition

221   to single cell pairs or chains (Fig. 2E). Lack of fimbriae did not appear to affect the

222   interaction of individual pairs or groups of *A. oris* Fim⁻ with *C. albicans* hyphae (Fig. 2F), or

223   interaction of the accompanying *S. oralis* cells with hyphal filaments. *S. oralis* RPS⁻ cells also

224   adhered to hyphal filaments (Fig. 2G) independently of *A. oris* T14V or Fim⁻ cells (Fig. 2H). A

225   single hyphal filament was able to support adhesion of both actinomyces and streptococcal

226   cells. Therefore the interactions of *A. oris* or *S. oralis* cells with *C. albicans* can occur

227   independently of fimbriae or RPS, respectively, and there is seemingly no direct competition

228   for binding sites on the candidal hyphae.

229   **Biofilm temporal development**

230   Development of monospecies biofilms or dual-species biofilms on salivary pellicle over 5-7 h

231   was visualized by light microscopy. *C. albicans* cells that were adhered to the pellicle

232   substratum had formed short hyphae at 2 h, and more extensive networks of hyphal

233   filaments at 5 h and 7 h (Fig. 3A) as previously reported (Dutton et al., 2014). *A. oris* T14V

234   cells formed a confluent biofilm by 5 h, but virtually no biofilm was formed by the *A. oris* Fim⁻

235   mutant (Fig. 3A), as reflected by biomass measurements (Fig. 4). When present in biofilms

236   with *C. albicans*, *A. oris* T14V cells were relatively evenly distributed over the pellicle, as well

237   as being associated with *C. albicans* hyphae (Fig. 3A). The *A. oris* Fim⁻ strain in this case

238   formed a dual-species biofilm with *C. albicans* by adhering to the hyphal filaments. *S. oralis*

239   34 and RPS⁻ mutant both formed confluent monospecies biofilms by 5 h, but the RPS⁻

240   mutant biomass was significantly lower than strain 34 biomass (Fig. 4). In dual-species

241   biofilms, the presence of the streptococci clearly stimulated formation of extensive networks

242   of hyphal filaments (Fig. 3B). These results confirm that *A. oris* fimbriae and *S. oralis* RPS

243   are not necessary for three-species biofilm formation with *C. albicans*, but that *A. oris*

244   fimbriae are required for attachment to salivary pellicle in the absence of coaggregation

245   partners.

Bacterial biofilm structure and architecture

246

247 Monospecies or dual-species bacterial biofilms developed on salivary pellicle were then

248 analyzed by confocal scanning laser microscopy (CSLM). After 6 h, *A. oris* T14V produced a

249 biofilm consisting of growth clusters (Fig. 5A), while the *A. oris* Fim⁻ mutant did not form a

250 biofilm on pellicle (Fig. 5B), as previously noted (Fig. 3A). *S. oralis* 34 formed a dense biofilm

251 of thickness ~15 μm that covered the pellicle surface uniformly (Fig. 5C), while the RPS⁻

252 mutant formed a more sparse and very much thinner biofilm (Fig. 5D). These images show

253 detail not deduced from the light microscopy images of fixed and stained biofilms (Fig. 3). In

254 dual-species bacterial biofilms the components were resolved by FISH. In *A. oris* T14V-*S.*

255 *oralis* 34 biofilms, actinomyces and streptococci were interdigitated within a confluent biofilm

256 (Fig. 5E). However, very little growth of the *A. oris* Fim⁻ mutant could be seen amongst *S.*

257 *oralis* 34 (Fig. 5F) and biomass values of crystal violet-stained biofilms were significantly

258 lower compared to dual species *A. oris* T14V-*S. oralis* 34 biofilms (Fig. 4). *A. oris* T14V

259 formed luxuriant communities on the pellicle amongst the RPS⁻ streptococcal biofilm (Fig.

260 5G). There were no interspecies interactions occurring in these biofilms, in the absence of

261 RPS, and the two strains coexisted side by side. In dual-species biofilms containing both

262 mutants, no *A. oris* Fim⁻ cells were visible within the less dense biofilm of *S. oralis* RPS⁻ cells

263 (Fig. 5H), and the biomass values of crystal violet-stained biofilms were significantly reduced

264 compared to dual-species *A. oris* T14V-*S. oralis* 34 biofilms (Fig. 4).

Interkingdom biofilm architecture

265

266 In biofilms of the bacterial strains together with *C. albicans* the first thing to note is that, in

267 the presence of *C. albicans*, all of the bacterial strains were able to form biofilms. *A. oris*

268 T14V was well integrated with *C. albicans* with both organisms attached to the substratum

269 (Fig. 6A), while *A. oris* Fim⁻ was also well established but not evidently bound to pellicle (Fig.

270 6B). Streptococcal strains covered the areas of pellicle not occupied by *C. albicans* to form

271 basal layers (Fig. 6C,D), with the *C. albicans* hyphae emanating into the environment.

272   Biovolume measurements of these biofilms clearly show that *A. oris* Fim⁻ is incorporated well

273   into biofilms with *C. albicans* (Fig. 7A). In addition, while *C. albicans* dominated the overall

274   biovolume in dual-species biofilms, it was possible to detect increase in biovolume of *C.*

275   *albicans* when *S. oralis* 34 cells were present (Fig. 7A).

276   In three-species biofilms, *C. albicans* and streptococci always predominated. *A. oris*

277   T14V and *S. oralis* 34 colonized the pellicle with the upper surface layers of the biofilms

278   almost completely consisting of *C. albicans* (Fig. 6E). This occurs presumably as a result of

279   dispersed *C. albicans* cells recolonizing the biofilm by adhering to *S. oralis*. Biovolume

280   measurements suggested that ~25% of the volume comprised streptococci while

281   actinomyces comprised <10% of total biovolume (Fig. 7B). In biofilms containing *A. oris* Fim⁻,

282   cells of the afimbrial mutant were associated with the *C. albicans* upper layers over a denser

283   streptococcal basal layer (Fig. 6F). In biofilms containing the *S. oralis* RPS⁻ mutant,

284   streptococci predominated within the basal layers together with clusters of *A. oris* T14V,

285   similar to the effect observed in Fig. 5G, and there were fewer *C. albicans* (Fig. 6G).

286   Incorporation of *A. oris* T14V into *C. albicans*-*S. oralis* RPS⁻ mutant biofilms on the basis of

287   biovolume measurements was ~10-fold reduced (Fig. 7B) compared with *C. albicans*-*S.*

288   *oralis* 34 biofilms. In three-species biofilms of *S. oralis* RPS⁻, *A. oris* Fim⁻ and *C. albicans*, the

289   *A. oris* Fim⁻ mutant was least well-incorporated of all the strains (Fig. 7B) and was

290   associated only with the top layer of *C. albicans*, while the *S. oralis* RPS⁻ mutant formed a

291   multi-layer biofilm on the pellicle substratum (Fig. 6H). Taken collectively the results suggest

292   that *A. oris*, *S. oralis* and *C. albicans* are able to form robust polymicrobial biofilms.

293   Furthermore, *C. albicans* can promote inclusion of bacteria into a biofilm even when the

294   bacteria themselves do not physically coaggregate, or when they are impaired in attachment

295   to salivary pellicle.

296

297   **DISCUSSION**

298   *S. oralis* is an early colonizer of the oral cavity in newborns (Pearce *et al*., 1995) and of the

299   salivary pellicle in dentate individuals (Diaz *et al*., 2006). *Actinomyces* spp. are also found

300   amongst the early colonizers of pellicle and are prominent components of the oral mucosal

301   microbiota (Ellen, 1976). Therefore the presence of these bacterial species will almost

302   always be an important consideration for *C. albicans* colonization of the oral cavity. A better

303   understanding of these intermicrobial mechanisms and their capacity to influence *C. albicans*

304   growth and persistence in the oral cavity may help to devise novel strategies to combat the

305   wide range of infections caused by *C. albicans* in humans of all ages.

306   It is well documented that *S. oralis* 34 produces a type 1Gn RPS with the repeating

307   structure [-6Gal*f*β1→6GalNAcβ1→3Galα1-PO_4^-→6GalNAcα1→3Rhaβ1→4Glcβ1→]$_n$

308   (Yoshida *et al*., 2006a), and that the GalNAcβ1-3Gal structure within the repeating unit is

309   recognized by *A. oris* type 2 fimbriae (Cisar *et al*., 1988). This mediates interbacterial

310   adhesion, and salivary flow biofilms of *A. oris* and *S. oralis* were diminished with type 2

311   fimbriae negative or RPS$^-$ mutants, respectively (Yoshida *et al*., 2006b). However, in other

312   studies utilizing salivary flow cell biofilms it was shown that *S. oralis* 34 or *A. oris* T14V were

313   able only to form dual-species biofilms. Neither strain alone could form a biofilm under

314   salivary flow, unlike the results presented here. Moreover *A. oris* and *S. oralis* were deficient

315   in planktonic growth in saliva. Therefore nutrient limitations in these experiments were

316   overcome by forming dual-species biofilms (Palmer *et al*., 2001) and, under the conditions,

317   interbacterial adhesion was essential for mutual metabolic benefit. By contrast, the

318   experiments conducted in this current paper with a minimal growth medium under static

319   conditions showed that RPS production by *S. oralis* was not essential for biofilm formation

320   on salivary pellicle. However, biofilms of *S. oralis* RPS$^-$ and *A. oris* T14V were comprised of

321   patches of actinomyces and streptococci, as opposed to being interdigitated as within the *S.*

322   *oralis-A. oris* wild-type biofilms. Thus, when nutritionally-sufficient conditions and available

323   pellicle receptors are present, the ability to employ interbacterial adhesion is not absolutely

324   necessary. On the other hand, afimbrial mutant strain *A. oris* Fim$^-$ was unable to form a

325   monospecies biofilm on salivary pellicle, and unable to form a mixed species biofilm with *S.*

326   *oralis* 34 or RPS⁻, consistent with the absence of both types of fimbriae. The *S. oralis* RPS⁻

327   biofilms were patchy and much thinner than wild-type, and so the RPS plays a role in cell-

328   cell interactions and biofilm formation by *S. oralis* 34.

329   Interestingly, we did notice some interbacterial coaggregation of *A. oris* Fim⁻ and *S.*

330   *oralis* 34 in the presence of *C. albicans* (Fig. 2). It is possible that there are additional

331   coaggregation mechanisms between *A. oris* T14V and *S. oralis* 34 that are secondary to the

332   fimbriae-RPS interaction. For example, we have recently shown that the *S. gordonii* SspB

333   (antigen I/II-family) protein binds to a polysaccharide receptor present on *A. oris* T14V (Back

334   *et al.*, 2015). *S. oralis* 34 also carries an antigen I/II protein-encoding gene, and so the *S.*

335   *oralis* antigen I/II protein might act similarly. In addition, *A. oris* T14V carries cell-surface

336   sialic acid (Jones *et al.*, 1986), which could be a target for other *S. oralis* adhesins such as

337   serine-rich repeat proteins (Zhou and Wu, 2009).

338   Wild-type and mutant strains of *A. oris* or *S. oralis* were all able to bind *C. albicans*

339   hyphal filaments. Fimbriae or RPS were clearly not requirements for interkingdom adhesion.

340   Typically the bacteria, present as single cells, groups or coaggregates, bound to various

341   sites along the *C. albicans* filaments. It is thought that *C. albicans* adhesins become

342   clustered as nanodomains following their activation (Lipke *et al.*, 2012) and so these sites

343   possibly represent adhesin clusters. The interaction of *S. gordonii* cells with *C. albicans*

344   involves the streptococcal antigen I/II protein SspB binding to Als3 protein present on hyphal

345   filaments (Silverman *et al.*, 2010). Again, since *S. oralis* 34 contains an antigen I/II family

346   protein-encoding gene, it is possible that this protein drives the *S. oralis*-*C. albicans*

347   interaction. Further work generating mutants and heterologous protein-expression strains

348   would be required to confirm this. Als3 appears to be receptor for a range of different

349   bacteria (Demuyser *et al.*, 2015) and could also be the target of *A. oris*. However, potential

350   adhesins of *A. oris* aside from type 1 and type 2 fimbriae are currently unknown.

351   *A. oris* and *S. oralis* strains, wild type and mutants, were all able to form biofilms with

352   *C. albicans*. The afimbriate mutant of *A. oris* was incorporated via interaction with *C.*

353   *albicans*, since *A. oris* Fim⁻ cells were unable to adhere to pellicle (Fig. 5). The *S. oralis* RPS⁻

354 mutant formed thicker biofilms in the presence of *C. albicans* than in monospecies (Fig. 3

355 and Fig. 5). One explanation for this is that *C. albicans* directly provides additional nutrients

356 or more conducive environmental conditions for the streptococci. Alternatively, it is possible

357 that RPS is normally an additional nutrient source for *C. albicans* thus providing the fungus

358 with a growth advantage. A wide range of hydrolases are produced by *C. albicans* in the

359 presence or absence of streptococci (Polacheck *et al.*, 1987; Molloy *et al.*, 1994; Bramono *et*

360 *al.*, 1995; Dutton *et al.*, 2015) and so the RPS could be broken down into sugar residues for

361 uptake by the fungus.

362   In summary, *C. albicans* and *S. oralis* provide sites on pellicle for colonization by *A.*

363 *oris*. In the absence of fimbriae production, *A. oris* is compromised in coaggregation with *S.*

364 *oralis* (via type 2 fimbriae) and in adherence to salivary pellicle (via type 1 fimbriae), but is

365 nevertheless incorporated into biofilms via binding to *C. albicans*. When *C. albicans* and *S.*

366 *oralis* are mainly occupying pellicle binding sites, the *A. oris* cells tend to be found in the

367 upper layers of the biofilm. Biofilm growth and hyphal filament production by *C. albicans* is

368 enhanced by *S. oralis*. Under these conditions, the bacterial cells mainly occupy the pellicle

369 binding sites and form a lower biofilm layer, while *C. albicans* forms the upper biofilm layers.

370 We did not detect inhibition of *C. albicans* by *A. oris* in dual-species biofilms (Fig. 6) that has

371 recently been shown for three *Actinomyces* species (Guo *et al.*, 2015). In fact there was

372 growth synergy between *A. oris* and *C. albicans*, and between *S. oralis* and *C. albicans*,

373 resulting in augmentation of total biofilm biomass in each case. We acknowledge that this

374 present study utilized only three specific strains of microorganisms, but our results may be

375 applicable generally to streptococci expressing RPS with Galβ1-3GalNAc or GalNAcβ1-3Gal

376 linkages that are recognized by *Actinomyces* type 2 fimbriae. There are many other

377 coaggregation groupings of *Streptococcus* and *Actinomyces* (Kolenbrander *et al.,* 1983) that

378 remain to be explored in the context of biofilm formation with *C. albicans*. The three-species

379 biofilms that we describe here are thus able to become metabolically coordinated, with the

380 adhesive interactions between the components determining their spatial distribution and

381 overall architecture. Clearly *C. albicans* is well endowed with the ability to form a biofilm

382   directly on salivary pellicle, or to benefit from two early colonizing bacterial species forming a

383   biofilm into which the fungus can be incorporated and thrive. Since *A. oris* and *S. oralis* are

384   major colonizers of the buccal mucosa, they may also enhance *C. albicans* retention and

385   growth at multiple oral cavity sites.

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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 (OD_{595}). 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)

626 few *A. oris* Fim⁻ cells are visible. In (G), *A. oris* T14V grows luxuriantly in communities

627 amongst the RPS⁻ streptococci, while in (H) no *A. oris* Fim⁻ cells are visible within the

628 sparser biofilm of *S. oralis* RPS⁻ mutant. Scale bars = 50 µm.

629 **Figure 6** CLSM images of dual- or three-species biofilms of *A. oris* and *S. oralis* wild type

630 and mutant strains with *C. albicans.* Biofilms were grown in YPTG medium as described in

631 Materials and Methods for 6 h at 37 °C. All biofilms contained *C. albicans.* In Panels A-D,

632 bacteria are stained green, *C. albcans* in red. Panels: A, *A. oris* T14V; B, *A. oris* Fim⁻; C, *S.*

633 *oralis* 34; D, *S. oralis* RPS⁻; Note the distribution of *C. albicans* and *A. oris* within the layers

634 of the biofilms (A), and in (B) where *A. oris* Fim⁻ is clearly incorporated, the predominance of

635 *C. albicans* within the layer in contact with pellicle. Two distinct cellular layers were seen in

636 combinations of *Candida* with streptococci (C and D). Panels E-H, three-species biofilms of

637 *S. oralis* (red), *A. oris* (green) and *C. albicans* (blue) detected by FISH. Panels: E, *A. oris*

638 T14V + *S. oralis* 34; F, *A. oris* Fim⁻ + *S. oralis* 34; G, *A. oris* T14V + *S. oralis* RPS⁻; H, *A. oris*

639 Fim⁻ + *S. oralis* RPS⁻. Note that the wild type strains of bacteria colonized the pellicle

640 together, with hyphal filaments of *C. albicans* predominantly in the upper layers (E). *A. oris*

641 Fim⁻ cells were only barely visible in (F) while cells of *A. oris* T14V colonized the pellicle

642 substratum in (G). *C. albicans* enabled the *A. oris* Fim⁻ strain to become incorporated into

643 the biofilm with *S. oralis* RPS⁻. Scale bars = 50 µm.

644 **Figure 7** Biovolume measurements of individual components of monospecies or dual-

645 species biofilms (panel A) and a three-species biofilm (Panel B), as shown in Figures 5 and

646 6. Since these are biovolume measurements from the individual biofilms there are no error

647 bars. 3D Micrograph of *C. albicans* SC5314 monospecies biofilm is not reproduced in this

648 paper as has been published elsewhere (Dutton *et al.*, 2014). Fluorescence was visualized

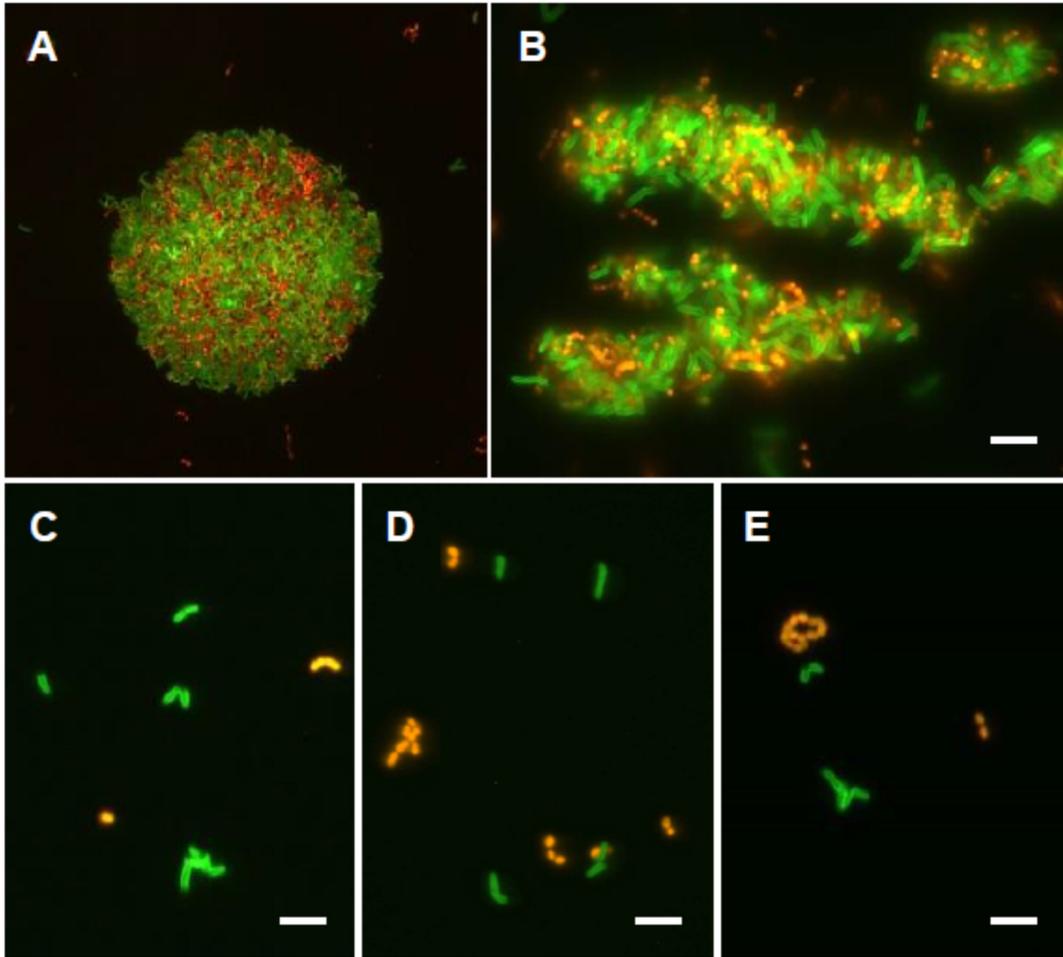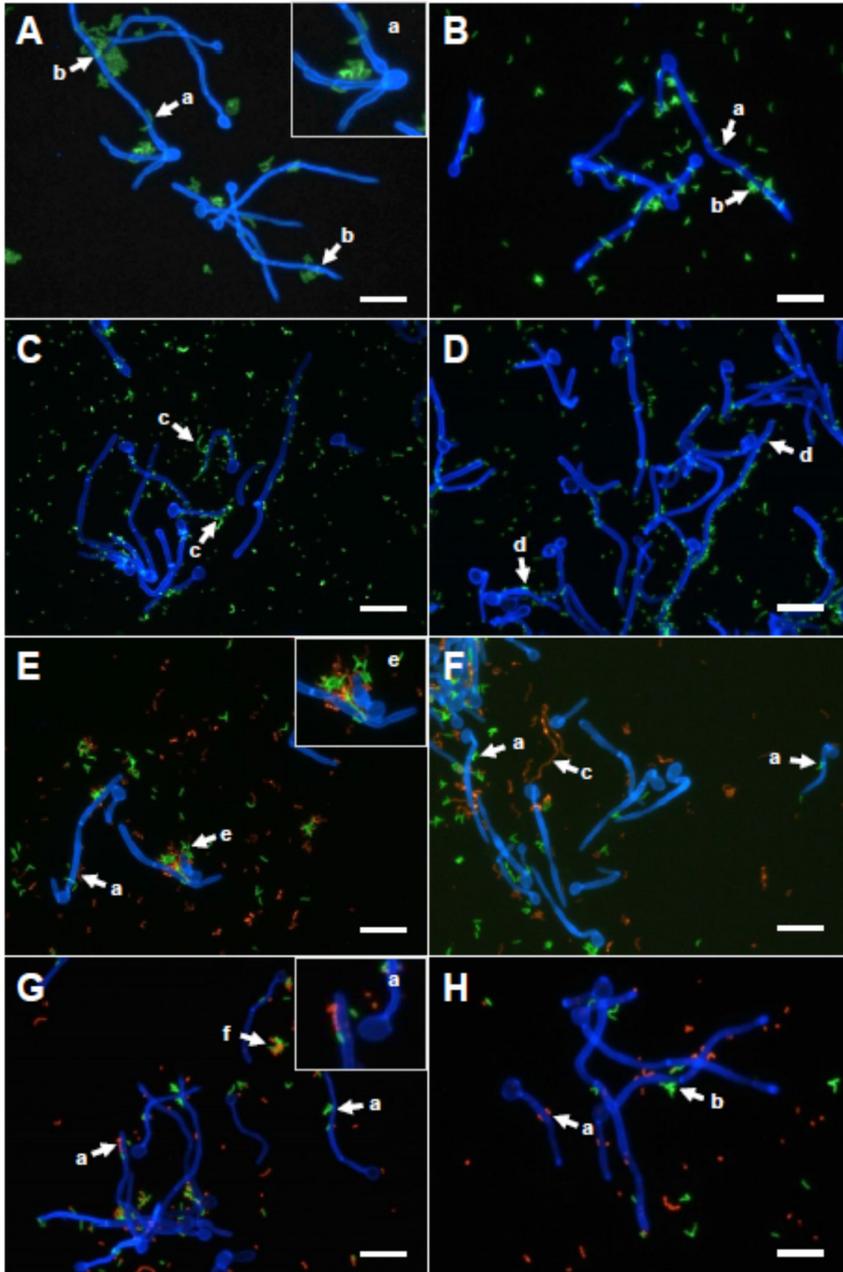649 by CSLM and quantified using Imaris® Bitplane software. *Ca*, *C. albicans*; *Ao*, *A. oris*; *So*, *S.*
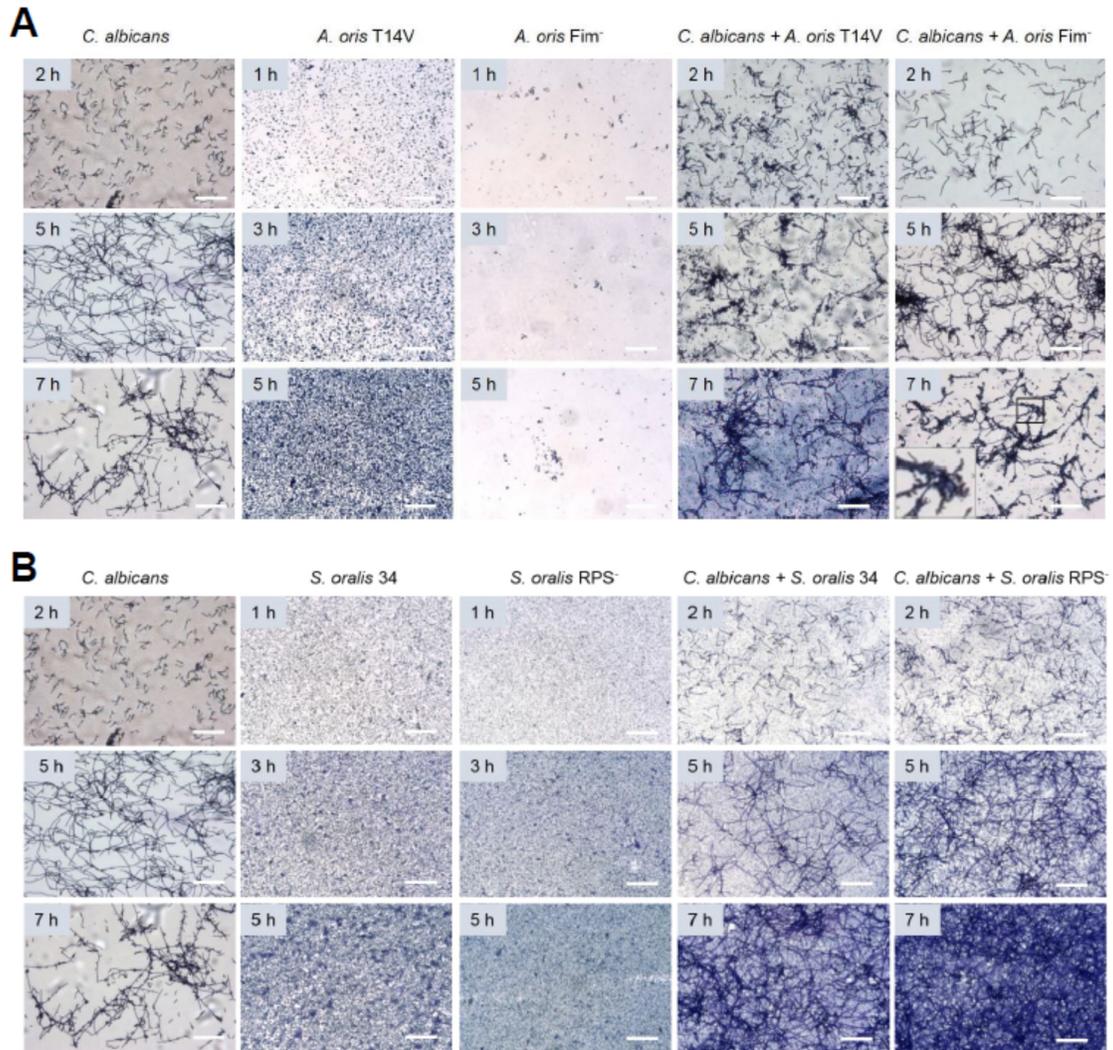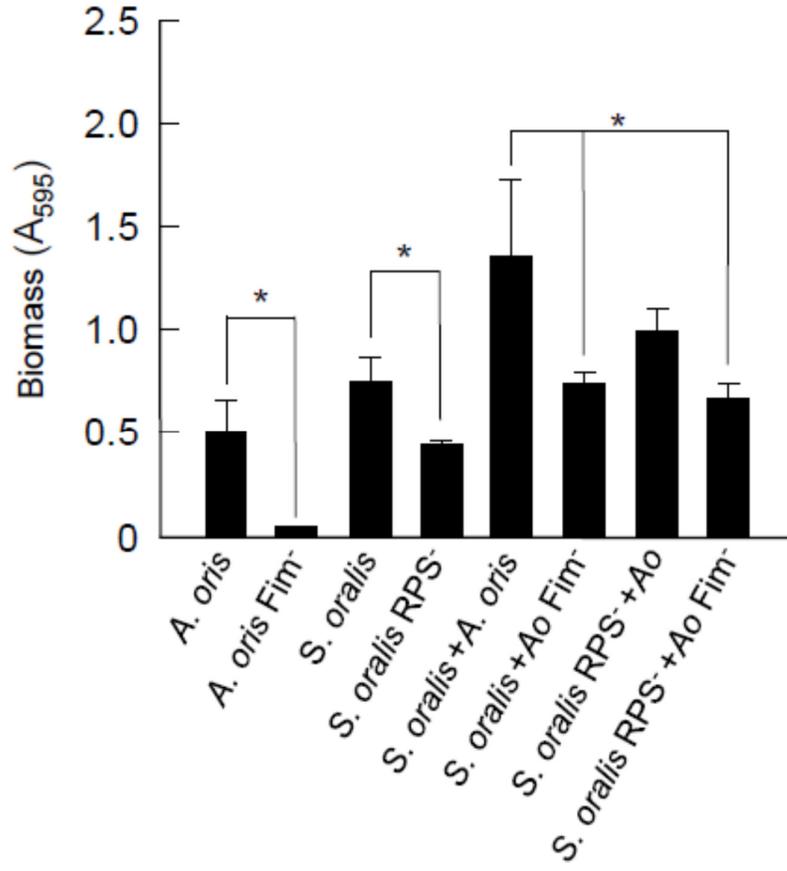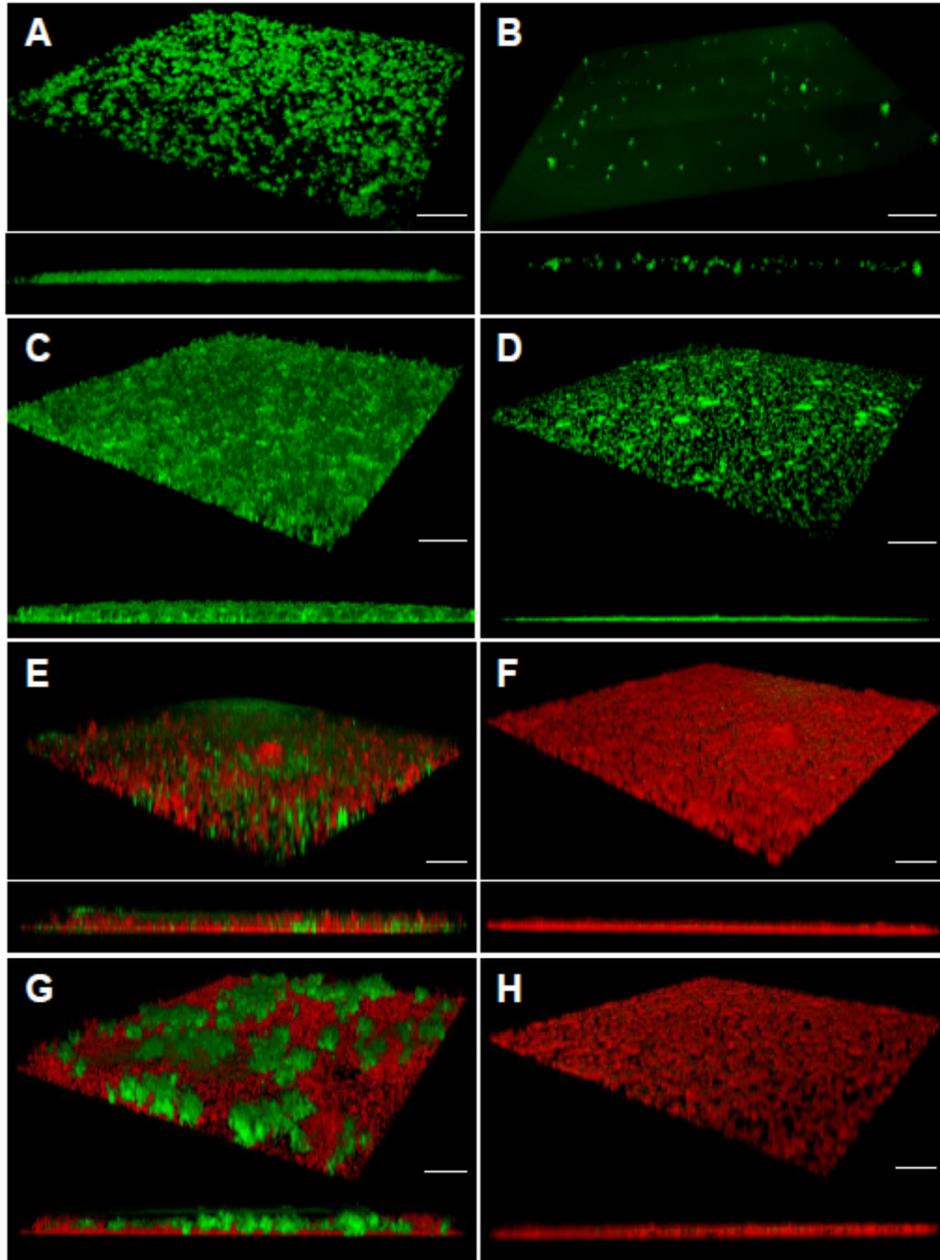
650 *oralis.*

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7