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Phytic acid demonstrates rapid antibiofilm activity and inhibits biofilm formation when used as a surface conditioning agent

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Running title: Antibiofilm properties of phytic acid

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Abstract

Root canal infections are associated with biofilms and treated with chemical irrigants with a high success rate. However, treatment failure does arise, which is attributed primarily to resistance exhibited by biofilms. Currently used irrigants in root canal treatment have disadvantages and there is therefore need for more biocompatible alternatives with antibiofilm properties, to reducing root canal treatment failure and complications. The aim of this study was to evaluate the *in vitro* antibiofilm properties of phytic acid (IP6) which is a potential alternative treatment agent. Single and dual-species biofilms of *Enterococcus faecalis* and *Candida albicans* were developed on hydroxyapatite (HA) coupons and exposed to IP6. In addition, selected coupons were preconditioned with IP6 prior to biofilm development. IP6 demonstrated bactericidal effects and altered the metabolic activity of biofilm cells. Confocal laser scanning microscopy showed that IP6 caused significant and rapid reduction in live biofilm cells. At sub-lethal concentration IP6 did not alter expression of tested virulence genes except for *C. albicans hwp1*, the expression of which was upregulated, but not reflected by a change in hyphal transformation. IP6-preconditioned HA coupons led to extensive inhibition of dual species biofilm formation. For the first time, the results of this study highlight the antibiofilm inhibition properties of IP6 and the potential for its exploitation in several clinical applications.

Importance

Root canal infections are biofilm-associated and despite mechanical and chemical treatment procedures infection recurrence occurs, and this is likely due to the high tolerance of associated biofilms to antimicrobials. The currently used treatment agents have several disadvantages which necessitates the search for new improved agents. In this study, the natural chemical, phytic acid, was found to exhibit antibiofilm activity against established mono and dual mature biofilms over a short contact time. Most importantly, phytic acid was found to cause significant inhibition of dual species biofilm formation when used as a surface preconditioning agent. The findings of this study identified a novel use of phytic acid as a potential antibiofilm agent that can be exploited in several clinical applications.

Introduction

Biofilms are microbial lifestyles that occur on biotic and abiotic surfaces in different settings (1). Eighty percent of human infections are estimated to have a biofilm origin (2). Biofilm microorganisms can be up to 1000-fold more tolerant to certain antimicrobials (3), and the infections they cause are therefore often persistent and recurrent, and present significant treatment challenges (4). Oral infections in humans, including those of the root canal are typical biofilm-associated infections. Both *Enterococcus faecalis* and *Candida albicans* are frequently recovered from persistent infections where endodontic treatment has failed (5-7). *Enterococcus faecalis* can grow as a biofilm on root canal walls in monotypic infections without synergistic support from other bacteria (8, 9). *Candida albicans* is primarily co-isolated from root canal persistent infections with other bacteria, especially *E. faecalis* (10, 11) and this could be attributed to synergistic interaction between these two species.

The mainstay of treatment of endodontic infections, involves mechanical debridement and chemical disinfectant for biofilm elimination (12). Ethylenediaminetetraacetic acid (EDTA) and sodium hypochlorite (NaOCl) are the most popular irrigants used for this purpose (13). At concentrations between 2.50-5.25%, NaOCl is the most frequently used disinfectant (14) in endodontic treatment despite the risk it presents to tooth integrity, the surrounding tissues and patient safety (15). Studies have shown that biofilm disinfection is significantly improved when using higher volumes and concentrations of NaOCl with an extended application time (16). However, this can negatively affect dentine strength and increase likelihood of tooth fracture and loss. The antimicrobial effect of NaOCl is also reduced in the presence of organic and inorganic matters (17-19). NaOCl cannot remove the organic smear layer formed during mechanical debridement (20) and thus a chelating agent is required. Proposed benefits of smear layer removal include better disinfection and penetration of endodontic sealers into the dental tubules (21, 22). Bacteria that reside within the smear layer, if not eliminated, can recolonise and cause recurrent infection. Hence it would be potentially beneficial to employ a chelating agent that has dual activity *i.e.,* an ability to remove the smear layer and antimicrobial activity. Importantly, use of a chelating agent with antimicrobial function would contribute to the disinfection process and help reduce the required NaOCl concentration and exposure time. EDTA (15-18%) is the most commonly used chelating irrigant (14), but has several drawbacks,

92 including host cell toxicity towards periapical tissue and a lack of antimicrobial activity
93 which have a negative impact on treatment outcome (23-25). EDTA is also not readily
94 biodegradable, and concerns exist about extrusion into the periapical tissue (26-28).
95 Therefore, given the recalcitrant nature of biofilms, identification of novel chelating
96 agent with antimicrobial and antibiofilm proprieties is required to aid in the
97 achievement of favourable treatment outcome.

98 Phytic acid (IP6) is a natural agent that has been proposed as a potential root canal
99 irrigant because of its ability to remove the smear layer and its higher biocompatibility
100 compared with EDTA (29). In general, the antimicrobial effect of IP6 has not been
101 studied in the context of dentistry and specifically in endodontics. Limited research has
102 investigated IP6 on foodborne microorganisms. Kim and Rhee studied its effect of
103 *Escherichia coli*, and the proposed antimicrobial mechanism was by cell membrane
104 disruption (30, 31). Zhou et al. also found IP6 was effective against *E. coli*,
105 *Staphylococcus aureus*, *Bacillus subtilis*, and *Salmonella typhimurium* (32). In a recent
106 study, IP6 showed inhibition of *Clostridium perfringens* spore germination and
107 vegetative cell growth (33). However, despite the potential antimicrobial activity of IP6
108 found in these previous studies, this activity was mainly investigated against planktonic
109 cultures of food-associated pathogens. We have previously reported the broad-
110 spectrum antibacterial activity of IP6 using planktonic cultures and antibiofilm effect on
111 early single-species biofilms (34). However, since biofilm tolerance to antimicrobial
112 agents is dependent on biofilm maturation, microbial composition and growth
113 conditions, it is important to investigate the effect of IP6 on mature biofilms under
114 conditions which better mirror those of endodontic infections. The aim of this study
115 was therefore to evaluate antibiofilm effectiveness of IP6 and the associated
116 mechanisms against mature biofilms on clinically relevant surfaces. Both static and
117 dynamic biofilms models were used to achieve the intended aims of this research.

118 **Methods and Materials**

119 **Microorganisms and growth conditions**

120 *Enterococcus faecalis* ATCC 29212, an oral strain of *E. faecalis* (35) and *Candida*
121 *albicans* ATTC 90028 were used to generate mature biofilms. *Enterococcus faecalis*
122 was cultured on tryptone soya agar (TSA) and incubated aerobically at 37°C for 24 h.

123 *Candida albicans* was cultured on Sabouraud Dextrose Agar (SDA) in 5% CO_2, at
124 37°C for 48 h.

125 **Static biofilm formation**

126 Mono and dual species biofilms were developed on the well surfaces of 12-well plates
127 that had been preconditioned with Dulbecco's Modified Eagle Medium (DMEM) for 24
128 h. Briefly, microbial colonies were used to prepare suspensions in tryptone soya broth
129 (TSB) which were then incubated at 37°C, with agitation (125 rev/min) in a shaker
130 incubator for 3-4 h. For mono species biofilms, bacterial suspensions were adjusted
131 to a 0.5 McFarland standard using a DensiCHEK™ meter (BioMerieux). For the dual
132 species inoculum, a 1.0 McFarland standard of *E. faecalis* ATCC 29212 was mixed
133 with an equal volume of a 1.0 McFarland standard of *C. albicans* ATTC 90028. One
134 ml of microbial suspension was added to each well of the 12-well plate. The biofilm of
135 the oral strain of *E. faecalis* was grown statically for 48 h, while biofilms of *E. faecalis*
136 ATCC 29212 and dual biofilms were grown for 72 h. Biofilms were cultured aerobically
137 at 37°C and the medium was changed every 24 h.

138 **Effect of IP6 on biofilm metabolic activity and biomass**

139 After biofilm formation, spent medium was discarded and biofilms were washed twice
140 with phosphate buffer saline (PBS). One ml of TSB and 1 ml of IP6 (Sigma),
141 concentration range (40%-0.31%) in water was added to designated wells. Untreated
142 biofilm and blank controls received 1 ml of TSB and 1 ml of sterile distilled water,
143 respectively. The biofilms were incubated aerobically for 24 h at 37°C. After IP6
144 treatment, test solutions were removed, and the biofilms washed twice with PBS. Two
145 ml of 10% Alamar Blue solution was added to each well and the plates incubated for
146 1 h at 37°C. Fluorescence of the wells was then measured using a microplate reader
147 (Hidex, Finland). To assess total biomass, Alamar Blue suspension was discarded and
148 2-ml volume of 0.1% crystal violet (CV) was added and incubated for 10 min at room
149 temperature. The CV was removed, and the wells washed twice with distilled water.
150 Excess water was removed, and the plates were allowed to air-dry for 30 min. Two ml
151 of 70% ethanol was added to each well to elute bound CV, and the plate was incubated
152 at room temperature for 10 min. The absorbance of the wells was then read using a
153 microplate reader at 570_{nm}.

154 **Effect of IP6 on microbial regrowth**

155 After treatment of the biofilms with IP6 for 24 h, test solutions were removed from the
156 wells, which were then washed twice with PBS. Two ml of sterile TSB was then added
157 to all wells, followed by 18-24 h aerobic incubation at 37°C. After incubation, regrowth
158 was assessed by reading the absorbance of the wells with a microplate reader (Hidex,
159 Finland) at 600_{nm}.

160 **Effect of IP6 on colony forming unit (CFU) reduction**

161 The IP6 concentrations that allowed bacterial regrowth were used to study effects of
162 IP6 on bacterial cell count reduction. After treatment of the biofilms with IP6 for 24 h,
163 the solution was removed from the wells which were then washed twice with PBS. The
164 biofilms were then detached by scraping and vigorous pipetting and subsequent
165 enumeration of viable cells was performed.

166 **Biofilms formation on hydroxyapatite coupons in a CDC bioreactor and**
167 **antibiofilm activity of IP6 on developed biofilms**

168 CDC bioreactors were assembled, and hydroxyapatite (HA) coupons were positioned
169 in designated sites in the support rods before sterilisation. The system flask was
170 aseptically filled with 325 ml of 3 g/L TSB. For mono species biofilms, the CDC
171 bioreactor was inoculated with 1 ml of a 0.5 MacFarland standard *E. faecalis* bacterial
172 suspension. One ml of a 0.5 MacFarland standard *E. faecalis* and 1 ml of a 0.5
173 MacFarland standard *C. albicans* were inoculated for dual species biofilms. The CDC
174 bioreactor was incubated as a batch system for 18-24 h at 37°C whilst placed over a
175 stirrer plate for baffle stirring to produce shear stress. After incubation, the bioreactors
176 were connected with a carboy containing sterile 1 g/L TSB to run a continuous flow
177 system for an additional 48 h at a flow rate of 1.1 ml/min. The biofilms formed on HA
178 coupons were then treated with three concentrations of IP6 (1.25, 2.5%, 5%) for 5 min
179 and the antibiofilm activity of IP6 was assessed as outlined below.

180 **Assessment of colony forming units (CFUs) from treated biofilms**

181 A single tube method (36) was used to assess the effect of IP6 on the number of
182 recovered CFUs from biofilms. Briefly, following biofilm development the coupon-
183 supporting rods were removed from the CDC bioreactor and washed using PBS. The
184 HA coupons were removed and placed in conical tubes containing 6 ml of IP6 (1.2%,

185 2.5%, 5%) or water. The conical tube had sterilised plastic splashguards to prevent
186 droplets accessing areas where IP6 could not reach. After 5 min, HA coupons were
187 washed in 35 ml PBS, transferred to 6 ml of PBS and exposed to two repeated cycles
188 of vortex mixing (30 s) and water bath sonication (30s), with a final vortex mix for 30
189 s. The recovered biofilm content was mixed by repeated (x10) pipetting. One ml of the
190 mix was transferred to a sterile plastic tube containing 9 ml of sterile PBS. After vortex
191 mixing at high speed for 10 s, CFU counts were measured.

192 **Confocal laser scanning microscopy (CLSM) assessment of IP6 treated biofilms**
193 After biofilm development in the CDC bioreactor, the rods were washed and treated
194 as described above. Biofilms were stained with Live/Dead® BacLight™ bacterial
195 viability mixture (Invitrogen Ltd., UK) for 10 min at room temperature and immediately
196 analysed by CLSM. Five random fields of view were imaged for each coupon using a
197 LSM880 Airyscan microscope (Zeiss). Quantitative image analysis was performed
198 using Comstat 2.1 to quantify live and dead biomass. CLSM micrographs were
199 generated using Imaris viewer software (9.9.1 version).

200 **Effect of preconditioning hydroxyapatite coupons with IP6 on biofilm formation**
201 To assess the effect of preconditioning HA coupons with IP6 on biofilm formation,
202 coupons were soaked in either 2.5% IP6 or sterile distilled water for 18 h under aseptic
203 conditions. The rods were removed and then placed in an assembled sterile CDC
204 bioreactor containing TSB. The CDC bioreactor was inoculated with 1 ml of a 0.5
205 McFarland standard level of both *E. faecalis* ATCC 28212 and *C. albicans* ATCC
206 90028 and incubated at 37°C under batch conditions and shear force, for 18-24 h.
207 Biofilm formation was assessed by CLSM and scanning electron microscopy (SEM).
208 For CLSM, coupons were processed as described above, and for SEM, coupons were
209 washed with PBS and placed immediately in 12-well plates containing 2.5%
210 glutaraldehyde and incubated overnight. After incubation, the biofilms were
211 dehydrated in 2 ml of an ethanol solution series (50%, 70%, 85%, 95% and 100%) for
212 10 min at each concentration. Dehydration with 100% ethanol was repeated twice.
213 The samples were then sputter coated with gold and imaged using a Tescan VAGA
214 SEM system at 5-10kV.

215 **Effect of IP6 on gene expression**

216 The effect of 0.16% IP6 on gene expression of 72 h old dual species biofilms
217 developed in 12 well plates, as described above, was assessed. Developed biofilms
218 were challenged with 0.16% IP6 for 24 h. The test solution was removed, and the wells
219 washed twice with PBS. One ml of RNA protect bacteria reagent (Qiagen) was added
220 and left at room temperature for at least 10 min. The biofilms were detached by
221 scraping and vigorous pipetting. The resulting suspension was processed for RNA
222 extraction using Qiagen kit, and RNA was then reverse transcribed as per
223 manufacturer protocol (Qiagen). Quantitative PCR was performed using SYBR green
224 mix (Biorad) with the following thermal cycling: 95°C for 2 min, followed by 40 cycles
225 of 95°C for 5 s, and 60°C for 20 s. Relative gene expression was normalised to
226 housekeeping genes and analysed using the $2^{-\Delta\Delta CT}$ relative expression method.
227 Target genes and primers sequences are provided in Supplementary tables S1 and
228 S.

229 **Effect of IP6 on *C. albicans* hyphal transformation**

230 To study effect of IP6 on hyphal production, 72 h dual species biofilms were prepared
231 and treated with 0.16% IP6 as described above. After treatment, medium was
232 retrieved, and the biofilm washed twice with PBS and fixed with 4% paraformaldehyde
233 at room temperature for 1 h. The wells were washed with PBS and stained with 0.2%
234 calcofluor white for 30 min at room temperature. Images were obtained using Olympus
235 IX53 fluorescent inverted microscope. The proportional of hyphae relative to total
236 *Candida* fungal units (yeast and hyphae) was determined using ImageJ software 1.48
237 version.

238 **Statistical analysis**

239 Statistical analysis was performed using Graphpad Prism 9.4.0. A parametric t-test
240 was used to compare differences between any two groups. Kruskal Wallis or ordinary
241 one-way ANOVA was used to compare more than two groups. A *p* value of less than
242 0.05 was considered significant. Retaliative fold-change in gene expression analysis
243 was performed using one sample t-test compared to a hypothetical mean of 1.

244 **Results:**

245 **Antibiofilm activity IP6 on mono and dual species biofilms developed using**
246 **static model**

247 The metabolic activity and total biomass of biofilms post treatment with different
248 concentrations of IP6 were determined using Alamar Blue and CV assays,
249 respectively. The means of relative fluorescence units (RFU) (metabolic activity) and
250 absorbance (OD570) (biomass) were calculated. Alamar blue is a redox indicator and
251 metabolically active cells reduce the blue color resazurin to resorufin which has
252 fluorescent pink color. The inability of cells to reduce Alamar Blue is indicative of
253 reduced metabolic activity. Generally, significant reduction in mean relative
254 fluorescence unit (RFU) in treated groups compared to untreated groups occurred
255 (Figure 1a). The exception was when *E. faecalis* mono species and dual species
256 biofilms of *E. faecalis* and *C. albicans* were treated with 0.16% IP6. Only the highest
257 concentration of IP6 (20%) significantly reduced the biomass of mono species *E.*
258 *faecalis* ATCC 29212 (p= 0.017) biofilms and dual species biofilms (p= 0.024). For
259 oral *E. faecalis* biofilms, significant biomass reduction was seen at lower IP6
260 concentrations (20%, 10% and 2.5%) (Figure 1b). After challenging biofilms with IP6,
261 the ability of cells to regrow after treatment was assessed. The assay revealed that
262 the lowest IP6 concentrations having 100% bactericidal effect (prevented regrowth)
263 on *E. faecalis* ATCC 29212 and the *E. faecalis* oral strain were 1.25% and 2.5%,
264 respectively. For dual species biofilms, IP6 at 10% and 20% prevented microbial
265 regrowth (Figure 1c). Since regrowth assessment usually underestimates
266 antimicrobial activity, the bacterial count reduction after treatment with IP6
267 concentrations deemed unsuccessful in completely eradicating the biofilm (in the
268 regrowth assessment) was assessed. Biofilm treatment with IP6 (0.31% and 0.63%)
269 led to a significant log reduction in recovered CFUs for mono species biofilms (Figure
270 2a and 2b). For dual species biofilms, significant reduction was only evident for *E.*
271 *faecalis* ATCC 28212 (Figure 2c).

272 **Antibiofilm activity of IP6 on mono and dual species biofilms developed on HA**
273 **coupon**

274 To assess antibiofilm activity of IP6 on more clinically relevant surfaces, HA coupons
275 were used for biofilm formation in a continuous flow of a diminishing nutrient medium
276 content along with exposure to shear force. The effect of IP6 over a clinically relevant
277 exposure time of 5 min was assessed based on recovered CFUs and CLSM. For *E.*
278 *faecalis* ATCC 29212 mono species biofilms, IP6 at 1.25%, 2.5%, and 5% led to a

significant reduction in log$_{10}$CFUs compared with untreated controls (Figure 3a) with relative percentage mean reductions of *E. faecalis* CFUs (compared to untreated control) being 96.79%, 98.98%, 99.70%, respectively (Table S3). For dual species biofilms, 1.25%, 2.5% and 5% IP6, led to a significant reduction in *E. faecalis* log$_{10}$CFUs (Figure 3b), with relative percentage mean reductions for *E. faecalis* CFUs of 97.32%, 99.09%, and 99.33%, respectively (Table S4). The presence of both *E. faecalis* and *C. albicans* in biofilms did not affect IP6 efficacy against *E. faecalis*. However, in all cases of IP6 treatment (1.25%, 2.5%, and 5%) no reduction in *C. albicans* ATCC 90028 log$_{10}$CFUs occurred (Figure 3b). Live biofilm biomass was significantly reduced with all the IP6 treatments (Figure 4e). The biomass of dead cells increased in treated groups but was only significant for biofilms treated with 2.5% and 5% IP6. Treatment with 5% IP6 led to the highest reduction in live biomass, which reached a mean of 98.31% and was significantly higher compared with 1.25% and 2.5% IP6 (Figure 4f). The same pattern of significant live biomass reduction was seen with dual species biofilms treated with 1.25%, 2.50% or 5% IP6 (Figure 5e). The relative reduction in live biomass of dual-species biofilms after IP6 treatment showed that 5% IP6 caused a mean reduction of 96.44% (Figure 5f). Comparison between the three treatments demonstrated that 5% IP6 achieved a statistically higher reduction in live biomass of dual species biofilm than the reduction caused by 1.25% IP6 (Figure 5f). CLSM images and associated graphs of live/dead mono-species and dual species are shown in figures 4 (a-d) and 5 (a-d).

Effect of preconditioning HA coupons with IP6 on biofilm formation

The effect of preconditioning HA coupons with 2.5% IP6 on biofilm formation of dual-species biofilm of *E. faecalis* and *C. albicans* was investigated by CLSM and SEM. CLSM quantitative image analysis revealed significant reduction in total and live cell biomass of biofilms formed on HA coupons preconditioned with 2.5% ($p < 0.001$ and $p < 0.0001$, respectively) (Figure 6c). This was also evident in CLSM and SEM images (Figure 6a and 6b). HA coupons preconditioned with water had a predominant number of live cells in the formed dual species biofilm.

308 **Effect of IP6 virulence genes expression and hyphal morphogenesis**

309 The relative expression of selected virulence genes of *E. faecalis* ATCC 29212 and *C.*
310 *albicans* ATCC 90028 in biofilms following treatment with 0.16% IP6 was quantified.
311 All analysed *E. faecalis* genes demonstrated no significant changes in expression
312 levels for IP6 treated biofilms compared to untreated biofilms. Expression of *C.*
313 *albicans* genes was unchanged in treated biofilms with the exception of *hwp1* gene
314 expression, which was upregulated (Figure 7). To evaluate whether *hwp1* gene
315 expression upregulation translated phenotypically, the percentage of hyphae post
316 treatment with 0.16% IP6 was compared with untreated controls. The results showed
317 that there was no significant change in hyphal development in treated biofilms (p=
318 0.40) (Figure 8).

319 **Discussion**

320 IP6 is a natural compound with high biocompatibility that has been proposed as an
321 alternative chelating agent to EDTA for root canal smear layer removal (29). Several
322 studies have assessed the potential of IP6 for use in dentistry (37). However, its
323 antimicrobial properties have not yet been adequately investigated. We previously
324 examined the broad-spectrum antimicrobial activity of IP6 and showed clear activity
325 against microorganisms (34). However, that work involved planktonic cultures and
326 immature biofilms, hence the antibiofilm activity of IP6 on mature robust biofilms, that
327 better represent persistent endodontic infections remained to be established. The
328 findings from this study are the first into the antibiofilm activity of IP6 on the eradication
329 of endodontic related biofilms and its effectiveness in inhibiting biofilm formation when
330 used as preconditioning agent on HA coupons.

331 Mono and dual species *in vitro* biofilms of *E. faecalis* and *C. albicans* were tested as
332 these species are frequently associated with persistent endodontic infection (38, 39).
333 IP6 exhibited bactericidal activity against constituent cells of these mature biofilms and
334 concentrations that did not lead to complete eradication of biofilm cells led to reduced
335 biofilm metabolic activity. It is worth highlighting that in general IP6 did not affect total
336 biofilm biomass, even for concentrations where total bactericidal effects were reported,
337 and this finding suggests that IP6 does not have 'surfactant' effects on tested biofilms.

338 IP6 antibiofilm effects against 72 h mono and dual biofilms on HA coupons with a
339 clinically relevant timing of 5 min were also determined. These biofilms were

340 developed under sheer force with a decreasing level of medium nutrients. This
341 arguably better represents the deprivation of nutrients that occurs as endodontic
342 infections progress. IP6 displayed antibacterial effects on biofilms even over this short
343 exposure time. Previous work revealed that IP6 is a potent chelating agent (29), a
344 property attributed to its ability to chelate multivalent cations via its multiple negative
345 charges (40, 41). Therefore, it might be hypothesised that the IP6 antibacterial activity
346 was through its high affinity for cations that are present in the bacterial cell envelope.
347 It has also been suggested that antibacterial effects of chelating agents arise through
348 disturbing essential metal metabolic processes (42). However, chelating agents have
349 different activities and mechanisms of action, based on their affinity for ions (43).

350 IP6's antibiofilm activity could be through bactericidal activity against constituent cells
351 and/or by acting as a biofilm matrix destabiliser by interacting with biofilm cations,
352 which are known to play a role in biofilm stability by crosslinking the negatively charged
353 polymers in a biofilm, thus reducing repulsion and promoting biofilm stability (44, 45).
354 Collectively, the presented findings clearly show an effect of IP6 on mature biofilms.
355 Although studies, from the food industry, had investigated IP6 antibacterial effects on
356 planktonic bacteria, the antibiofilm activity and the associated mechanisms remain
357 poorly researched. In the present study, IP6 had effects on bacterial cells, but no
358 activity was evident against *C. albicans*. This observation might relate to the
359 biocompatibility of IP6 towards eukaryotic cells (29) and could support the hypothetical
360 antibacterial mechanism of action of IP6 given cell envelope differences between
361 bacteria and fungus.

362 It should be noted that the inability of a compound to completely eradicate biofilms
363 might not necessarily preclude its value in managing biofilm-associated infections. It
364 is possible that even at sub-lethal concentrations an impact upon microbial fitness and
365 modulation of virulence factors could arise (46, 47). Some studies have even shown
366 that at sub-lethal concentrations, some antimicrobials can upregulate virulence gene
367 expression (48). Therefore, an evaluation of targeted gene expression of *E. faecalis*
368 and *C. albicans* dual-species biofilms following exposure to sub-lethal IP6
369 concentrations was investigated. Expression of tested genes was not affected by
370 exposure to sub-lethal levels of IP6. *Als1* and *als3 C. albicans* adhesin genes were
371 also not altered by IP6 treatment. In contrast, expression of *C. albicans hwp1* was

372 upregulated. However, associated changes in hyphal transformation were not
373 detected.

374 Another notable observation was IP6's ability to completely inhibit biofilm formation
375 when used as a preconditioning agent. This property could be exploited in prevention
376 strategies, where root canal walls could be treated with IP6 to inhibit microbial
377 adherence and biofilm formation. This would be important in preventing secondary
378 infections due to microbial leakage from the oral cavity into the root canal system. This
379 characteristic could also be exploited in conditioning obturation materials, or implants,
380 to prevent microbial adherence to surfaces thus increasing treatment success rates.
381 It might be logical to assume that surface topographical changes induced by IP6 were
382 instrumental in these effects occurrence. However, SEM images demonstrated that
383 HA surfaces became more irregular and with more undulations following IP6
384 conditioning. This might contradict previous research where 'rougher' surfaces
385 typically were thought to increase surface area and offer protection from shear forces
386 for bacterial adhesion and biofilm formation (49). A recent study reported the high
387 adsorption of IP6 into HA, where it was absorbed as monolayer (50). It could be that
388 IP6 affects HA surface wettability and resulting charge, possibly due to IP6's
389 negatively charged phosphate group. A negatively charged surface might reduce
390 bacterial adhesion through repulsion, given the negative net charge of bacteria. It is
391 worth highlighting that there remains a lack of clarity regarding the effect of surfaces
392 charge on bacterial adhesion (49) and some studies have shown that bacteria can
393 overcome electrostatic repulsion through cation bridging (49, 51). More studies are
394 needed to understand, not only the observed antibacterial activity of IP6 on formed
395 biofilms, but also to its biofilm inhibition for different surfaces incorporating a wide
396 range of microbial species.

397 In conclusion, for the first time, the present study demonstrates the antibiofilm efficacy
398 of IP6 against mature mono and dual species biofilms as well as its high-level biofilm
399 formation inhibition activity when used as surface conditioning. The findings of this
400 study highlight the unique antibiofilm properties of IP6 which could be exploited in
401 dental applications as well as other areas where biofilm inhibition and management
402 are needed.
403

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409 The authors declare no conflict of interest.

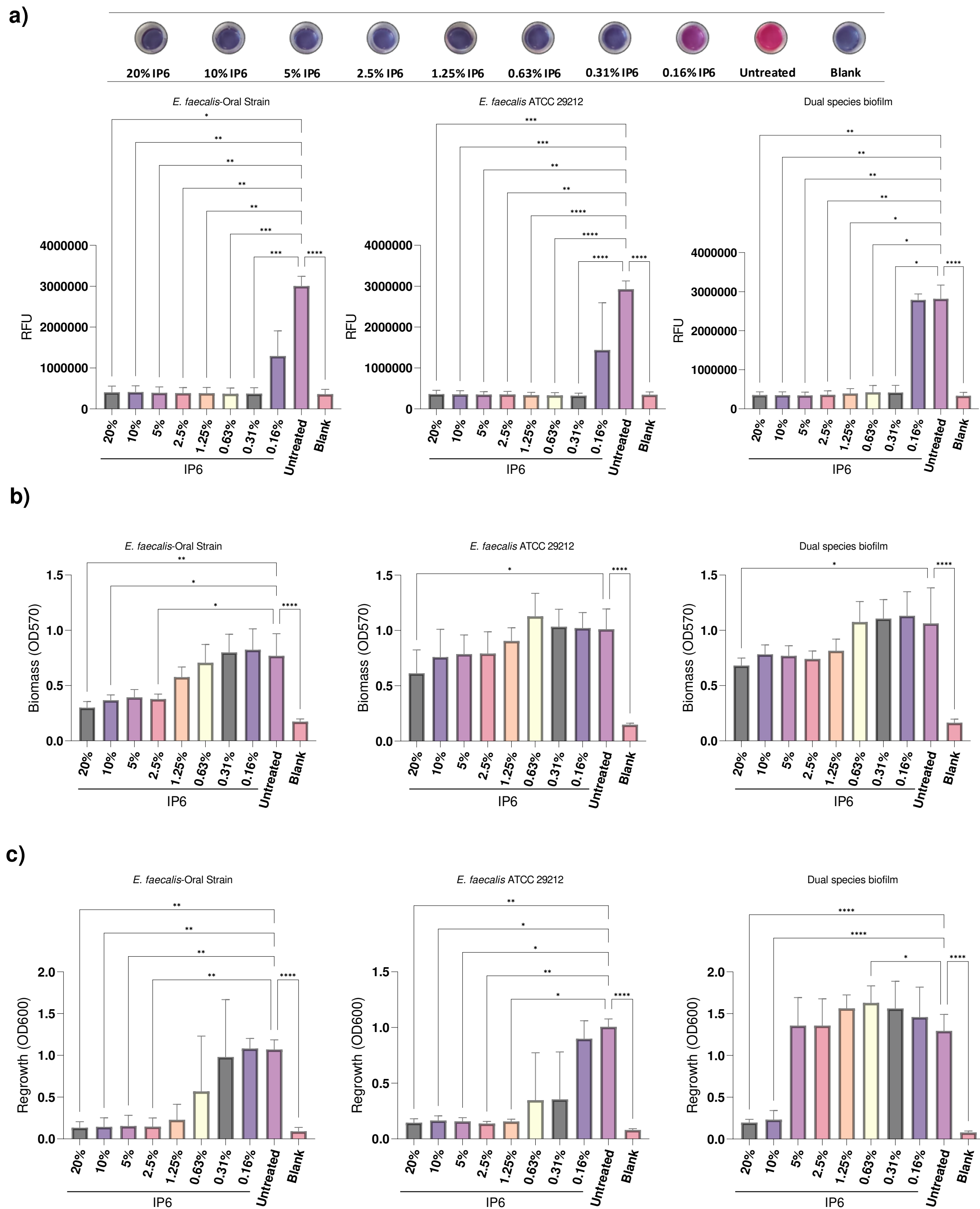


Figure 1 Effect of phytic acid on metabolic activity (a), biomass (b) and complete eradication (c) of mono and dual species biofilms developed in the wells of a 12-well plate and treated for 24 h. Data is expressed as mean of at least three independent experiments, each including at least two replicates. Error bars represent SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

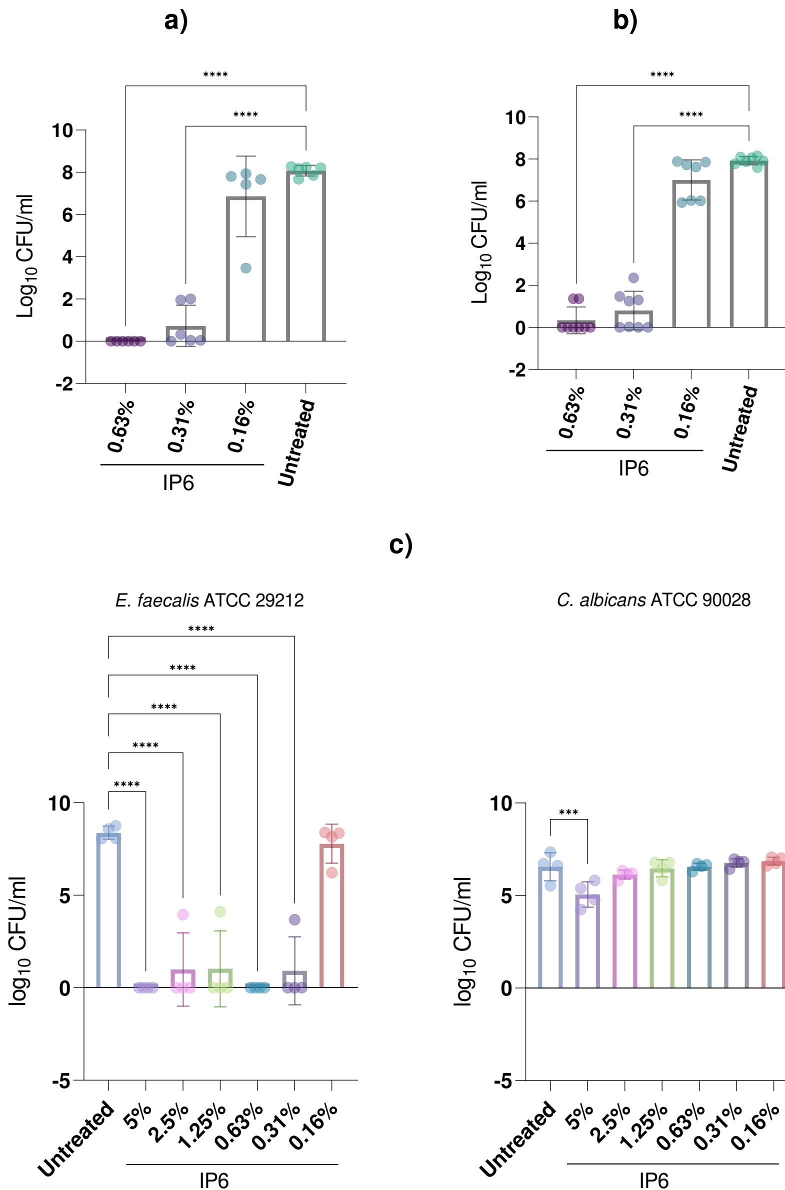


Figure 2 Effect of IP6 on recovered CFU/ml from 24 h mono species biofilms of **a)** *E. faecalis* oral strain **b)** *E. faecalis* ATCC 29212 and **c)** dual species biofilm of *E. faecalis* ATCC 28212 and *C. albicans* ATCC 90028 developed in the wells of a 12-well plate and treated for 24 h. Data is expressed as mean of at least three independent experiments, each including at least one replicate. Error bars represent SD. *** $P < 0.001$ and **** $P < 0.0001$

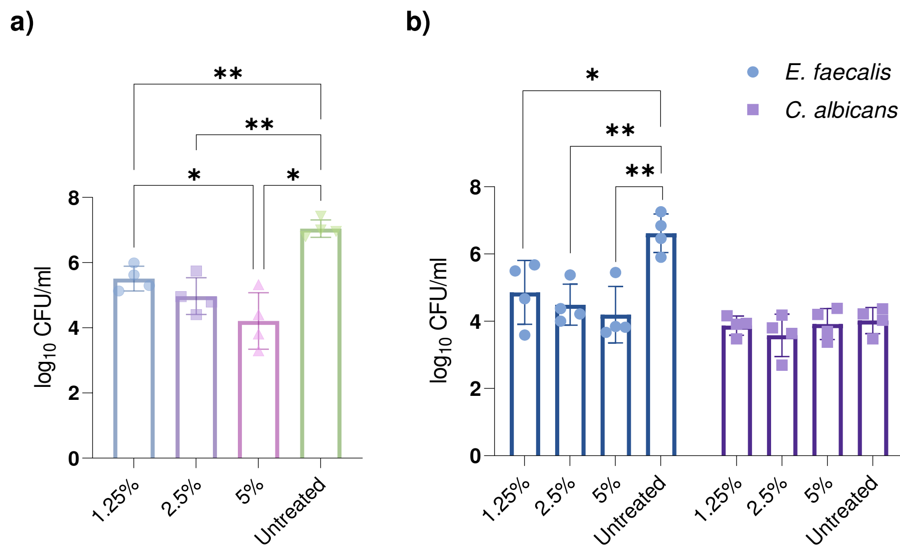


Figure 3 Effect of 5 min IP6 treatment on recovered CFU/ml of **a)** mono-species biofilm of *E. faecalis* ATCC 29212 and **b)** dual-species biofilm of *E. faecalis* ATCC 28212 and *C. albicans* ATCC 90028 developed on HA coupons. Data is expressed as mean of four independent experiments. Error bars represent SD. * $P < 0.05$ and ** $P < 0.01$.

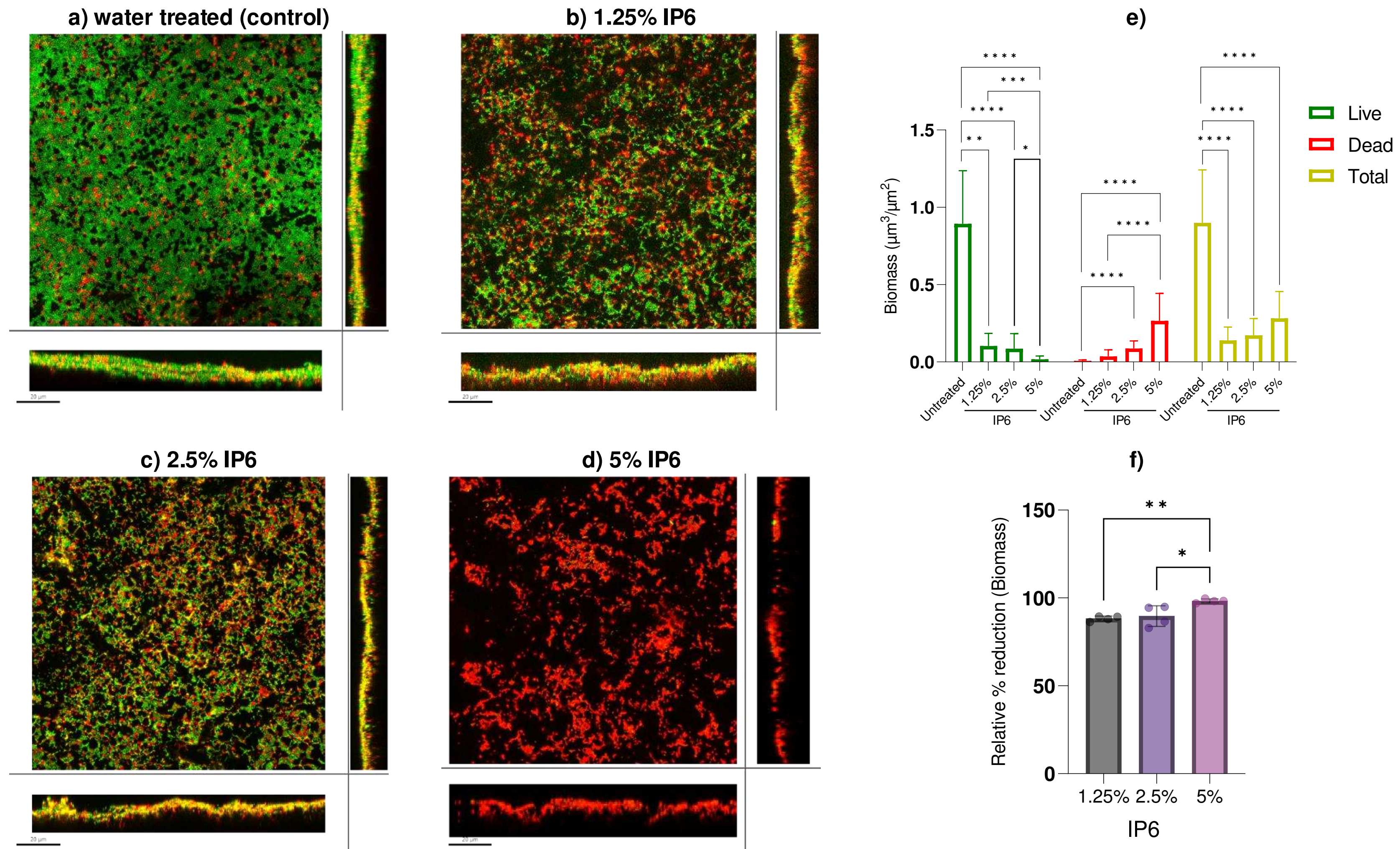
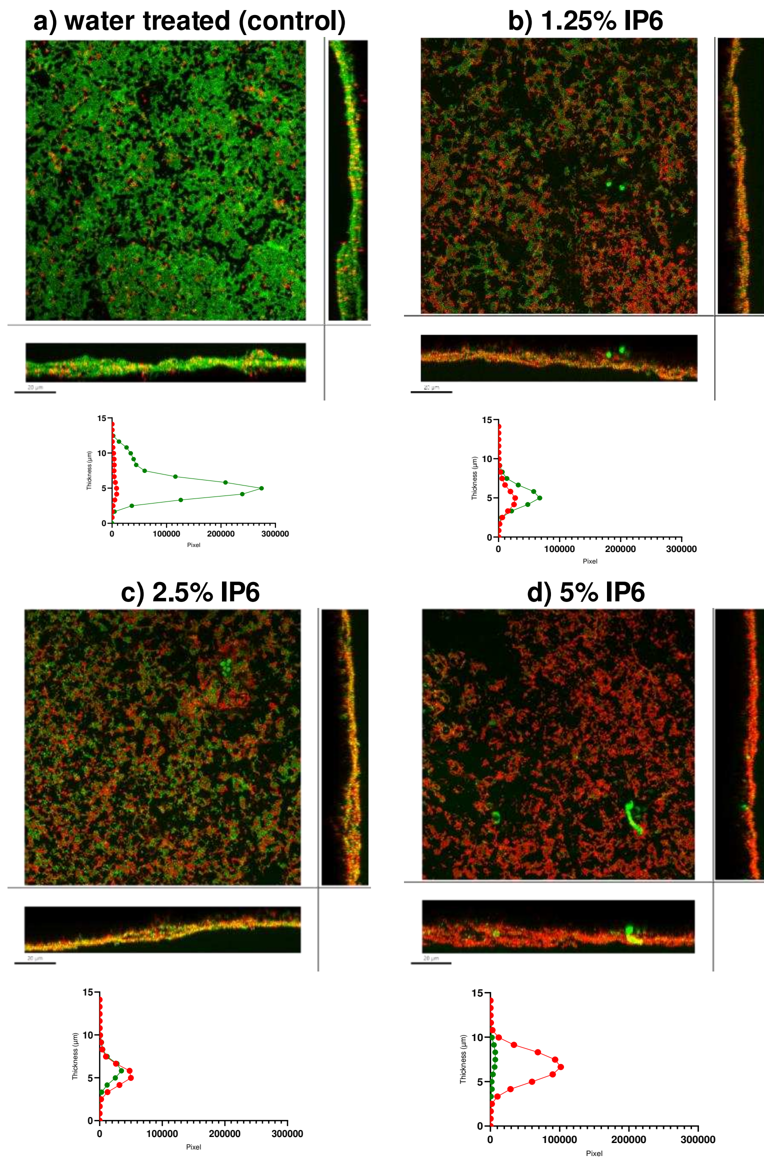
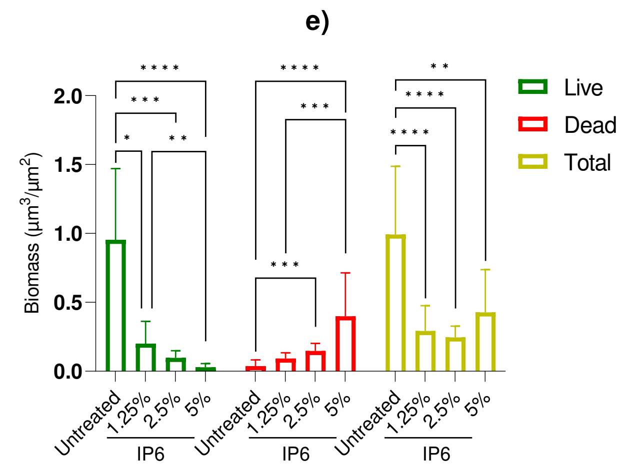


Figure 4 Confocal laser scanning microscopy (CLSM) of Live (green)/Dead (red) stained 72 h *E. faecalis* mono-species biofilms that were developed on HA coupons were treated with either water (a), 1.25% (b), 2.5% (c) or 5% IP6. The square images show biofilm

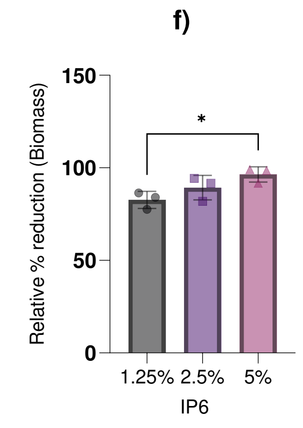
projection through the x-y plane, the bottom rectangle shows x-z projection and the right rectangle shows the y-z plane. Scale bar is 20 µm. (e) represent biomass (green channel and red channel) of these mono species biofilms treated with phytic acid for 5 min. (f) shows biomass (green channel) relative percent reduction (in comparison to untreated biofilms) of these mono species biofilms. Data is expressed as mean of four independent experiments. Error bars represent SD. * $P < 0.05$, ** $P < 0.01$ and **** $P < 0.0001$.



a) water treated (control)
b) 1.25% IP6
c) 2.5% IP6
d) 5% IP6



e)
Biomass (µm³/µm²)
Live
Dead
Total
Untreated
1.25%
2.5%
5%
IP6



f)
Relative % reduction (Biomass)
1.25%
2.5%
5%
IP6

Figure 5 Confocal laser scanning microscopy (CLSM) of Live (green)/Dead (red) stained 72 h *E. faecalis* and *C. albicans* dual-species biofilms that were developed on HA coupons were treated with either water (a), 1.25% (b), 2.5% (c) or 5% IP6. The square images show biofilm projection through the x-y plane, the bottom rectangle shows x-z projection and the right rectangle shows the y-z plane. Scale bar is 20 µm. The graphs below CLSM images represent distribution of pixel average of both stains along the biofilm thickness. Pixel average was calculated from three independent experiments. (e) represent biomass (green channel and red channel) of these dual species biofilms treated with phytic acid for 5 min. (f) shows biomass (green channel) relative percent reduction (in comparison to untreated biofilms) of these dual species biofilms. Data is expressed as mean of three independent experiments for CLSM and four independent experiments for CFU analysis. Error bars represent SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$

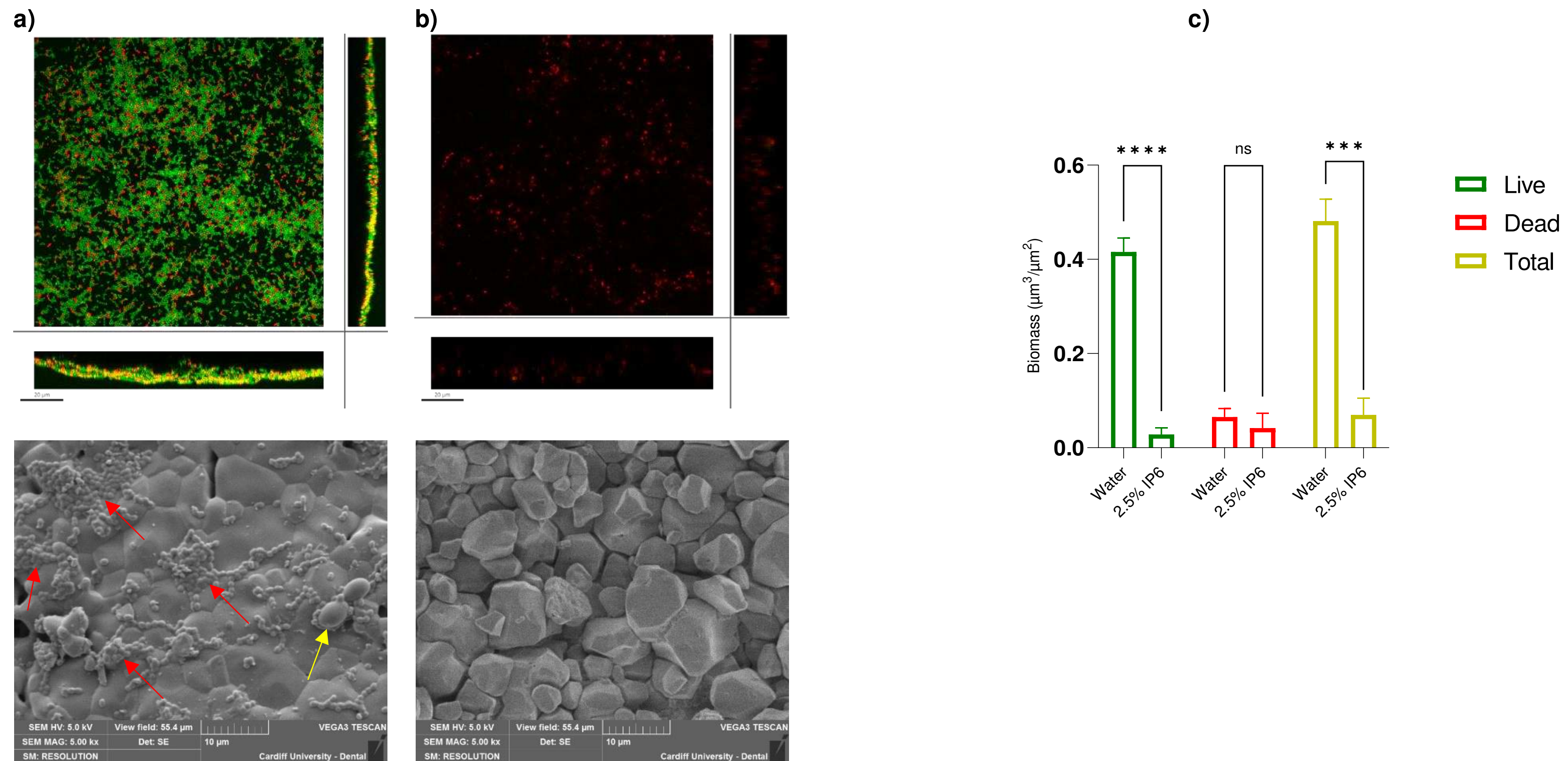


Figure 6 Dual species biofilms of *E. faecalis* ATCC 29212 and *C. albicans* ATCC 90028 developed on HA coupons for 24 h that were either preconditioned with water (control) or 2.5% IP6. Scanning electron microscopy (lower) and Confocal laser scanning microscopy (upper) of Live/Dead stained dual-species biofilms that were developed on HA coupons preconditioned with either water (a) or 2.5% IP6 (b). Red arrows indicate *E. faecalis* communities while yellow arrows indicate *C. albicans*. Graph bars (c) represent biomass of developed biofilms on HA coupons that either preconditioned with water (control) or 2.5% IP6. Data is expressed as the mean of

three independent experiments. Error bars and values in parenthesis represent SD. **** $P < 0.0001$, *** $P < 0.001$ and ns is not significant.

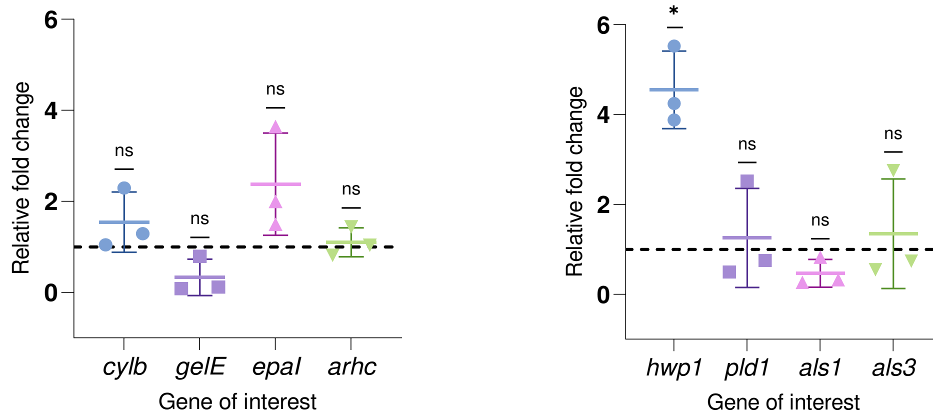


Figure 7 Effect of IP6 (0.16%) on *E. faecalis* ATCC 29212 (left) and *Candida albicans* ATCC 90028 (right) genes expression. Analysis performed by $2^{-\Delta\Delta Ct}$ method. Data is expressed as mean of three independent experiments. Error bars represent SD. * $P < 0.05$ and ns is not significant.

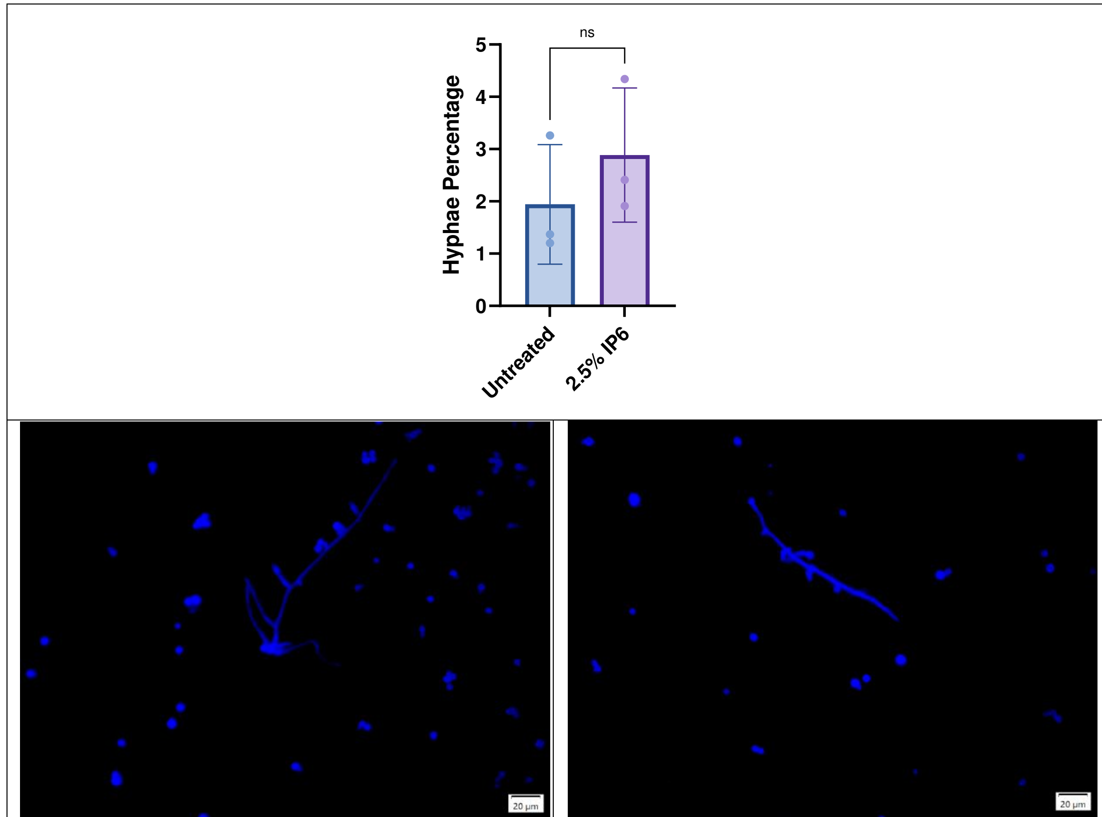


Figure 8 *Candida albicans* ATCC 90028 hyphal percentage in dual species biofilm treated with 0.16% phytic acid for 24 h compared to non-treated (control) biofilm. Data expressed as mean of three independent experiments. Error bars represent standard deviation. ns is not significant. Typical fluorescent images of dual species biofilm stained with calcofluor white used to quantify *C. albicans* hyphae. *Candida albicans* hyphal presence in untreated control biofilm (bottom left) and 0.16% IP6 treated biofilm (bottom right).

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