Interaction between *Staphylococcus aureus* and *Pseudomonas aeruginosa* is beneficial for colonisation and pathogenicity in a mixed-biofilm

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Keywords : polymicrobial, wound-infection, biofilm, aggregation, invasion, adhesion

Abstract

Debate regarding the co-existence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in wounds remains contentious, with the dominant hypothesis describing a situation akin to niche partitioning, whereby both microorganisms are present but occupy distinct regions of the wound without interacting. In contrast, we hypothesised that these microorganisms do interact during early co-colonisation in a manner beneficial to both bacteria. We assessed competitive interaction between *S. aureus* and *P. aeruginosa* in biofilm cultured for 24-72 h and bacterial aggregates analogous to those observed in early (<24h) biofilm formation, and interaction with human keratinocytes. We observed that *S. aureus* predominated in biofilm and non-attached bacterial aggregates, acting as a pioneer for the attachment of *P. aeruginosa.* We report for the first time that *S. aureus* mediates a significant ($P<0.05$) increase in the attachment of *P. aeruginosa* to human keratinocytes, and that *P. aeruginosa* promotes an invasive phenotype in *S. aureus.* We show that co-infected keratinocytes exhibit an intermediate inflammatory response concurrent with impaired wound closure that is in keeping with a sustained pro-inflammatory response which allows for persistent microbial colonisation. These studies demonstrate that, contrary to the dominant hypothesis, interactions between *S. aureus* and *P. aeruginosa* may be an important factor for both colonisation and pathogenicity in the chronic infected wound.

38 **Introduction**

39

40 The prevention and management of biofilm in wounds is a priority for clinicians and
41 researchers, and is allied with the increased global focus on antimicrobial
42 stewardship due to the increasing number of multi-drug resistant bacteria (Mashburn
43 et al., 2005; Tümmler et al., 2014; Barnabie et al., 2015; WUWHS, 2016). Biofilms
44 are thought to be present in most wounds and are known to be comprised of multiple
45 bacterial species; moreover, their incidence is documented as having a deleterious
46 impact on wound healing.

47 There are a number of different guidelines supporting the diagnosis and
48 treatment of biofilms in wounds, but detecting biofilm is challenging in clinical
49 practice (Dohmen et al., 2008; Rhoads et al., 2008; Hoiby et al., 2010; Gottrup et al.,
50 2011; Metcalfe et al., 2016; WUWHS, 2016). Most guidelines are predicated on
51 expert opinion, yet in reality little is known about the impact of bacterial interaction on
52 the development and severity of co-infected wounds, from which *Staphylococcus*
53 *aureus* and *Pseudomonas aeruginosa* are most commonly isolated (Serra et al.,
54 2015).

55 An extensive body of evidence exists describing the relationship between *S.*
56 *aureus* and *P. aeruginosa* using cystic fibrosis (CF) lung infection models (Baldan et
57 al., 2014; Filkins et al., 2015; Maliniak et al., 2016). The lungs of children with CF are
58 readily colonised by *S. aureus* during the early years of life, with colonisation by *P.*
59 *aeruginosa* during mid to late teenage years. Colonisation with *S. aureus* is
60 associated with a higher propensity for secondary colonisation with *P. aeruginosa*,
61 and once present the latter rapidly establishes and eventually predominates (Ahlgren
62 et al., 2015; Limoli et al., 2016). Co-infection with *P. aeruginosa* and *S. aureus*

63 ultimately results in a poor clinical outcome for the patient correlated with increased
64 inflammatory markers.

65 Less is understood about the relationship between *S. aureus* and *P.*
66 *aeruginosa* in the chronic infected wound. Chronic wounds are often co-colonised
67 by *S. aureus* and *P. aeruginosa,* and once contaminated with *P. aeruginosa* the
68 infected wound becomes highly recalcitrant to treatment (Serra et al, 2015). Biopsies
69 of established chronic infected wounds have indicated that *S. aureus* and *P.*
70 *aeruginosa* exist apart in distinct, separate niches with *P. aeruginosa* found within
71 the deeper tissues and *S. aureus* at the surface of the wound (Fazli et al., 2009).
72 Hypotheses currently describing co-colonised wounds therefore reject the idea that
73 they are truly polymicrobial based on this pattern of colonisation (Woods et al., 2012;
74 Phalak et al., 2016).

75 Ecological theories of competitive exclusion and the niche concept support
76 current observations of *S. aureus* and *P. aeruginosa* in chronic infected wounds
77 (Davies, 2006). These principles state that two-species competing for the same
78 resource cannot stably co-exist if other ecological factors are constant.
79 Consequently, one of the two competitors will become dominant and drive the other
80 either to extinction or another ecological niche. However, this is rarely observed in
81 nature, where multiple species co-exist in a single environment.

82 We hypothesise that the niche partitioning observed in chronic wounds occurs
83 over a long period of time, and these two pathogens initially co-exist and interact in a
84 beneficial manner in much the same way as oral microorganisms in plaque
85 development. Furthermore, we suggest that inter-bacterial interactions between *S.*

86 *aureus* and *P. aeruginosa* impact upon temporal colonisation, pathogenicity, and
87 inflammatory responses associated with infection.

88 Therefore the objectives of this study were to assess the relative competition
89 between *S. aureus* and *P. aeruginosa* in co-cultured biofilm, and to determine
90 whether co-culture resulted in synergy of growth and virulence concurrent with
91 enhanced damage, diminished immune response and impaired repair of a human
92 keratinocyte cell line.

93 **Materials and Methods**

94 *Bacterial strains*

95 *Pseudomonas aeruginosa* reference strain ATCC 9027 (NCIMB 8626) and
96 *Staphylococcus aureus* NCTC 13142 (EMRSA-15) were used throughout the study.
97 Both strains are associated with skin/wound infections. All strains were cultured
98 aerobically at 37°C in nutrient broth (NB; Oxoid). Selective media were used to
99 recover the isolated from co-culture experiments, these were Pseudomonas
100 Selective Agar (Sigma Aldrich, UK) and Baird Parker Agar (Sigma Aldrich, UK).

101

102 *Preparation of bacterial extracts*

103 Biofilms of *S. aureus* and *P. aeruginosa* were cultured in 5ml NB in sterile plastic
104 Petri dishes for 24, 48 or 72h at 37°C, media was not changed during this time.
105 Spent media (containing constituents secreted by biofilm bacteria) from these three
106 time points was aspirated, centrifuged at 9,000 *g* and heat treated at 60°C for 1h.
107 Cell extracts were prepared by washing the biofilm three times with phosphate
108 buffered saline (PBS; Oxoid) scraping biofilm cells from the Petri dish using a cell
109 scraper and re-suspending them in 1ml sterile PBS prior to heat treatment as
110 described above. Secreted and cell extracts were stored at -20°C.

111

112 *Static biofilm model*
113

114 Co-cultured biofilms were prepared by culturing planktonic *P. aeruginosa* and *S.*
115 *aureus* (separately) aerobically for 16h in NB at 37°C, which were subsequently
116 harvested by centrifugation at 3,000 *g*, at 4°C for 5 minutes. Bacterial cell pellets

117 were re-suspended in 1ml of PBS (Oxoid) and the optical density at 600nm (OD_{600};
118 SPECTROstar^{Nano}, BMG Labtech) of the culture was adjusted to 0.1 (± 0.05;
119 equivalent to $1x10^8$ cfu ml^{-1}); 100µl total volume of bacterial cells from both cultures
120 were mixed in a 1:1 ratio and 5µl used to inoculate wells of a 96-well microtitre plate
121 (MTP), containing 50µl NB per well. Following incubation at 37°C for 24, 48 or 72h,
122 media and non-adherent cells were aspirated and the biofilm biomass was
123 determined by staining with 0.5% (w/v) crystal violet, which was re-solubilized with
124 7% (v/v) acetic acid and its concentration determined by its absorbance at 595nm.

125 For analysis of the effect of bacterial extracts on biofilm formation and
126 disruption, 10µl of either secreted or cell extract was added to developing (at time
127 zero) biofilm or pre-cultured (24h) biofilm. In the latter case, pre-cultured biofilm was
128 incubated with the extract for 1h at 37°C prior to staining for biomass.

129 For sequential biofilm culture, single species biofilms were grown as
130 described above and following 24, 48 or 72h incubation, the second microorganism
131 was inoculated at $1x10^8$ CFU ml^{-1} and growth allowed to continue for a further 24h at
132 37°C. Biomass was assessed as described above.

133

134 *Total viable count and assessment of viability*

135

136 Biofilms were grown as described above. Media and planktonic cells were aspirated
137 and the biofilms scraped off the surface of the plate using a cell scraper; 100µl of
138 PBS was added to each well and the bacterial aggregates re-suspended by vigorous
139 pipetting. Cell suspensions were transferred to sterile microcentrifuge tubes,
140 vortexed to ensure the cell suspension was homogenous and, serially diluted from
141 10^{-1} to 10^{-7} using PBS. The Miles & Misra technique was used to determine the CFU

142 ml^{-1} and was performed in triplicate (Miles et al., 1938). Plates were incubated at
143 37°C for 24h.

144

145 *Protein binding assay*
146

147 To determine the adherence of co-cultured *S. aureus and P. aeruginosa* to
148 immobilized fibronectin, fibrinogen and collagen (Sigma Aldrich) 1µg of each protein
149 was used to coat the surface of a 96-well MTP; proteins were dissolved in coating
150 buffer (20mM sodium bicarbonate buffer, pH 9.3) and wells were blocked with 1%
151 (w/v) bovine serum albumin (BSA) to prevent non-specific binding. *P. aeruginosa*
152 and *S. aureus* were grown and a 1:1 ratio was prepared as previously described.
153 Bacterial cells were adhered to protein coated wells for 2h at 37°C. Following
154 incubation, non-adherent cells were aspirated from the wells; the plate was washed
155 with PBS and adherent cells fixed with 25% (v/v in water) formaldehyde for 30min at
156 room temperature. Adherent bacteria were stained with 0.5% (w/v) crystal violet
157 which was then re-solubilized and analysed as described above. The assay included
158 a control set of wells containing only coating buffer without protein ligands to
159 determine whether the wells of the plate were sufficiently coated. The experiments
160 used three biological replicates and each assay was performed in triplicate.

161 Assays to determine affinity of *S. aureus* and *P. aeruginosa* for bacterially-
162 derived cellular extracts utilized the same protocol except that wells were coated with
163 50µl aliquots of extract diluted in an equal volume of coating buffer. Blocking assays
164 were carried out as described above, however the single species biofilm was
165 blocked by the addition of an anti-staphylococcal antibody (1:200 in PBS;
166 ThermoScientific) for 1h at room temperature, prior to the addition of *P. aeruginosa.*

167 *Aggregation assay*

168

169 Bacterial suspensions and ratios were prepared as previously described, to achieve
170 a final volume of 1ml in co-aggregation buffer (1mM Tris-HCl [pH 8], 150mM NaCl,
171 0.1mM $CaCl_2.2H_2O$, 0.1mM $MgCl_2.6H_2O$). Fibronectin, fibrinogen or collagen (final
172 concentration 1 μg ml^{-1}) were added separately to the aliquot of bacterial cells. In an
173 untreated control, the proteins were replaced with PBS to maintain the appropriate
174 volume and concentration. Triplicate samples were thoroughly mixed and incubated
175 at 37°C. The OD_{600} was measured after 210min. The composition of aggregates was
176 determined by TVC, using the method of Miles and Misra, previously described. The
177 experiments used three biological replicates and each assay was performed in
178 triplicate.

179

180 *Gentamicin protection assay*
181

182 HaCaT (an immortalised keratinocyte cell line)cells were maintained in Dulbecco's
183 Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated Foetal
184 Bovine Serum (FBS) (Biosera, East Sussex, UK), 2mM glutamine and 2.5g/L
185 glucose (all supplements were purchased from Gibco BRL, Paisley, UK). Cells were
186 cultured at 37°C in a humidified atmosphere with 5% CO_2 for optimal growth and
187 proliferation.

188 The gentamicin protection assay followed the method of Rasigade *et al.* with
189 some modifications (Rasigade et al., 2011). HaCaT cells were seeded at 50,000
190 cells/well in 24-well plates and incubated at 37°C with 5% CO_2 for 48h in culture
191 medium, as described above. Bacterial strains were cultured and 1:1 ratios

192 prepared as previously described (using DMEM). HaCaT cells were washed twice
193 with DMEM and were infected with bacterial cells at a multiplicity of infection of
194 200:1. Cells were incubated at 37°C for 2h to allow for adhesion and internalization
195 of bacteria, and then washed with PBS to remove any unbound bacterial cells. For
196 adhesion assays, cells were osmotically shocked using pure water and extensively
197 pipetted to release all cell-associated bacteria. For invasion assays, infected cells
198 were incubated for a further 1h in culture medium containing 200 µg ml^{-1} gentamicin
199 to kill extracellular, but not internalized, bacteria. Cells were washed twice in PBS
200 and treated with pure water as described above to release internalized bacteria. For
201 both assays bacterial cells were enumerated using selective media as described
202 previously. The number of adherent bacteria was calculated by subtracting the
203 number of internalized bacteria from the total number of cell-associated bacteria.

204 *ELISA for pro-inflammatory cytokines*

205 Cell culture media was harvested from scratch assays and analysed by ELISA to
206 determine the concentration of IL-1β, IL6 and TNF-α. These included untreated
207 (control) cells, and these treated with bacterial extracts derived from single and two-
208 species biofilms cultured for 24, 48 and 72h. ELISA's were carried out according to
209 the manufacturer's instructions (Novex ELISA Kits; ThermoFisher Scientific). Briefly,
210 50µl of cell supernatant (or standard) was added to each well of a pre-coated 96well
211 MTP followed by 50µl of biotinylated antibody reagent and incubated at room
212 temperature for 3h. The plate was washed three times with wash buffer (provided by
213 the manufacturer) and 100µl of Streptavidin HRP solution added and, the plate
214 incubated at room temperature for 30min. The plate was washed three times and
215 100µl of developer added to each well. After 30min 100µl of stop solution was

216 added to each well and the absorbance recorded at 450/550nm using a

217 SPECTROstar^{Nano} reader (BMG Labtech).

218 *In vitro scratch wound healing assays*

219

220 HaCaT cells were seeded at 50,000 cells/well in a 12-well plate, and grown to 90%

221 confluence prior to beginning scratch assays. Once confluence was reached media

222 was removed and a scratch wound made using a 10μl pipette tip; each well was

223 washed with warm PBS (37°C) and cells incubated for a further 24h in the presence

224 and absence of secreted or cellular components derived from single or two-species

225 biofilms (as previously described). Images were taken at 24h to observe wound

226 closure.

227 *Statistical analysis*

228

229 Statistical analysis of data used ANOVA followed by Tukeys post-hoc test, using

230 Minitab v14.

231

232 **Results**

233 *S. aureus predominates in early biofilm*
234

235 Early biofilm formation occurs via a combination of bacterial auto/co-aggregation,
236 and attachment to a sub-stratum. Two-species biofilms comprised of *S. aureus* and
237 *P. aeruginosa,* cultured for 24-72h, showed a statistically significant ($P<0.05$)
238 increase in the amount of accumulated biofilm (Figure 1A) when stained with crystal
239 violet, relative to single species biofilms of *S. aureus*, cultured for the same amount
240 of time (Figure 1B). However, the biomass increases observed between *P.*
241 *aeruginosa* mono-species and dual-species biofilm, did not equate to a statistically
242 significant fold-change when compared to single-species biofilms of *P. aeruginosa*
243 over the same period (Figure 1B). Fold changes were calculated relative to single-
244 species biofilms for each bacterium. Analysis of the composition of two-species
245 biofilm by CFU (using selective media as indicated in materials and methods) and
246 subsequent calculation of competitive relative index, indicated that *S. aureus*
247 predominated at each time point with higher competitive relative indices than *P.*
248 *aeruginosa* (Figure 1C). Competitive relative indices are derived from CFU and
249 therefore higher competitive indices signify higher numbers of bacteria. This
250 suggests that it is advantageous for *S. aureus* to be part of a two-species biofilm in
251 terms of synergistic growth.

252 Early biofilm development for both *S. aureus* and *P. aeruginosa* relies on the
253 formation of bacterial aggregates in suspension, which subsequently attach to a solid
254 substratum (Birkenhauer et al., 2014). Relative to auto-aggregation of each
255 bacterium alone, co-aggregation of both bacteria together in suspension, led to
256 reduced total aggregation after 210 min, relative to *S. aureus* alone (auto-
257 aggregated), but was not statistically different compared to *P. aeruginosa* alone

258 (auto-aggregated) over the same time period (Figure 2A). By comparing the extent
259 of co-aggregation to auto-aggradation, for each bacterium it was apparent that co-
260 aggregation negatively impacted on *S. aureus* (i.e. the propensity to aggregate was
261 less), but positively impacted on the propensity for *P. aeruginosa* to aggregate (i.e.
262 *P. aeruginosa* aggregated more readily) (Figure 2A). Competitive relative indices
263 derived from TVC analysis of bacterial aggregates indicated that despite the reduced
264 aggregation observed for *S. aureus* when co-aggregated with *P. aeruginosa*, *S.*
265 *aureus* comprised the largest proportion of the bacterial aggregates (Figure 2B).

266 Collectively these data indicate that during the early stages of biofilm
267 development, *S. aureus* has a competitive advantage over *P. aeruginosa* in terms of
268 aggregation, attachment and growth, but also promotes increased aggregation of *P.*
269 *aeruginosa*. This could be advantageous since the latter has fewer known surface
270 adhesins and attachment to *S. aureus* could provide a means of integrating into the
271 competitive environment of the early biofilm (Prince, 1992; Clarke & Foster, 2006).

272

273 *S. aureus early biofilm influences the attachment and maintenance of P. aeruginosa*
274 *biofilm*

275 Clinical analysis of wound infection shows a typical “Gram-negative shift” over time,
276 (Altoparlak et al., 2004; Dalton et al., 2011; Guggenheim *et al*., 2011; Pastar et al.,
277 2013). We investigated whether Gram positive *S. aureus,* through provision of a
278 conditioning layer, promoted this shift by enhancing attachment of *P. aeruginosa,* as
279 is observed for other human biofilms (Kolenbrander et al., 2010). Using mono-
280 species biofilm of each respective bacterium as a base-line for comparative biomass
281 accumulation and fold-changes in biofilm mass, it was evident that biofilm of *S.*
282 *aureus* cultured for 24, 48 and 72h, subsequently inoculated with *P. aeruginosa*

283 accumulated a significantly (P<0.05) higher mass of biofilm (Figure 3A and B)
284 relative to biofilm of *S. aureus* alone. Conversely, mono-species biofilms of *P.*
285 *aeruginosa* inoculated with *S. aureus* as a secondary coloniser did not show any
286 significant increase in biofilm mass relative to single-species biofilm of *P. aeruginosa*
287 (Figure 3A and B). Of note is the statistically significant (P<0.05) decrease in the
288 accumulation of biofilm for 24h established biofilm of *P. aeruginosa* following
289 inoculation with *S. aureus* and further incubation for 24h (Figure 3A and B).

290 To ascertain whether increased biofilm formation of *P. aeruginosa* when
291 inoculated onto pre-formed biofilm of *S. aureus* was a consequence of direct
292 attachment to *S. aureus,* microtitre plates were coated with protein extracts derived
293 from 24, 48 and 72h old biofilms of *S. aureus.* This resulted in a 2-2.6-fold increase
294 in *P. aeruginosa* biofilm relative to *P. aeruginosa* mono-species biofilm. This effect
295 was not observed when the *S. aureus* proteins coating the MTP were blocked with
296 an anti-staphylococcal antibody (Figure 3C), and biofilm accumulation was
297 significantly (P<0.05) reduced. Addition of spent media aspirated from *S. aureus*
298 biofilm cultured for 24, 48 and 72h, to 24, 48 and 72h old biofilm of *P. aeruginosa*
299 showed a similar effect to the addition of live *S. aureus* to pre-formed biofilm of *P.*
300 *aeruginosa*, in that the amount of biofilm was significantly (P<0.05) less (Figure 4A)
301 compared to the untreated control and biofilms treated with extracts from 48 and 722
302 biofilm. There was a concurrent reduction in recoverable bacteria (by CFU) (Figure
303 4B) from 24h *P. aeruginosa* biofilms treated with secreted extracts derived from 24h
304 *S. aureus* biofilm indicating the possibility of biofilm dispersal or killing of biofilm
305 bacteria by *S. aureus*.

306 This suggests both competitive and mutual interactions between *S. aureus*
307 and *P. aeruginosa* during early biofilm establishment whereby *S. aureus* directly

308 disrupts immature biofilm of *P. aeruginosa*, but once it is itself established it allows
309 for secondary attachment of *P. aeruginosa.* Furthermore, these data support clinical
310 observations that persons who are pre-colonised with *S. aureus* have a higher
311 predisposition for secondary infection with *P. aeruginosa* (Ahlgren et al., 2015; Limoli
312 et al., 2016).

313 *S. aureus and P. aeruginosa do not competitively attach or adhere to human plasma*
314 *proteins*

315 Human tissue proteins such as collagen and hyaluronin can influence polymicrobial
316 biofilm development (Biyikoglu et al., 2012; Birkenhauer et al., 2014). Co-cultured *S.*
317 *aureus* and *P. aeruginosa* showed impaired adherence to immobilised fibronectin,
318 fibrinogen and collagen, that was not statistically significant ($P>0.05$) (less than one-
319 fold), relative to mono-cultured bacteria (Figure 5A). Calculated competitive relative
320 indices based on TVCs recovered for each organism, indicated that despite less
321 attachment of both bacteria overall, *S. aureus* was more adept at adhering to
322 immobilised proteins than *P. aeruginosa* (Figure 5B).

323 Soluble fibrinogen, or collagen resulted in negligible co-aggregation (Figure
324 5C) over the time course of the experiment; soluble fibronectin did not mediate any
325 increase or decrease in co-aggregation relative to co-aggregation in co-aggregation
326 buffer alone over the time course of the experiment (Figure 5C; Figure 2A).
327 Furthermore, analysis of the composition of bacterial aggregates by TVC, and
328 calculated competitive relative indices, indicated little if any competition for
329 attachment (Figure 5D) as similar numbers of each bacteria were recovered.

330 Therefore, these data suggest that the early interactions between *S. aureus*
331 and *P. aeruginosa* that precede biofilm establishment and maturation are not

332 dependent upon the host proteins fibronectin, fibrinogen or collagen; this contrasts
333 with other biofilm forming organisms that thrive as polymicrobial communities within
334 the human host, and rely on host extracellular matrix proteins for biofilm
335 establishment (Rickard et al., 2003; Peters et al, 2012).

336 *Co-infection of human keratinocytes mediates increased bacterial invasion*

337 Given the differential capacity for attachment, aggregation and interaction using a
338 combination of microtitre-based assay and purified human proteins, we next aimed
339 to establish whether such interactions occurred using a human cell line to represent
340 the skin. Using an immortalised human keratinocyte infection model infected with
341 either *P. aeruginosa* or *S. aureus*, or both organisms together in a ratio of 1:1, we
342 observed a statistically significant (P<0.05) increase in the number of *P. aeruginosa*
343 adhered to the cell monolayer, in co-culture than in monoculture (Figure 6A). The
344 number of internalised, and therefore invasive, *S. aureus* were significantly higher
345 (P<0.05) in co-culture compared to mono-culture (Figure 6A). Repeat experiments
346 using either spent media (containing secreted constituents) or cellular constituents
347 derived from washed biofilm were undertaken; for *S. aureus* these were concerned
348 only with invasion; for *P. aeruginosa* these were concerned only with attachment.
349 There were statistically significant (P<0.05) fold-increases in invasion of
350 keratinocytes by *S. aureus* (calculated relative to untreated controls) in response to
351 both secreted and cellular material derived from *P. aeruginosa* biofilms cultured for
352 24, 48 and 72h. Despite being statistically significant (P<0.05) relative to untreated
353 controls, but there was no significant difference between culture times (6B). This
354 indicates that the increased invasion could be mediated by soluble or cell-bound
355 factors. For *P. aeruginosa* infected keratinocytes treated with secreted or cellular
356 material derived rom *S. aureus* biofilms, there was no statistically significant fold-

357 increase in attachment irrespective of culture time (Figure 6C). This suggests that
358 intact or live *S. aureus* are likely necessary to serve as a pioneer or bridging
359 organism when attached to human cells.

360 *Co-cultured bacteria impair the pro-inflammatory response of damaged keratinocytes*

361 Having demonstrated the interaction between *S. aureus* and *P. aeruginosa* impacted
362 upon adhesion and invasion into human keratinocytes, we then explored the effect
363 that this might have on the pro-inflammatory response of these cells since they are
364 the first to encounter pathogens during skin/wound infection. Treatment of
365 immortalised human keratinocytes with extracts derived from mono- and co-cultured
366 biofilm indicated a general trend in support of an impaired pro-inflammatory
367 response for co-cultured biofilm, which would in turn mediate reduced neutrophil
368 migration (Figure 7). Significantly, higher levels of IL-6 were evident for co-cultured
369 biofilm compared to *S. aureus* mono-cultured biofilm ($P<0.05$) but the converse was
370 true for mono-cultured *P. aeruginosa,* suggesting collectively that co-culture resulted
371 in an intermediate response. However, the elevated production of IL-6 compared to
372 the untreated control (20pg ml^{-1} [untreated] compared to 210pg ml^{-1} [co-culture])
373 suggest that this level of IL-6 production would still favour a pro-inflammatory state,
374 synonymous with chronic infection.

375

376

377 *Co-cultured biofilm perpetuates a non-healing state that is no more recalcitrant than*
378 *mono-cultured biofilm*

379 The pro-inflammatory response can perpetuate a state of persistent, localised
380 damage that impairs healing and promotes the maintenance of infection. Based on
381 our observations of differential cytokine production in response to co-cultured biofilm,
382 monolayers of immortalised human keratinocytes were damaged by scratching, and
383 subsequently treated with spent supernatant or cellular material derived from mono-
384 or co-cultured biofilm. Application of secreted biofilm components derived from co-
385 cultured biofilm (24 and 48h) had a significant effect ($P<0.05$) on scratch repair
386 compared to mono-cultured biofilm of both *S. aureus* and *P. aeruginosa* (Figure 8A).
387 Specifically healing was impaired for a greater extent by extracts derived from co-
388 culture compared to *S. aureus* in mono-culture, but healing was impaired to a lesser
389 extent for *P. aeruginosa* compared to mono- culture. Reminiscent of the cytokine
390 production profiles (Figure 7), this indicated an intermediate result for co-cultured
391 biofilm, but repair was still impaired compared to the untreated control; this supports
392 the model of impaired healing associated with wound chronicity. Cell derived
393 components had no significant effect on scratch repair suggesting that secreted
394 factors such as toxins or pyocyanin facilitated the observed effect, rather than cell-
395 structures such as LPS or LTA.

396 **Discussion**

397 Pre-colonisation with *S. aureus* is known to be a risk factor for colonisation with *P.*
398 *aeruginosa* in patients with chronic lung infection, such as that seen in cystic fibrosis.
399 Competition between these microorganisms within the chronic infected lung is well
400 documented and associated with worse patient outcomes (Baldan et al., 2014;
401 Fugere et al., 2014; Limoli et al., 2017). Information derived from the study of
402 chronic infected wounds lags that of chronic lung infection, but it is known that both
403 *S. aureus* and *P. aeruginosa* can co-exist within the chronic wound environment;
404 these microorganisms are the two species most commonly isolated from such
405 wounds (Hotterbeekx et al., 2017).

406 Disparate evidence has described scenarios in which these two pathogens
407 co-exist in a co-operative or competitive manner, or alternatively that they inhabit
408 discrete sites within the wound meaning that they are unlikely to interact (Hendricks
409 et al., 2001; Pastar et al., 2013; DeLeon et al., 2014). The latter hypothesis is based
410 on the observation of these bacteria in biopsies of chronic infected wounds and the
411 former have been reliant on *in vitro* and/or *in vivo* models (Fazli et al., 2009;
412 Hotterbeekx et al., 2017). We hypothesise that during the early phases of co-
413 colonisation *S. aureus* and *P. aeruginosa* interact, and the distinct colonisation
414 pattern observed in wound biopsies occurs over a long period of time, during which
415 competition for space and nutrients, drives the population towards separation. We
416 suggest that during those early stages of co-colonisation, bacterial interaction might
417 mediate heightened host-damage but that over time the pathoadapted population
418 undergoes niche partitioning with a concurrent reduction in host damage, conducive
419 to maintenance of a stable microbial population.

420 The findings presented here demonstrate that a two-species biofilm
421 comprised of *S. aureus* and *P. aeruginosa* produces a significantly ($P<0.05$) and
422 consistently greater biofilm accumulated over a period of 72h, and that within these
423 biofilms *S. aureus* is most numerous as a consequence of successful competition
424 against *P. aeruginosa,* evidenced by analyses of relative competitive indices.
425 Examination of early co-aggregation events that precede biofilm establishment infers
426 advantageous circumstance for *P. aeruginosa* which aggregates more effectively in
427 the presence of *S. aureus*. Despite this, bacterial aggregation was diminished
428 overall, but *S. aureus* was most numerous within the aggregates. Soluble human
429 serum proteins did not enhance aggregation nor attachment, contrary to other
430 studies which have demonstrated that collagen and hyaluronin at wound sites can
431 influence early polymicrobial adhesive events (Birkenhauer et al., 2014).
432 Contemporary biofilm research has described the important role of bacterial
433 aggregates in biofilm formation, indicating that it is possible that biofilms are seeded
434 wholly or in part from aggregates (Kragh et al., 2016; Melaugh et al., 2016). This
435 being the case our results indicate that by co-aggregating with *S. aureus* the
436 likelihood of colonisation by *P. aeruginosa* is elevated.

437 Further to these data, we demonstrate that when *S. aureus* is employed as a
438 pioneer coloniser in biofilm growth assays it augments the attachment of *P.*
439 *aeruginosa*. This effect is not reciprocal, moreover *S. aureus* added as a secondary
440 coloniser to biofilms of *P. aeruginosa* established for 24h, results in diminished
441 overall biofilm biomass indicating that *S. aureus* disrupts biofilm of *P. aeruginosa* in
442 some way, possibly either by dispersal or through direct bactericidal activity.
443 Additionally, secreted components derived from biofilm of *S. aureus* replicated this
444 effect suggesting that biofilm dispersal relies on a secreted factor. *S. aureus*, but

445 not *S. epidermidis,* is known to produce nucleases that either prevent or disrupt
446 biofilm growth for a number of bacterial pathogens, including *P. aeruginosa* (Pihl et
447 al., 2010; Tang et al., 2011; Yang et al., 2011; Pihl et al., 2013). Furthermore, the
448 secreted protein SpA (staphylococcal protein A) is known to interact with *P.*
449 *aeruginosa* to impair biofilm formation and enhance the propensity to persist
450 (Armbruster et al., 2016); it is therefore likely that the effects we have observed are
451 mediated by either of these two proteins.

452 In a human cell model we demonstrate that *S. aureus* increases the numbers
453 of *P. aeruginosa* that attach to the surface of keratinocytes. The same effect was not
454 observed when keratinocytes are inoculated with *P. aeruginosa* in a suspension
455 containing cellular components or spent media derived from staphylococcal biofilm
456 suggesting that whole staphylococci, attached to human keratinocytes are required
457 for augmented adhesion of *P. aeruginosa*. This synergy is reciprocal; in co-culture
458 *P. aeruginosa* results in significantly ($P<0.05$) larger numbers of *S. aureus* invading
459 the keratinocyte cell line, an effect which is also observed when *S. aureus* was
460 inoculated into the cell line in the presence of spent media or cell-derived material
461 from *P. aeruginosa* biofilm. Synergy of virulence between *S. aureus* and *P.*
462 *aeruginosa* has been previously reported but appears strain-specific with no
463 conserved interactions described (Hendricks et al., 2001; Pastar et al., 2013;
464 DeLeon et al., 2014; Kumar and Ting, 2015; Serra et al., 2015). These include
465 upregulation of *hla* and *pva*, encoding α-haemlysin and Panton-Valentine leucoidin
466 and production of staphyloxantin, but, to our knowledge we report for the first time,
467 an increased propensity for invasion. Of note are the disparate consequences of
468 interaction between *S. aureus* and *P. aeruginosa* on virulence which highlights the
469 importance of co-adaptation between these two organisms.

470 Damaged or infected keratinocytes produce a number of cytokines including
471 IL-6, IL-8 and TNF-α. Keratinocytes respond to these cytokines by proliferating and
472 differentiating, a secondary function of these cytokines is to promote inflammation
473 and the recruitment of macrophage to the site of infection. Co-infected keratinocytes
474 showed an altered cytokine profile compared to single-species infection. Generally,
475 the response was heightened when compared to *S. aureus* alone, but dampened
476 when compared to *P. aeruginosa* alone. This intermediate response to co-infection
477 is conducive to the maintenance of low-level tissue damage associated with
478 chronicity, prolonged colonisation and impaired wound repair. Scratch assay
479 corroborated these findings and those of others, in which co-culture impaired
480 healing. For some secreted components this was statistically significant, but for
481 cellular derived extract, healing was impaired to no greater extent than for mono-
482 culture. Certainly, studies of intra-abdominal wound infection indicate that
483 polymicrobial infection does not impair the rate of healing and recovery compared to
484 mono-microbial infection (Shah et al., 2015). Conversely other studies have shown
485 that re-epithelialisation is delayed by polymicrobial infection comprising *S. aureus*
486 and *P. aeruginosa* (Pastar et al., 2013). During this study we were not able to
487 establish whether the impaired healing observed was due to arrested keratinocyte
488 proliferation or migration.

489 Collectively, the findings of this study indicate clear interactions between *S.*
490 *aureus* and *P. aeruginosa* that are both competitive and reciprocally beneficial to
491 each organism, in terms of pathogenicity and colonisation. Several studies have
492 shown that these bacteria interact in polymicrobial infection resulting in differential
493 immune response and enhanced virulence. Indeed alginate production by *P.*
494 *aeruginosa* has been shown to promote co-existence, and the immune protein

495 calpropectin induced co-colonisation of the lung by these two pathogens (Wakeman
496 et al 2016; Limoli et al., 2017). But analysis of wound biopsies show clear
497 segregation between the two species (Fazli et al., 2009). Therefore within the
498 context of the broader body of research describing both *in vitro* and *in vivo*
499 interaction between *S. aureus* and *P. aeruginosa,* we suggest that these bacteria
500 interact early on to afford the best chance of colonisation and to augment virulence,
501 but that once established, both facets diminish and the two bacteria separate into
502 distinct niches. Recent work using laser electrospray ionisation mass spectrometry
503 has indeed shown significant co-localisation between these microorganisms in
504 biofilm cultured for 24 hours, rather than self-segregation, which might be in part due
505 to dynamic phenotypic changes that occur in *S. aureus* during early attachment to
506 biotic and abiotic surfaces (Williams et al., 1997; William's et al, 1999; Dean et al.,
507 2015).

508 Our findings also support the pre-colonisation hypothesis that is well
509 established for cystic fibrosis lung, whereby persons pre-colonised with *S. aureus*
510 are pre-disposed to secondary colonisation with *P. aeruginosa*. There is an
511 emerging international consensus that most of the current guidelines on the
512 prevention and treatment of biofilms are of limited utility in chronic wounds as
513 bacteria are present in biofilms as aggregated communities (Hoiby et al., 2015;
514 WHO, 2015; WUWHS, 2016). Clinicians could prioritise patient groups who are at
515 a higher risk of colonisation with *P. aeruginosa* by screening chronic wounds for *S.*
516 *aureus* and subsequently be in a position to intervene at the earliest opportunity.
517 Globally, diagnostic labs are equipped to routinely culture and identify *S. aureus*
518 therefore such an intervention could be utilised in both developed and transitional

519 countries to help to manage the burden of infection disease and concurrently support
520 antimicrobial stewardship.

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524 **Acknowledgements**

525 We would like to thank the Leonardo Da Vinci program for enabling PMA to

526 undertake this work. We would also like to thank Raymond Samuriwo for helpful

527 insights.

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