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1 ***Enterococcus faecalis* demonstrates pathogenicity through increased**
 2 **attachment in an *ex vivo* polymicrobial pulpal infection**

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 14 Running Head: *E. faecalis* pulpal pathogenicity and attachment

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26 **Abstract**

27 This study investigated the host response to a polymicrobial pulpal infection
28 consisting of *Streptococcus anginosus* and *Enterococcus faecalis*, bacteria
29 commonly implicated in dental abscesses and endodontic failure, using a validated
30 *ex vivo* rat tooth model. Tooth slices were inoculated with planktonic cultures of *S.*
31 *anginosus* or *E. faecalis* alone or in co-culture at ratios of 50:50 and 90:10 *S.*
32 *anginosus* to *E. faecalis*. Attachment was semi-quantified by measuring area
33 covered by fluorescently labelled bacteria. Host response was established by viable
34 histological cell counts and inflammatory response using RT-qPCR and
35 immunohistochemistry. A significant reduction in cell viability was observed for single
36 and polymicrobial infections, with no significant differences between infection types
37 (≈ 2000 cells/mm² for infected pulps compared to ≈ 4000 cells/mm² for uninfected
38 pulps). *E. faecalis* demonstrated significantly higher levels of attachment (6.5%)
39 compared to *S. anginosus* alone (2.3%) and mixed species infections (3.4% for
40 50:50 and 2.3% for 90:10), with a remarkable affinity to the pulpal vasculature.
41 Infections with *E. faecalis* demonstrated the greatest increase in TNF- α (47.1 fold for
42 *E. faecalis*, 14.6 fold for *S. anginosus*, 60.1 fold for 50:50 and 25.0 fold for 90:10)
43 and IL-1 β expression (54.8 fold for *E. faecalis*, 8.8 fold for *S. anginosus*, 54.5 fold for
44 50:50 and 39.9 fold for 90:10) when compared to uninfected samples.
45 Immunohistochemistry confirmed this with the majority of inflammation localised to
46 the pulpal vasculature and odontoblast regions. Interestingly, *E. faecalis* supernatant
47 and heat killed *E. faecalis* treatment was unable to induce the same inflammatory
48 response, suggesting *E. faecalis* pathogenicity in pulpitis is linked to its greater ability
49 to attach to the pulpal vasculature.

50

51 Introduction

52 The dental pulp is a complex environment composed of soft connective tissue,
53 nerves, blood vessels and a variety of cells, such as dental pulp stem cells,
54 fibroblasts and odontoblasts (1). When the pulp becomes inflamed in response to
55 bacterial infection or other stimuli, this is known as pulpitis. Early stages are
56 considered “reversible” and treatment involves removal of the stimulus, such as
57 carious lesions, in order to maintain pulp vitality. If untreated however, the microbial
58 invasion may progress into the deeper dentin and subsequently the pulpal chamber
59 resulting in severe tissue degradation and necrosis. This condition, known as
60 “irreversible pulpitis”, requires a challenging and difficult endodontic or root canal
61 treatment, which involves the removal of the pulp and obturation with an inert
62 material. The success rate of root canal treatments is highly variable, ranging from
63 31% to 96% depending on clinical considerations (2) and studies across a range of
64 countries have shown a high percentage (up to 67.9%) of patients who have
65 undergone this treatment subsequently develop apical periodontitis (3, 4). An
66 alternative endodontic treatment is vital pulpotomy, which involves removal of the
67 coronal pulp, leaving the radicular pulp vital and free of any pathological alterations
68 (5). Although this procedure is thought to require shorter appointment times and can
69 be accomplished in one visit, the efficacy of this technique is debated with success
70 rates of clinical studies ranging from 70% to 96% (6). Accurate models to better
71 understand the process of pulpal infection and to test the efficacy of novel
72 therapeutics will aid in the development of more effective vital pulp treatments. *In*
73 *vitro* monolayer cell culture models lack the complexity of the pulpal matrix, whilst *in*
74 *vivo* studies suffer from systemic factors, high costs and ethical considerations. To
75 overcome these limitations, Roberts et al. (7) developed an *ex vivo* co-culture

76 system to model pulpal infections on rat tooth slices. This study focused
77 predominantly on the *Streptococcus anginosus* group (SAG), consisting of *S.*
78 *anginosus*, *S. constellatus* and *S. intermedius*, Gram-positive cocci which are part of
79 the body's commensal flora. This group are known to be primary colonisers of the
80 oral cavity due to their ability to attach to the salivary pellicle and other oral bacteria
81 (8). They are considered opportunistic pathogens and have been reported to form
82 dental abscesses (9). The study by Roberts et al. demonstrated a significant
83 reduction in viable pulp cells, an increase in cytokine expression and bacterial
84 attachment over 24 hours as a result of *S. anginosus* infections (7).

85 Although Roberts et al. demonstrated invasion of the dental pulp by *S.*
86 *anginosus* group species, the number of microbial species encountered in the oral
87 cavity is far more diverse, with studies identifying between 100 to 300 different
88 species from different regions of the oral cavity of healthy individuals (10). It is
89 therefore unsurprising that complex mixed species microbiomes are often detected
90 in cases of pulpitis (11). As lesions progress into the tooth, a shift in microbial
91 species has been well documented due to environmental and nutritional changes
92 (12). Of particular interest is the *Enterococcus faecalis* species, a Gram-positive
93 facultative anaerobic coccus, also part of the normal human commensal flora (13). *E.*
94 *faecalis* has been shown to be pathogenic, particularly in endodontic failure (14) with
95 prevalence in such infections ranging from 24% up to 77% (15). Although highly
96 implicated in persistent endodontic failure, molecular studies have recently revealed
97 this species is frequently present in necrotic pulps, highlighting its potential role in
98 late-stage pulpitis (16, 17).

99 This study aims to use a validated *ex vivo* co-culture model to quantify and
100 better understand the host tissue response to mixed species pulpal infections

101 caused by *S. anginosus* and *E. faecalis*. Understanding the mechanism of complex
102 pulpal infections and the host inflammatory response may elucidate potential targets
103 for more effective vital pulp therapies.

104

105 **Results**

106 *Mixed species culture does not significantly influence S. anginosus and E. faecalis*
107 *growth rate.*

108 Growth characteristics in a simple mixed species planktonic broth culture were
109 investigated to ensure potential competitive growth between *S. anginosus* and *E.*
110 *faecalis* would not influence the *ex vivo* experiments investigating host tissue
111 response.

112 Clinical isolates of *S. anginosus* and *E. faecalis* species were selected from the
113 culture collection of the Oral Microbiology Unit, School of Dentistry at Cardiff
114 University. Species identity was confirmed by standard microbial identification tests
115 and 16S rRNA sequencing as described in the methods and supplemental materials
116 (Fig. S1 and S2).

117 Fig. 1 shows the planktonic growth curves for *S. anginosus* and *E. faecalis* alone
118 and in combination at ratios of 50:50 and 90:10 respectively over 24 hours in BHI. *E.*
119 *faecalis* reached mid-log phase earlier than *S. anginosus* (8 hours for *E. faecalis*
120 compared to 10 hours for *S. anginosus*). When cultured at a ratio of 50:50 however,
121 *S. anginosus* reached mid-log at a similar time to *E. faecalis* (10 hours). When the
122 bacteria were cultured at an *S. anginosus* to *E. faecalis* ratio of 90:10, *S. anginosus*
123 reached mid-log at approximately 8 hours and *E. faecalis* at approximately 12 hours.
124 Growth rate calculations during the log phase demonstrated no significant

125 differences between *E. faecalis* and *S. anginosus* under all culture conditions
126 ($p>0.05$, Table 1).

127

128 *E. faecalis* demonstrates greater levels of attachment to dental pulp than *S.*
129 *anginosus* at 24 hours, with particular affinity to the pulpal vasculature.

130 To assess differences in bacterial attachment to the dental pulp, the *ex vivo* rat
131 tooth model was infected with planktonic cultures of *S. anginosus* and *E. faecalis*
132 individually or as mixed species infections. Gram staining and fluorescent labelling
133 of bacteria were undertaken to localise and semi-quantify bacterial attachment.

134 High levels of bacterial attachment to the pulp were detected for tooth slices
135 incubated with *E. faecalis* (Fig. 2A) and mixed species of *S. anginosus* and *E.*
136 *faecalis* (Fig. 2B to 2C). Attachment was predominantly observed in intercellular
137 spaces within the pulpal matrix and around the pulpal vasculature. Bacteria were
138 also observed attached to soft tissue surrounding the tooth and within dentinal
139 tubules (Fig. 2D and 2E). Attachment of bacteria was not detected using Gram
140 staining on tooth slices incubated with *S. anginosus* alone.

141 Control samples demonstrated low levels of background fluorescence (Fig. 3A).
142 Infections consisting of *E. faecalis* alone had the greatest fluorescent signal, in
143 particular centred near the pulpal vasculature (Fig. 3B). *S. anginosus* demonstrated
144 low bacterial attachment, spread evenly across the pulp (Fig. 3C). When combining
145 *E. faecalis* and *S. anginosus*, higher levels of attachment were observed compared
146 to *S. anginosus* alone (Fig. 3D to 3E), with attachment again localised predominantly
147 to the pulpal vasculature. When the percentage bacterial coverage was semi-
148 quantified (Fig. 3F), the single species *E. faecalis* infection had significantly higher
149 levels of bacterial attachment when compared to *S. anginosus* alone (approximately

150 6.5% compared to 2%, $p=0.00021$) and the mixed species infections (50:50,
151 $p=0.0235$ and 90:10, $p=0.0032$).

152

153 *S. anginosus* and *E. faecalis* infections significantly reduce pulp cell viability with *E.*
154 *faecalis* infections inducing a significantly greater inflammatory response.

155 To establish the dental pulp host response to *S. anginosus* and *E. faecalis*
156 infections alone and as mixed species infections, histomorphometric analysis was
157 performed alongside RT-qPCR and immunohistochemistry for TNF- α and IL-1 β
158 expression.

159 Histological cell counts of the infected tooth sections demonstrated a significant
160 reduction ($p\leq 0.05$) in viable cells due to infection by both *E. faecalis* and *S.*
161 *anginosus* alone and in combination (Fig. 4A). There were no significant differences
162 in cell numbers between single species infections and multi-species infections.

163 All infected samples had significantly higher pro-inflammatory cytokine
164 expression, tumour necrosis factor alpha (TNF- α , Fig. 4B) and interleukin 1 beta (IL-
165 1 β , Fig. 4C), when compared to the control samples ($p\leq 0.05$). The single species
166 infection of *E. faecalis* resulted in significantly higher levels of TNF- α and IL-1 β
167 expression when compared to *S. anginosus* ($p=0.0276$ and $p=0.0234$ for TNF- α and
168 IL-1 β respectively). Combining *E. faecalis* and *S. anginosus* together did not result
169 in a significantly higher inflammatory response from the pulp when compared to *E.*
170 *faecalis* alone (for TNF- α $p=0.493$ and $p=0.096$ for 50:50 and 90:10 respectively and
171 for IL-1 β $p=0.988$ and $p=0.400$ for 50:50 and 90:10 respectively).

172 Negative controls replacing the primary TNF- α antibody with a nonimmune
173 immunoglobulin G control showed no immunopositivity (Fig. S3). Similarly, primary
174 exclusion controls were negative for staining, indicating specific binding of the

175 secondary antibody (Fig. S3). Control samples demonstrated low expression of
176 TNF- α and interestingly *S. anginosus* alone did not induce a high TNF- α response
177 (Fig. 4D). Samples incubated with *E. faecalis* alone or in combination with *S.*
178 *anginosus* had the most pronounced staining, both within the pulp (around the
179 vasculature) and the odontoblast layer. The level of TNF- α staining in these samples
180 was similar to those encountered in the rat lung positive control (Fig. S3).

181 Immunohistochemistry staining for IL-1 β , showed no positive signal for IgG
182 and primary exclusion controls (Fig. S3). Similar to the TNF- α
183 immunohistochemistry, the control sample and the sample incubated with *S.*
184 *anginosus* alone had few positively stained cells, whilst samples incubated with *E.*
185 *faecalis* alone and in combination with *S. anginosus* had more positively stained cells
186 (Fig. 4D). Although the level of staining was not as pronounced as observed with
187 TNF- α , the positive cells were again located adjacent to the pulpal vasculature and
188 similar in staining to the positive lung control (Fig. S3).

189
190 *Greater host inflammatory response to E. faecalis is not due to differences in water*
191 *soluble cell wall proteins or culture supernatants.*

192 To establish whether the increased host inflammatory response to *E. faecalis*
193 was due to specific water soluble cell proteins or components of the culture
194 supernatant, SDS-PAGE was performed to identify proteins in water soluble cell wall
195 proteins and culture supernatants. Similarly, heat killed *E. faecalis* and *E. faecalis*
196 supernatant was used to stimulate the pulp in order to assess the host response.

197 Few differences were observed between the water soluble cell wall proteins of *S.*
198 *anginosus* and *E. faecalis* when cultured alone and in combination with each other
199 (Fig. S4A). In terms of the culture supernatant, there was one band at approximately

200 35kDa observed with the *E. faecalis* cultures that was not observed with *S.*
201 *anginosus* (Fig. S4B).

202 When culturing the rat tooth slices with the *E. faecalis* supernatant or the heat
203 killed *E. faecalis*, no significant differences were observed in TNF- α expression when
204 compared to the untreated controls (Fig. 5A, $p=0.196$ and $p=0.152$ for supernatant
205 and heat killed *E. faecalis* respectively). A significant increase was observed in IL-1 β
206 expression for the tooth slices cultured with heat killed *E. faecalis* when compared to
207 the untreated controls (Fig. 5B, $p=0.041$) but not for *E. faecalis* supernatant
208 ($p=0.148$).

209 The negative controls (IgG control and primary exclusion) and the control sample
210 for the TNF- α immunohistochemistry did not show staining (Fig. S5). The tooth
211 slices incubated with *E. faecalis* supernatant had few cells stained positive for TNF- α ,
212 the majority of which was concentrated at the pulpal vasculature and odontoblast
213 layer (Fig. 5C). Similarly, the heat-killed *E. faecalis* had few cells expressing TNF- α
214 (Fig. 5C), whilst the lung positive control stained positive for TNF- α (Fig. S5).

215 The IgG control, the primary exclusion control and the untreated sample (Fig. S5)
216 did not stain positive for IL-1 β . Fewer cells were positive for IL-1 β than TNF- α (Fig.
217 5C). Samples treated with *E. faecalis* supernatant showed some cells stained
218 positive within the pulpal vasculature, whilst heat-killed *E. faecalis* showed few
219 positively stained cells. The positive lung control demonstrated cells stained positive
220 for IL-1 β expression (Fig. S5).

221

222 Discussion

223 This study has successfully employed an existing *ex vivo* rat tooth infection
224 model to study the effect of mixed species *E. faecalis* and *S. anginosus* pulpal
225 infections on cell viability, bacterial attachment and host inflammatory response.

226 By studying simple planktonic growth kinetics, it was established that *E. faecalis*
227 caused the *S. anginosus* bacteria to reach log phase at a more rapid rate. This
228 concept of polymicrobial synergy has been highlighted in recent work, which
229 investigated metabolite cross-feeding, whereby metabolic end-products produced by
230 one bacterium are consumed by a second community member (18-20). In particular,
231 this has been demonstrated for a similar oral pathogen, *Streptococcus gordonii*.
232 Lactate produced by *S. gordonii* as the primary metabolite during catabolism of
233 carbohydrates was found to support the growth of *Aggregatibacter*
234 *actinomycetemcomitans* (20). Interestingly, in a study using a primate model, the
235 addition of *E. faecalis* to a four-strain mixed species culture resulted in higher levels
236 of survival of all four bacteria than in the absence of *E. faecalis* (21). Another
237 mechanism of coordinating activities and communicating between microbial species
238 is quorum sensing, which has been shown to occur between different groups of
239 Streptococci (22). Although the rate of growth during the log phase was not altered
240 during mixed species planktonic culture in this study, it is important to appreciate that
241 under mixed species biofilm conditions, alterations in growth are likely to occur.

242 The mixed species infection did not result in higher levels of bacterial attachment
243 when compared to *E. faecalis* alone. The data suggests that *E. faecalis* is capable
244 of attaching to the dental pulp to a greater extent than *S. anginosus*, with a particular
245 affinity to the pulpal vasculature. This was not attributed to a more rapid rate of
246 growth or higher number of bacteria as a similar number of *S. anginosus* was

247 counted after 24 hours in planktonic broth culture. Similarly in the mixed species
248 culture where *S. anginosus* achieved log phase at an earlier time point, attachment
249 was not as high when compared to *E. faecalis* alone. The increased attachment
250 may therefore be due to differences between the species in terms of motility, sensing
251 or cell surface adhesins. *E. faecalis* and *S. anginosus* are classified as groups D
252 and F respectively using Lancefield grouping (23), a method of grouping based on
253 the carbohydrate antigens on the cell wall. These differences in surface
254 carbohydrates could mediate changes in attachment to epithelial cells as
255 demonstrated by Guzman et al. (24). A review by Fisher and Phillips (25)
256 highlighted *E. faecalis* specific cell wall components which play a vital role in
257 pathogenic adhesion. Aggregation substance (Agg) increases hydrophobicity and
258 aids adhesion to eukaryotic and prokaryote surfaces and also encourages the
259 formation of mixed-species biofilm through adherence to other bacteria.
260 Extracellular surface protein (ESP) promotes adhesion, antibiotic resistance and
261 biofilm formation. Adhesin to collagen of *E. faecalis* (ACE) is a collagen binding
262 protein belonging to the microbial surface components recognizing adhesive matrix
263 molecules (MSCRAMM) family. ACE plays a role in the pathogenesis of
264 endocarditis and *E. faecalis* mutants which do not express ACE have been shown to
265 have significantly reduced attachment to collagens type I and IV but not fibrinogen
266 (26, 27). Whilst *S. anginosus* has been shown to adhere to the extracellular matrix
267 components fibronectin, fibrinogen and laminin, binding to collagens type I and IV
268 was much less prominent (28). This is of particular interest in explaining differences
269 in pulpal adherence and the affinity of *E. faecalis* to localise near the pulpal
270 vasculature, as collagen fibres are often found in higher density around blood
271 vessels and nerves (29).

272 Although the level of cell death was the same between the groups tested,
273 infections consisting of *E. faecalis* alone produced a greater inflammatory response
274 when compared to *S. anginosus* and mixed species infections. This increase in
275 inflammation was not due to supernatant or water-soluble cell wall virulence factors
276 of *E. faecalis* as treatment of the dental pulp with these isolated factors did not yield
277 high levels of TNF- α and IL-1 β expression both at gene and protein level. Basic
278 analysis of supernatant and water soluble cell-wall proteins by SDS-PAGE showed
279 similar bands, however this may be due to the absence of serum or collagen
280 (present in the co-culture model) which has been shown to influence virulence factor
281 production, such as ACE (27). These results indicate the pulpal inflammation
282 caused by *E. faecalis* is likely due to the higher levels of attachment to the dental
283 pulp. Similar pathogenic traits have been established for *E. faecalis* in urinary tract
284 infections and endocarditis (30). Increased attachment to the dental pulp would
285 allow direct contact between cells and cell wall components such as lipoteichoic acid
286 (LTA), which induces activation of cluster of differentiation 14 (CD-14) and toll-like
287 receptor 2 (TLR-2) (31). An *in vivo* study, which infected canine pulp with
288 lipopolysaccharides (LPS) from *Escherichia coli* and lipoteichoic acid (LTA) from *E.*
289 *faecalis*, demonstrated LTA treatment led to pulp destruction, albeit to a lesser extent
290 than LPA (32). *In vitro* studies investigating macrophage responses to *E. faecalis*
291 LTA found that TNF- α expression was significantly increased in a dose-dependent
292 manner (33), with one study attributing it to the NF- κ B and p38 MAPK signalling
293 pathways (34). These studies however were performed using monolayer cultures,
294 allowing easy access for LTA to activate toll-like receptors, whereas the presence
295 extracellular matrix would limit penetration of virulence factors into the dental pulp *in*
296 *vivo*. Furthermore macrophages are normally present as monocytes in normal

297 healthy pulp and require a stimulus to become activated (35). Studies using
298 immunohistochemistry have shown these monocytes as well as dendritic cells to be
299 located predominantly around blood vessels, with few distributed throughout the pulp
300 (36, 37).

301 High levels of TNF- α expression were also observed in the odontoblast region
302 using immunohistochemistry. Due to its anatomical location, odontoblasts are the
303 first cells to encounter foreign antigens either through infiltration of virulence factors
304 through dentinal tubules or the breakdown of enamel and dentine. Through Gram
305 staining in this study, *E. faecalis* was observed within the dentinal tubules of the
306 infected tooth slices. This phenomenon has been previously reported in human
307 teeth (38). Odontoblasts, which line the dentine, have been shown to express TLRs
308 and play a role in the pulp's immune response, in particular to bacterial exotoxins
309 (39-41). This explains the high inflammatory response observed for both infections
310 and supernatant treatments when assessed using immunohistochemistry. Cytokine
311 gene expression using RT-qPCR however did not demonstrate higher levels when
312 treating the dental pulp with supernatants or heat killed bacteria. This may be
313 attributed to the fact that the methods employed for pulp extraction would be unlikely
314 to fully remove the odontoblast cells.

315 Although the host response to a mixed species infection consisting of *S.*
316 *anginosus* and *E. faecalis* has been established and the potential pathogenicity of *E.*
317 *faecalis* in pulpal infections has been elucidated, there are several limitations to this
318 study. The methods employed to fluorescently localise the bacteria could potentially
319 result in diffusion-related artefacts. More specific post-processing techniques, such
320 as fluorescent in-situ hybridization (FISH) probes may allow for more specific
321 identification, quantification and localisation of mixed species pulpal infections.

322 Whilst the *ex vivo* model offers a 3D organotypic culture setting, the static nature,
323 which lacks blood flow does not allow full observation of the systemic immune
324 response. Potential methods to overcome this may involve addition of monocytes
325 directly to the culture media and prolonged incubation times to stimulate repair
326 mechanisms. Closer examination of attachment mechanisms using ACE negative *E.*
327 *faecalis* mutants and purified LTA would also help fully establish the pathogenicity of
328 *E. faecalis* in pulpal infections. This will allow the model to be used to develop more
329 effective treatments for pulpitis by assessing the efficacy of antimicrobial and anti-
330 inflammatory treatments to inhibit bacterial colonisation.

331 In conclusion, this study has modelled a mixed species pulpal infection consisting
332 of *S. anginosus* and *E. faecalis* using a validated *ex vivo* rat tooth model. Although *E.*
333 *faecalis* caused *S. anginosus* to reach log growth phase more rapidly, the mixed
334 species infection did not result in higher cell death, attachment or inflammatory
335 response from the dental pulp. *E. faecalis* was found to elicit a much greater
336 inflammatory response, which was due to higher levels of attachment to the dental
337 pulp, with a particular affinity to the pulpal vasculature. Future work will focus on
338 assessing the mechanisms and attachment kinetics in order to elucidate the
339 molecular process and rate at which *E. faecalis* colonises the pulp.

340

341 **Materials and Methods**

342 *Materials*

343 All reagents including culture media, broths and agars were purchased from
344 Thermo Scientific (Leicestershire, UK) unless otherwise stated.

345

346 *Bacterial identification*

347 The *S. anginosus* and *E. faecalis* species studied were clinical isolates selected
348 from the culture collection of the Oral Microbiology Unit, School of Dentistry at Cardiff
349 University. To confirm the identity of the species, standard microbial identification
350 tests were performed by assessing: colony appearance on blood agar, Gram
351 staining, haemolysis, presence of catalase, lactose fermentation (MacConkey agar),
352 Lancefield grouping and bile aesculin agar growth.

353 16S rRNA sequencing was also performed on the *S. anginosus* and *E. faecalis*
354 clinical isolates to validate species identity. *S. anginosus* and *E. faecalis* were
355 cultured overnight in brain heart infusion (BHI) broth at 37°C, 5% CO₂. DNA was
356 extracted from using a QIAamp DNA Mini Kit (Qiagen, Manchester, UK), according
357 to the manufacturer's instructions. DNA was used in a PCR reaction using 16S
358 rRNA bacterial universal primers D88 (F primer; 5'-GAGAGTTTGATYMTGGCTCAG-
359 3') and E94 (R primer; 5'-GAAGGAGGTGWTCCARCCGCA-3') (42) and sequencing
360 of the products was performed by Central Biotechnology Services (Cardiff University)
361 using a 3130xl Genetic Analyser (Applied Biosystems). DNA sequences were
362 aligned with GenBank sequences using BLAST (NCBI) to establish percentage
363 sequence identity.

364

365 *Growth curves*

366 Overnight cultures of *S. anginosus* and *E. faecalis* in BHI broth were prepared
367 and diluted to 10⁸ colony forming units/mL (CFU/mL, absorbance at 600nm=0.08-
368 0.1). The inoculum was diluted in BHI to give a starting concentration of 10²
369 CFU/mL. Mixed species planktonic cultures with a total of 10² CFU/mL were
370 prepared consisting of 50% *S. anginosus* and 50% *E. faecalis* (herein referred to as
371 50:50) and 90% *S. anginosus* and 10% *E. faecalis* (herein referred to as 90:10). The

broths were incubated at 37°C, 5% CO₂ and 1mL aliquots removed every 4 hours for 24 hours. The absorbance of the aliquots was measured at 600nm using an Implen OD600 DiluPhotometer (München, Germany) and 50µL spiral plated on tryptic soya agar using a Don Whitley Automated Spiral Plater (West Yorkshire, UK). The remaining aliquot was then heat treated at 60°C for 30 minutes prior to spiral plating on bile aesculin agar containing 6.5%w/w sodium chloride. Heat treatment and the presence of high concentrations of bile and sodium chloride would only permit the growth of *E. faecalis* but not *S. anginosus* (43). Plates were incubated at 37°C, 5% CO₂ for 24 hours prior to counting. *E. faecalis* counts were subtracted from total counts to give the number of *S. anginosus* bacteria. Specific growth rate was calculated using the log phase of each growth curve and Equation 1, where μ is the growth rate in CFU/mL per hour, x is the CFU/mL at the end of the log phase, x_0 is the CFU/mL at the start of the log phase and t is the duration of the log phase in hours.

$$\mu = \frac{\ln(x-x_0)}{t} \quad [1]$$

Co-culture model

The co-culture rat tooth infection model was prepared as described by Roberts et al. (7). 28-day-old male Wistar rats were sacrificed under schedule 1 of the UK Animals Scientific Procedures Act, 1986 by a qualified technician at the Joint Biological Services Unit, Cardiff University for harvesting of tissue. Upper and lower incisors were extracted and the incisors were cut into 2mm thick transverse sections using a diamond-edged rotary bone saw (TAAB, Berkshire, UK). The sections were transferred to fresh sterile Dulbecco's Modified Eagle Medium (DMEM) for no more than 20 minutes before being cultured in 2mL DMEM, supplemented with 10%v/v

397 heat-inactivated fetal calf serum, 0.15mg/mL vitamin C, 200mmol/L L-glutamine,
398 100U/mL penicillin, 100µg/mL streptomycin sulphate and 250ng/mL amphotericin B
399 at 37°C, 5% CO₂ for 24 hours. Tooth slices were then washed in 2mL of phosphate
400 buffered saline (PBS), transferred to supplemented DMEM without antibiotics and
401 incubated overnight to remove traces of antibiotic. *S. anginosus* 39/2/14A and *E.*
402 *faecalis* were cultured to the log phase in BHI for 8-12 hours before dilution to 10²
403 CFU/mL in BHI. The bacteria were then used alone or combined for mixed species
404 infections (*S. anginosus* to *E. faecalis* ratios of 50:50 and 90:10 respectively). Forty
405 µL of 1%w/v fluorescein diacetate (FDA) in acetone was added to 2mL of the
406 bacterial suspension and incubated for 30 minutes at 37°C, 5% CO₂ before being
407 passed through a 0.22µm syringe-driven filter unit (Millipore, Oxford, UK). Bacteria
408 captured on the filter were then resuspended in 2mL sterile supplemented DMEM
409 without antibiotics and with 10%v/v BHI (herein referred to as DMEM-BHI) and used
410 to inoculate one tooth slice. Tooth slices were incubated with the bacteria at 37°C, 5%
411 CO₂ for 24 hours under constant agitation at 60rpm in the dark. Sterile DMEM-BHI
412 was used as a control. After incubation the tooth slices were processed for histology
413 in the dark. Tooth slices were fixed in 10%w/v neutral-buffered formalin at room
414 temperature for 24 hours. Slices were demineralized in 10%w/v formic acid at room
415 temperature for 72 hours; dehydrated through a series of 50%v/v, 70%v/v, 95%v/v,
416 and 100%v/v ethanol followed by 100%v/v xylene for five minutes each; and
417 embedded in paraffin wax. Sections 5µm thick were cut and viewed under a
418 fluorescent microscope with a FITC filter, with images captured using a Nikon digital
419 camera and ACT-1 imaging software (Nikon UK Ltd, Surrey, UK). To quantify cell
420 viability and structural degradation, sections were stained with hematoxylin and
421 eosin (H&E) prior to capturing images with a light microscope.

422

423 *Gram stain of tissue sections*

424 Gram stains of tooth slices were performed using a modified Brown and Brenn
425 method (44). Paraffin-embedded tooth slices were cut using a microtome into 5µm
426 sections and rehydrated through a series of xylene, 100, 95 and 70%v/v ethanol for
427 five minutes each. Sections were immersed in 0.2%w/v crystal violet for 1 minute,
428 rinsed with distilled water, immersed in Gram's iodine for 1 minute, rinsed with
429 distilled water, decolourised with acetone for 5 seconds and counterstained for 1
430 minute with basic fuchsin solution prior to washing with distilled water and mounting.
431 Light microscopy images were captured at x100 magnification using a Nikon digital
432 camera and ACT-1 imaging software (Nikon UK Ltd, Surrey, UK).

433

434 *Semi-quantification of cell viability by cell counts*

435 ImageJ (National Institutes of Health, Maryland USA) was used to count the
436 number of nuclei per pulp on stained histological sections. For each time point,
437 sections were cut from 5 tooth slices. Images were captured at x20 magnification
438 and combined using ImageJ software (Fig. S6). The blue field was extracted from
439 the images and the moments threshold method was applied to separate the pulp
440 cells. The watershed function was applied to split adjacent cell nuclei and the
441 number of particles ranging in size from 3 to 100µm² were counted. The data was
442 normalised to the pulpal area and standard errors of the mean were calculated.

443

444 *Semi-quantification of bacterial coverage*

445 ImageJ was used to quantify the area of the pulp inoculated with fluorescent
446 bacteria. The green field of the fluorescent image was extracted and the image

447 converted into a binary form using the moments threshold method. The pulpal area
448 was manually selected and the total area of the pulp measured. The area covered
449 by the fluorescent bacteria was then measured and calculated as a percentage of
450 the selected pulp area (Fig. S7).

451

452 *RT-qPCR of cytokines*

453 Four mm thick tooth slices were cultured as previously described for 24 hours
454 with either sterile DMEM-BHI as a control; DMEM-BHI inoculated with 10^2 CFU/mL
455 *S.anginosus* or *E. faecalis* or DMEM-BHI with a mixed species of *S.anginosus* or *E.*
456 *faecalis* (50:50 and 90:10 ratios respectively). After incubation, the tooth slice was
457 transferred to sterile PBS and the pulp removed by flushing the pulpal cavity with
458 PBS using a 0.1mm needle and syringe. RNA was extracted using TRIzol® Reagent
459 (ThermoFisher Scientific, Loughborough, UK) followed by RNase treatment
460 (Promega, Southampton, UK) according to the manufacturers' instructions.

461 Analysis of gene expression was performed in accordance to the Minimum
462 Information for publication of Quantitative real-time PCR Experiments (MIQE)
463 guidelines (45). RNA concentrations were determined using a NanoVue
464 Spectrophotometer (GE Healthcare Life Sciences, Buckinghamshire, UK). RNA
465 purity was determined by ensuring the ratio of absorbance at 260/280nm was above
466 1.8 and RNA quality checked by separating 1µg of RNA electrophoretically on a 2%
467 agarose gel containing SafeView (NBS Biologicals, Cambridgeshire, UK) in
468 Tris/Borate/EDTA buffer to ensure intact 28S and 18S rRNA bands using a Gel
469 Doc™ EZ System (BioRad, Hertfordshire, UK). Fig. S8 demonstrates RNA integrity
470 following extraction for samples tested.

471 Complementary DNA (cDNA) was synthesized by reverse transcription using
472 Promega reagents (Southampton, UK) in a G-Storm GS1 thermocycler (Somerton,
473 UK). One μg extracted RNA was combined with 1 μL random primer in a 15 μL
474 reaction in nuclease free water at 70°C for 5 minutes. This suspension was added to
475 5 μL MMLV reaction buffer, 1.25 μL deoxyribonucleotide triphosphates (10mM stock
476 dNTPSs), 0.6 μL RNasin, 1 μL MMLV enzyme and 2.15 μL nuclease free water and
477 incubated at 37°C for 1 hour.

478 The resultant cDNA was diluted 1:10 in nuclease free water (25ng cDNA).
479 Forward and reverse primers used are listed in Table 2. Ten μL of PrecisionFAST
480 qPCR SYBR Green MasterMix with low ROX (Primerdesign, Chandler's Ford, United
481 Kingdom) was combined with 2 μL of forward and 2 μL of reverse primers (3 μM) with
482 1 μL nuclease-free water prior to addition of 5 μL cDNA in BrightWhite Real-time PCR
483 FAST 96-well plates (Primerdesign, Chandler's Ford, United Kingdom). The plates
484 were subsequently heated to 95°C for 20 seconds; then 40 cycles of: 95°C for 1
485 second and 55°C for 20 seconds; followed by melt-curve analysis at 95°C for 15
486 seconds, 60°C for 60 seconds and 95°C for 15 seconds in a QuantStudio™ 6 Flex
487 Real-Time PCR System with QuantStudio Real-Time PCR Software (ThermoFisher
488 Scientific, Loughborough, UK). Relative TNF- α and IL-1 β gene expression was
489 calculated with beta actin (β -actin) as the reference gene and uninfected samples as
490 the control using the Livak method (46).

491 Primer specificity was ensured by the presence of single melt curve peaks (Fig.
492 S9) and by running products on agarose gels, as previously described, to confirm
493 single bands and correct product lengths (Fig. S10). Primer efficiency was between
494 90-110% for all primers used (Fig. S11) and determined using total rat RNA
495 converted to cDNA, as previously described, and serially diluted 1:4 in nuclease-free

496 water. Reference gene validation was performed by comparing gene stability across
497 all samples using NormFinder software (47). β -actin was found to be the most stable
498 reference gene (Fig. S12).

499

500 *TNF- α and IL-1 β Immunohistochemistry*

501 Immunohistochemical staining of the tooth slices for TNF- α and IL-1 β was
502 performed based on methods used by Smith et al (48). Rat lung was used as a
503 positive control for TNF- α and IL-1 β following fixation in 10%w/v neutral-buffered
504 formalin at room temperature for 24 hours, dehydration through a series of 50%v/v,
505 70%v/v, 95%v/v, and 100%v/v ethanol followed by 100%v/v xylene for five minutes
506 each; and embedding in paraffin wax. Paraffin-embedded tooth slices and lung
507 samples were cut using a microtome into 5 μ m sections and incubated on glass
508 slides at 65°C for one hour. The samples were subsequently rehydrated through a
509 series of xylene, 100%v/v, 95%v/v and 70%v/v ethanol and double-distilled water for
510 5 minutes each. Endogenous peroxidase activity within the tissue sections was
511 quenched by incubation in 3%w/v hydrogen peroxide for 10 minutes, followed by 2
512 washes for 2 minutes in tris-buffered saline (TBS). Non-specific binding was blocked
513 with 3%v/v normal horse serum (Vector laboratories, Peterborough, UK) in TBS for
514 30 minutes. Sections were incubated for 1 hour with primary antibodies for TNF- α
515 and IL-1 β (Santa Cruz Biotechnology, Heidelberg, Germany) diluted 1:50 in TBS
516 containing 1%w/v bovine serum albumin (Sigma Aldrich, Gillingham, UK).
517 Immunoreactivity was then performed using a Vectastain ABC peroxidase detection
518 kit (Vector laboratories, Peterborough, UK). Negative controls included omission of
519 the primary antibody and replacements of the primary antibody with immunoglobulin
520 G isotype diluted to the working concentration of the primary antibody. Sections

521 were counterstained with 0.05% light green for 30 seconds, dehydrated with 100%
522 ethanol and xylene for 10 minutes each and mounted using VectaMount Permanent
523 Mounting Medium (Vector laboratories, Peterborough, UK) prior to imaging using a
524 Nikon digital camera and ACT-1 imaging software (Nikon UK Ltd, Surrey, UK).

525

526 *SDS-PAGE of bacterial proteins*

527 An overnight culture of *S. anginosus* and *E. faecalis* in BHI was prepared and
528 diluted to 10^2 CFU/mL. *S. anginosus* and *E. faecalis* were cultured at 37°C, 5% CO₂
529 for 24 hours alone or in combination at a ratio of 50:50 and 90:10 respectively. The
530 suspensions were centrifuged at 5000g for 5 minutes. The supernatant was used for
531 analysis of supernatant proteins. The pellet was lysed in RIPA buffer by vortexing
532 for 30 seconds followed by 30 seconds ultrasonication at 50 Joules using a Branson
533 SLPe sonifier (Connecticut, USA). Protein concentrations in the supernatant and the
534 bacterial pellet were quantified using a BCA assay (ThermoFisher Scientific,
535 Loughborough, UK) and 20µg of protein in Laemmli buffer (Biorad, Hertfordshire, UK)
536 separated by SDS-PAGE at 200V for 40 minutes. Gels were stained using a Biorad
537 Silver Stain Plus Kit according to the manufacturer's instructions and imaged using a
538 Gel Doc™ EZ System (Biorad, Hertfordshire, UK).

539

540 *E. faecalis* supernatant and heat-killed *E. faecalis* treatments

541 An overnight culture of *E. faecalis* was diluted in 20mL DMEM-BHI media to give
542 a starting inoculum of 10^2 CFU/mL as previously described. After incubation for an
543 additional 24 hours at 37°C, 5% CO₂, the suspension was centrifuged at 5000g for 5
544 minutes. The supernatant was filtered through a 0.22µm syringe filter and frozen
545 overnight at -20°C before freeze drying for 24 hours using a ScanVac CoolSafe

546 freeze dryer (LaboGene, Lynge, Denmark). The pellet of bacteria was resuspended
547 in 20mL of PBS and centrifuged at 5000g for 5 minutes. This step was repeated
548 again to ensure minimal carryover of culture supernatant. The pellet was then
549 resuspended in 20mL DMEM-BHI and heated to 100°C for one hour. The solution
550 was then frozen overnight at -20°C before freeze drying as previously described.
551 20mL of sterile DMEM-BHI was also frozen and freeze dried as a control. All freeze
552 dried samples were individually resuspended in 20mL of sterile DMEM-BHI and used
553 to culture rat tooth slices for RT-qPCR of cytokines and immunohistochemistry of
554 TNF- α and IL-1 β as previously described.

555

556 *Statistical analysis*

557 A one-way analysis of variance (ANOVA) was performed using the data analysis
558 package in Excel (Microsoft, Reading, UK) to determine the relative significance of
559 the difference between the infected groups and the controls in terms of cell counts,
560 bacterial coverage and cytokine expression. The Tukey-Kramer test was used in
561 conjunction with ANOVA to compare the significant difference between all possible
562 pairs of means. $P \leq 0.05$ was considered significant.

563

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567

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730

Figure legends

732 Fig. 1: Growth curves of (A) *E. faecalis*, (B) *S. anginosus*, *E. faecalis* and *S.*
733 *anginosus* combined at a ratio of (C) 50:50 and (D) 90:10 respectively. Mean values
734 of three experimental repeats shown with error bars indicating standard deviation.

735

736 Fig. 2: Gram stain of tooth slices infected with (A) *E. faecalis*, (B) 50:50 *S.*
737 *anginosus* : *E. faecalis* and (C to E) 90:10 *S. anginosus* : *E. faecalis*. Arrows
738 highlight areas of bacterial attachment, P represents dental pulp, D represents
739 dentine and S represents soft tissue surrounding the tooth. Representative images
740 of three experimental repeats shown.

741

742 Fig. 3: Localisation of bacterial attachment by fluorescent microscopy for tooth slices
743 infected with: (A) No bacteria control, (B) *E. faecalis*, (C) *S. anginosus*, (D) 50:50 *S.*
744 *anginosus* : *E. faecalis* and (E) 90:10 *S. anginosus* : *E. faecalis*. P represents the
745 dental pulp, O the odontoblast region and D the dentine. Representative images of

746 three experimental repeats shown. (F) Bacterial coverage as quantified by area of
747 fluorescence relative to total pulp area (* $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$). Mean
748 values of three experimental repeats shown with error bars indicating standard error
749 of the mean.

750

751 Fig. 4: (A) Viable cells counted per mm^2 of pulp. Tooth slices infected with *E.*
752 *faecalis* and *S. anginosus*, both alone and in combination after 24 hours all resulted
753 in a significant reduction in viable cell number in the pulp when compared to the non-
754 infected control (* $p \leq 0.05$). Mean values of three experimental repeats shown with
755 error bars indicating standard error of the mean. Fold change in (B) TNF- α and (C)
756 IL-1 β gene expression as a result of *E. faecalis* and *S. anginosus* infections, alone
757 and in combination (* $p \leq 0.05$, ** $p \leq 0.01$ compared to control samples and * $p \leq 0.05$ and
758 ** $p \leq 0.01$). Mean values of three experimental repeats shown with error bars
759 indicating standard error of the mean. (D) Immunohistochemistry of TNF- α and IL-
760 1 β for control samples and tooth slices infected with *S. anginosus*, *E. faecalis*, 50:50
761 *S. anginosus* : *E. faecalis* and 90:10 *S. anginosus* : *E. faecalis*, Representative
762 images of three experimental repeats shown.

763

764 Fig.5: Fold change in (A) TNF- α and (B) IL-1 β gene expression relative to β -actin as
765 a result of treating tooth slices with *E. faecalis* supernatant and heat-killed *E. faecalis*
766 (* $p \leq 0.05$ compared to control samples). Mean values of three experimental repeats
767 shown with error bars indicating standard error of the mean. (C)
768 Immunohistochemistry of TNF- α and IL-1 β for control samples and tooth slices
769 infected with *E. faecalis* supernatant and heat-killed *E. faecalis*. Representative
770 images of three experimental repeats shown.

Fig. 1

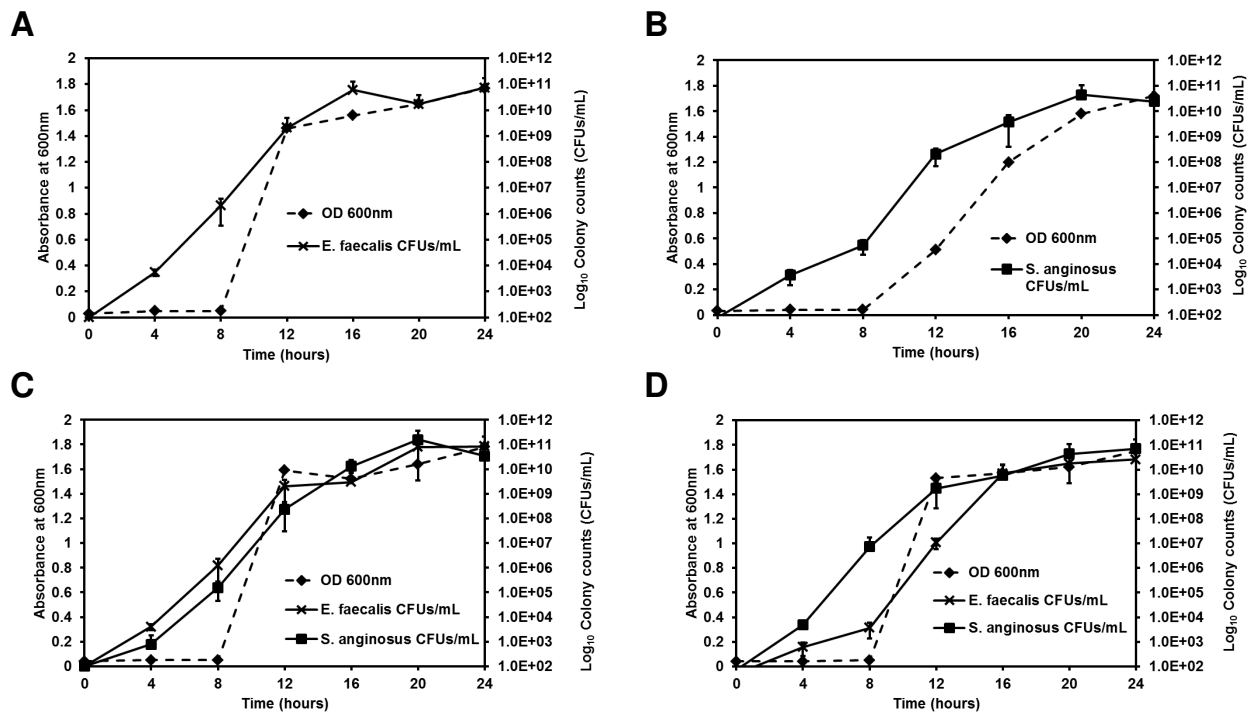


Fig. 2

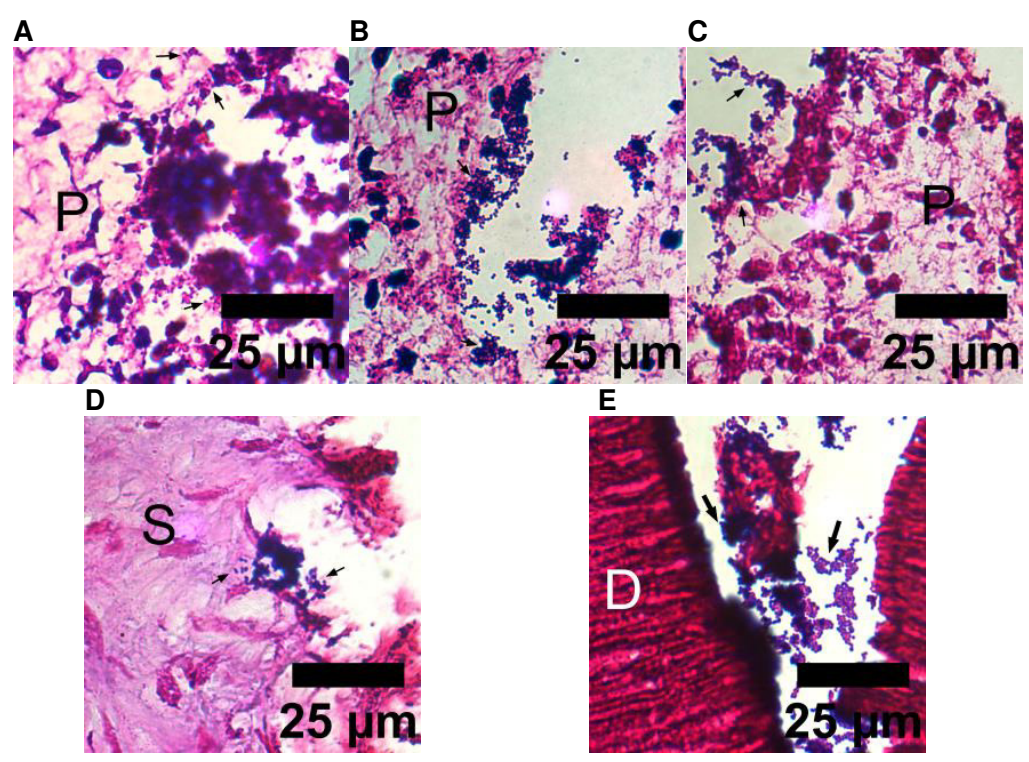


Fig. 3

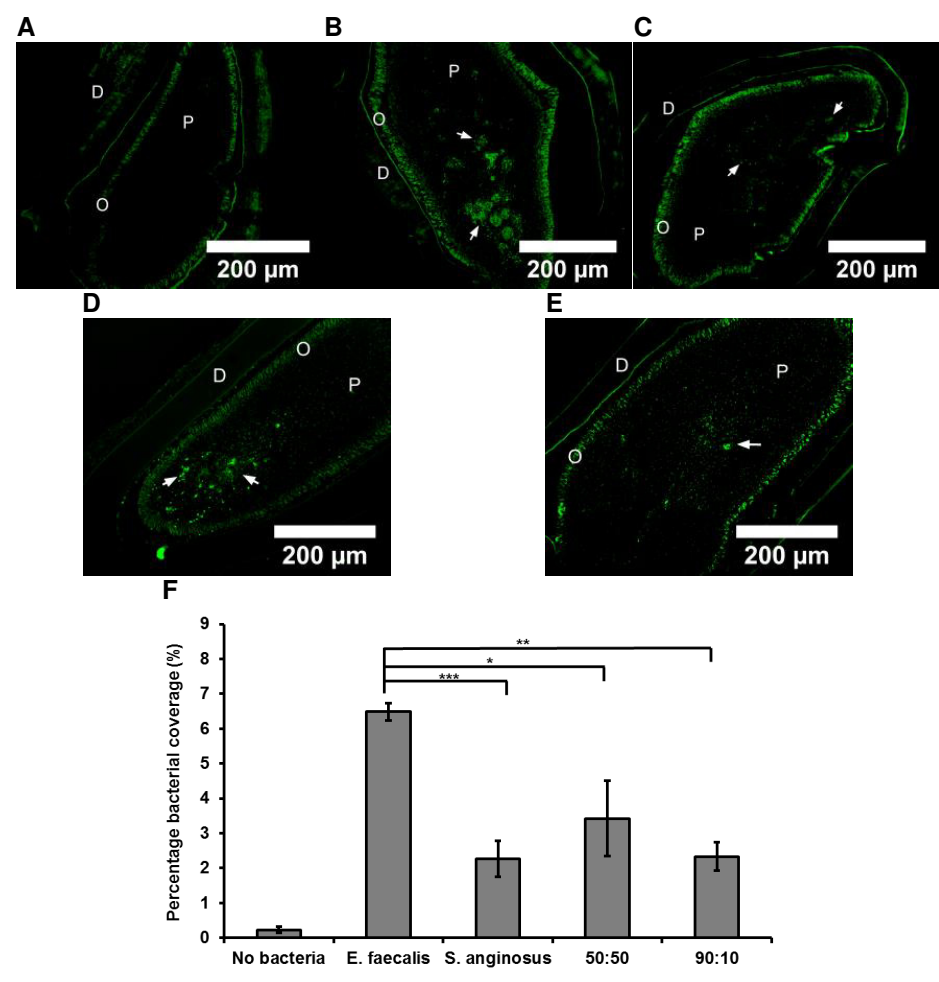


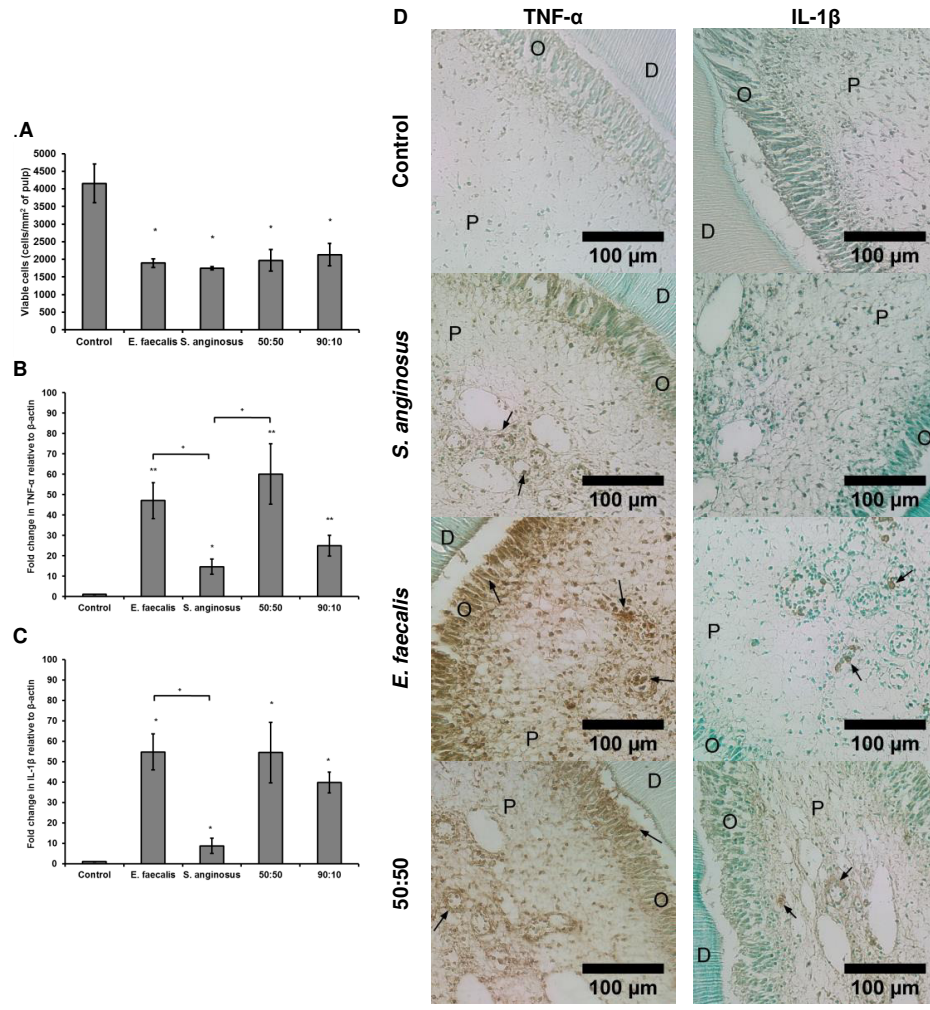
Fig. 4

Fig. 5

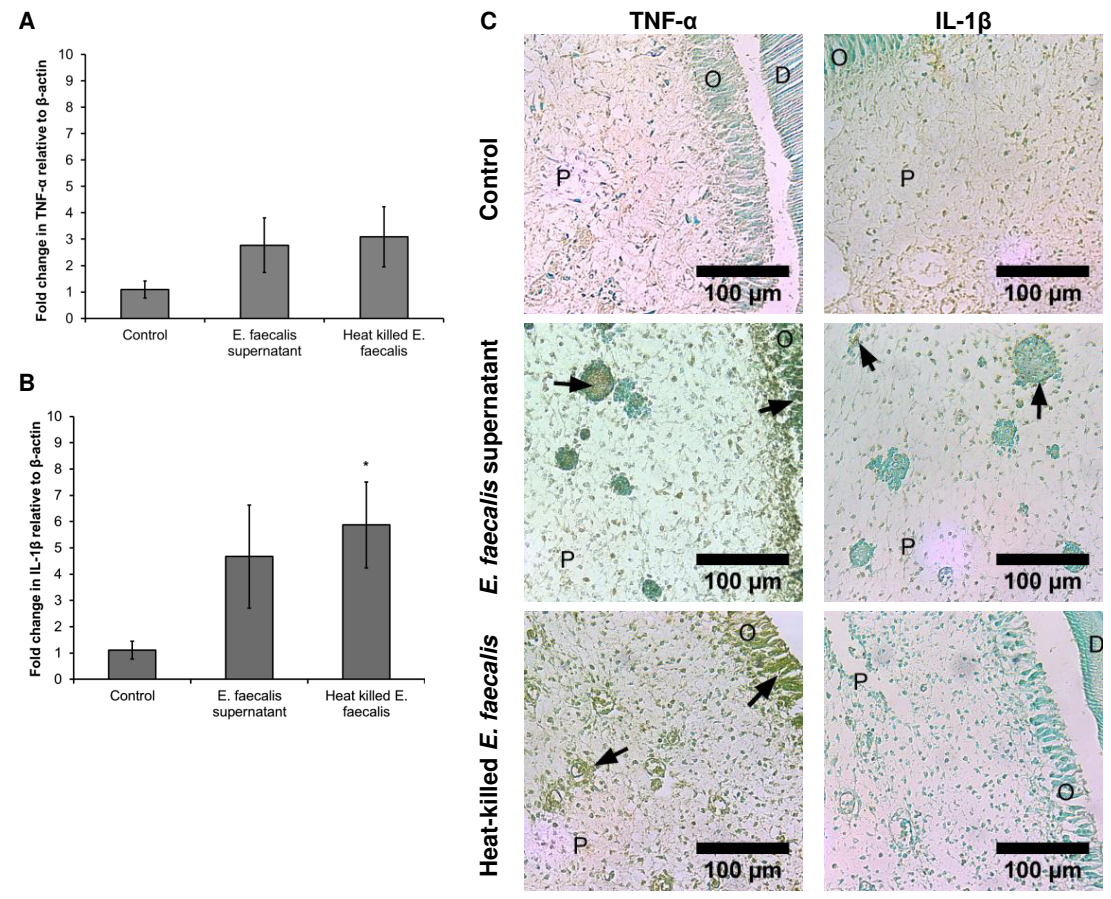


Table 1: Growth rates during the log phase of *S. anginosus* and *E. faecalis* alone and in combination at ratio of 50:50 and 90:10 respectively.

	Average growth rate during log phase (CFUs/mL per hour)	Standard deviation
<i>E. faecalis</i> (alone)	1.51	0.20
<i>S. anginosus</i> (alone)	2.00	0.25
<i>E. faecalis</i> (50:50)	1.62	0.10
<i>S. anginosus</i> (50:50)	1.53	0.14
<i>E. faecalis</i> (90:10)	1.98	0.12
<i>S. anginosus</i> (90:10)	1.57	0.12

Table 2: Primer sequences used for qPCR analysis

Gene	Primer sequence (5'-3')	Product length (Bp)	Melting temperature (°C)	Efficiency (%)	Source
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH - NM_017008.4)	Forward – GCA AGA GAG AGG CCC TCA G Reverse – TGT GAG GGA GAT GCT CAG TG	74	61.0 59.4	106.37	(48)
Beta-actin (β-actin - NM_031144.3)	Forward – TGA AGA TCA AGA TCA TTG CTC CTC C Reverse – CTA GAA GCA TTT GCG GTG GAC GAT G	155	60.69 64.37	108.56	(49)
Hypoxanthine Phosphoribosyltransferase 1 (HPRT-1 - NM_012583.2)	Forward – TGT TTG TGT CAT CAG CGA AAG TG Reverse – ATT CAA CTT GCC GCT GTC TTT TA	66	60.24 59.43	91.71	(50)
Ribosomal Protein L13a (RPL13a - NM_173340.2)	Forward – GGA TCC CTC CAC CCT ATG ACA Reverse – CTG GTA CTT CCA CCC GAC CTC	131	61.8 63.7	99.99	(51)
18s ribosomal RNA (18s rRNA – V01270)	Forward – AAA CGG CTA CCA CAT CCA AG Reverse – TTG CCC TCC AAT GGA TCC T	159	57.3 56.7	90.22	(52)
Tumor necrosis factor alpha (TNF-α - NM_012675.3)	Forward – AAA TGG GCT CCC TCT CAT CAG TTC Reverse – TCT GCT TGG TGG TTT GCT ACG AC	111	62.7 62.4	90.28	(53)
Interleukin 1 beta (IL-1β - NM_031512.2)	Forward – ATG CCT CGT GCT GTC TGA CCC ATG TGA G Reverse – CCC AAG GCC ACA GGG ATT TTG TCG TTG C	135	70.06 70.16	94.80	