

1 **Title**

2 Propionic acid enhances the virulence of Crohn's disease-associated  
3 adherent-invasive *Escherichia coli*

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40 **Abstract**

41 Short chain fatty acids (SCFA), such as propionic acid (PA), are natural human intestinal antimicrobials  
42 and immune modulators that are also used in Western food production and agriculture. Here we examine  
43 the effect of PA on the pathogenicity of the Crohn's disease-associated microbe, adherent-invasive  
44 *Escherichia coli* (AIEC). We show that AIEC is insensitive to the antimicrobial effects of PA and adapts  
45 to utilize it for efficient growth. Repeated exposure of AIEC to PA significantly increases AIEC  
46 adherence to human intestinal epithelial cells, acid tolerance and biofilm formation. RNA-sequencing  
47 identified transcriptional changes in response to PA with upregulation of genes involved in biofilm  
48 formation, stress responses, metabolism, membrane integrity and use of alternative carbon sources.  
49 Finally, after pre-exposure to PA, AIEC demonstrated an increased ability to colonize (>5-fold) and  
50 persist (>50-fold) in an *in vivo* model where the low murine intestinal PA concentrations were increased  
51 to mimic those found in humans. Our data indicates that exposure of AIEC to PA evolves bacteria that are  
52 both resistant to this natural human intestinal antimicrobial, and increasingly virulent in its presence.

53 **Importance**

54 Propionic acid (PA) is a common agricultural and industrial antimicrobial in the Western world. Its use is  
55 increasing as efforts intensify to reduce reliance on antibiotics, particularly in food production. Here we  
56 show that exposure of adherent-invasive *Escherichia coli* (AIEC) to PA induced significant virulence  
57 associated phenotypic changes. AIEC exhibited greater adherence and biofilm formation *in vitro*,  
58 translating into greater colonization and persistence in an *in vivo* mouse model. RNAseq analysis  
59 identified transcriptional changes directly related to these phenotypic changes. While PA is generally  
60 regarded as safe for both human and animal use, the significance of SCFAs such as PA to maintaining  
61 human intestinal immune health means their widespread use requires further scrutiny. This will allow us  
62 to understand if, in a similar manner to antibiotics, SCFAs are facilitating horizontal transmission of  
63 microorganisms.

## 64 **Introduction**

65           The effect of increased preservative use on bacteria, particularly in agriculture and food  
66 producing animals (FPAs), has remained largely unexplored. While there is increasing evidence for  
67 antibiotic driven enhanced genome wide mutation rates [1] and horizontal transmission of bacteria from  
68 FPAs to humans, the role of alternative antimicrobials in driving a similar form of horizontal transmission  
69 of microbes has not been addressed [2–4].

70           One such group of alternative antimicrobials are short chain fatty acids (SCFAs). SCFAs are  
71 naturally produced within the gut as inflammatory mediators as the microbiota facilitate the breakdown of  
72 undigested carbohydrates and starches into small carbon molecules with acetic (AA), butyric (BA) and  
73 propionic acids (PA) accounting for approximately 90% of these. SCFAs are also easy to manufacture on  
74 an industrial scale and are now commonly used in agriculture, as in addition to their anti-inflammatory  
75 effects, are antimicrobial. SCFAs have exhibited particular successes in reducing pathogen numbers in  
76 poultry, with PA particularly effective in reducing *Salmonella* and *Campylobacter* carriage [5–8]. PA is  
77 also an effective antimicrobial agent used in Western food production and agriculture to reduce the need  
78 for antibiotic use amidst growing antibiotic resistance concerns [9–11]. Preservation of animal feed, grain,  
79 and food accounted for almost 80% of PA consumption across the world in 2016 with Western Europe  
80 (40% of total use), North America (30%) and Asia (23%), the main consumers (17). However, the success  
81 of SCFAs in reducing antibiotic dependence is now seeing their use spread to countries across Africa, the  
82 Middle East and Central and South America [12].

83           PA has attracted recent interest, as PA supplementation in murine models has been shown to  
84 reduce the severity of Crohn’s disease (CD) and the associated colitis [13,14]. CD is a debilitating and  
85 incurable inflammatory disease of a multi-factorial nature that occurs in genetically susceptible  
86 individuals. The mechanisms underlying the disease are not fully understood however it is thought that  
87 defects in the immune response to the gut microbiota are a contributing factor [15–18]. Sudden changes in  
88 diet have been shown to result in rapid changes in the gut microbiota [19–21], while the inflammation  
89 associated with CD results in markedly decreased microbial diversity [22–24]. Levels of

90 *Enterobacteriaceae* in particular are higher in intestinal samples from CD patients when compared to  
91 healthy controls [25–27]. One group of *Enterobacteriaceae* that is of particular interest is the *Escherichia*  
92 *coli* pathotype, adherent-invasive *E. coli* (AIEC). These bacteria are overrepresented in the intestinal  
93 microbiota in the inflamed gut of CD patients being present in 51.9% of mucosal samples from CD  
94 patients compared with 16.7% in healthy controls [28]. Key features of the AIEC pathotype that  
95 distinguish them from non-invasive commensal strains include; adherence to and invasion of the intestinal  
96 epithelium, an increased ability to form biofilms, and the ability to survive and replicate within  
97 macrophages without inducing cell death [28]. While AIEC strains harbour genetic similarity, in terms of  
98 phylogenetic origin and virulence genotype, with extra-intestinal pathogenic *E. coli* (ExPEC), they have  
99 few known virulence factors [29]. This apparent lack of virulence factors, and the discovery of AIEC  
100 strains across all five major diverse phylogroups of *E. coli* mean that an overarching explanation for the  
101 origin and virulence of AIEC has remained out of reach. This is of significant interest given that PA is a  
102 natural human intestinal antimicrobial; and AIEC is both phylogenetically and genomically linked to  
103 avian pathogenic *E. coli* (APEC) species [28,30–34] which doubtless come into contact with SCFAs that  
104 are used extensively in poultry production [9,11]. Additionally, there is mounting evidence of horizontal  
105 transmission of *E. coli* with poultry considered a potential vehicle or reservoir for other human disease  
106 causing *Escherichia coli*, such as extraintestinal pathogenic *E. coli* (ExPEC) [3,35–37].

107         In this study, we show that exposure of AIEC to PA results in an ability to utilize PA as a carbon  
108 source whilst simultaneously enhancing the virulent adherent and invasive phenotype of AIEC. The basis  
109 of these phenotypic changes was determined by RNA sequencing and the phenotype was determined to  
110 be reversible by removal of PA, suggesting an epigenetic basis for the changes. Exposure to PA also  
111 increases the colonization and long-term persistence of LF82 within a humanized murine model where  
112 PA levels have been increased from the low levels of the mouse intestine to those closer to that seen in the  
113 human intestine. This is the first time that a direct link has been made between bacteria over-represented  
114 in CD and a common SCFA preservative. Our work indicates that further research is needed to

115 understand the consequence of the use of SCFA antimicrobials especially when, as in the case of PA, they  
116 play such important roles in maintaining human intestinal health.

## 117 **Results**

### 118 *Short chain fatty acids as antimicrobials*

119 To examine the impact of PA on the phenotype of AIEC, LF82 was grown in minimal media  
120 supplemented with PA as the sole carbon source (20 mM). PA concentration increases across the human  
121 intestine from 1.5 mM in the ileum to 27 mM in the colon, with the greatest inhibitory effect on bacterial  
122 colonization seen where PA is highest [5,38]. Therefore 20 mM was chosen as a physiologically relevant  
123 concentration to grow LF82, as AIEC are known to colonize the ileal/colonic junction [25]. While LF82  
124 was able to grow in PA, the human commensal *E. coli* strain, F-18 (38), replicated extremely poorly.  
125 Following successive sub-culturing over five growth cycles in PA supplemented minimal media, a ‘PA-  
126 adapted’ strain of LF82 was generated termed LF82-PA. LF82-PA had a significantly reduced doubling  
127 time (3.98 h) in PA when compared to wild type LF82 (25.59 h; Fig. 1a). We were unable to generate a  
128 corresponding PA-adapted version of F-18 due to its reduced ability to metabolize and grow in PA. The  
129 increased rate of growth was specific to PA and did not impact growth of LF82-PA in nutrient rich  
130 Lysogeny broth (LB; Fig. 1b).

### 131 *Propionic acid exacerbates the adherent and invasive and biofilm forming phenotypes of AIEC*

132 AIEC adherence to, and invasion of, the intestinal epithelium and their increased ability to form  
133 biofilms are key features of this pathotype that distinguish them from non-invasive commensal strains  
134 [28]. To better understand any effects of increased growth on PA on these attributes in LF82-PA a  
135 number of virulence assays were performed. LF82-PA showed a significant increase in adherence to  
136 Caco-2 human intestinal epithelial cells when compared to wild type LF82 (>7.45 fold; p-value = 0.0371;  
137 Fig. 1c), whilst a trend towards increased invasion by LF82-PA was also observed but was not significant  
138 (>5.26 fold; p-value = 0.3297; Fig. 1d). Biofilm formation by LF82-PA was also significantly enhanced  
139 under anaerobic conditions (>1.72 fold; p-value = 0.00019; Fig. 1e). Finally, LF82-PA was more acid

140 tolerant than LF82 despite adaptation to PA being carried out at pH 7.4 (Fig. 1f). A reduction in cell  
141 number was seen for both strains in response to acidic conditions (pH 3) however the LF82-PA strain  
142 survived in greater numbers and for longer periods of time (At 20 min, LF82-PA recovered >30.4 fold  
143 higher than LF82 [p-value = 0.0286]; at 40 min, LF82-PA was >22.7 fold higher [p-value = 0.028]).

144 We hypothesized that this increased virulence was likely to be mediated by changes in the  
145 composition of the bacterial membrane following growth on PA. Direct incorporation of PA into the  
146 membrane is a mechanism employed by bacteria to minimize the toxic effects of excess PA in the  
147 environment [40–42]. Gas chromatography coupled to isotope ratio mass spectrometry (GC-IRMS) using  
148 <sup>13</sup>C labelled PA (1-<sup>13</sup>C sodium propionate) revealed that PA was not incorporated into odd chain long  
149 chain fatty acids (LCFAs). However, there appeared to be significant <sup>13</sup>C enrichment in 12 fatty acid  
150 methyl esters (FAMEs) that did not correspond to any of 37 FAMEs in our reference standard (see  
151 *Supplementary experimental procedures*). However we speculated that the proximity of these labelled  
152 peaks to LCFAs was due to incorporation of PA into methylated or branched chain fatty acids (BCFAs)  
153 as described previously during *Mycobacterium tuberculosis* growth on and detoxification of PA [40].

#### 154 ***A transcriptional basis for enhanced LF82-PA virulence***

155 Genome sequencing of three independently adapted biological replicates of LF82-PA revealed a  
156 number of single nucleotide polymorphisms (SNPs; Supplementary File S1). However, no SNPs were  
157 conserved across all strains. Detailed analysis of the genes and pathways in which the SNPs were  
158 identified did not lead to the identification of any candidate pathways that may explain the changes in  
159 virulence observed. However, we cannot exclude the possibility that different combinations of small  
160 genomic changes could result in the same outcome at the transcriptional level.

161 Therefore, given no mutational basis for the observed increase in virulence was detected, we  
162 employed a comparative RNA-sequencing (RNA-seq) approach to probe the global transcriptional  
163 profiles of LF82 and LF82-PA grown on PA. RNA-sequencing revealed 25 differentially expressed genes  
164 (DEGs; P-value ≤0.05) between LF82 and LF82-PA (Fig. 2; Supplementary Table S2); 24 were

165 upregulated in the LF82-PA strain and one (*mcbR*; -20.85 fold) was downregulated. Of the 25 DEGs  
166 identified by RNA-seq, 21 including *mcbR*, were validated as significantly altered by PA using qRT-PCR  
167 (Supplementary Fig. S1). Functional grouping of these 21 DEGs revealed their roles in diverse processes  
168 including biofilm formation, stress responses, metabolism, membrane integrity and transport of  
169 alternative carbon sources (Fig. 2c; Supplementary Table S2). Eight DEGs have well described roles in  
170 biofilm formation further adding to our *in vitro* findings indicating that PA was a driver of adhesion and  
171 biofilm formation (Fig. 1c & e). Upregulation of another DEG, a regulator of membrane fatty acid  
172 composition *yibT*, adds further evidence for potential detoxification of PA through membrane  
173 incorporation [40,41]. This RNA-seq analysis indicates that prior exposure of LF82 to PA can result in  
174 significant changes in transcription leading to an altered virulence phenotype.

#### 175 ***The enhanced LF82-PA phenotype is reversible***

176 Recently, a long-term epigenetic memory switch with a role in controlling bacterial virulence  
177 bimodality was identified in enteropathogenic *E. coli* (EPEC) [43]. This ‘resettable phenotypic switch’  
178 results in populations of virulent and hypervirulent genetically identical subpopulations that are retained  
179 through generations. As the virulent phenotype persists over a number of generations, and was not  
180 explained by genetic analysis, we hypothesised that the phenotype we see may be as a result of a similar  
181 epigenetic memory switch in LF82-PA. In order to examine this hypothesis, the LF82-PA strain was  
182 repeatedly passaged through rich (LB) media with no PA selective pressure. After five successive  
183 subcultures, the strain (LF82-PA-LB) no longer exhibited an increased growth rate in PA as LF82-PA  
184 did; and its virulence phenotype with regards adherence and invasion reverted to that of the wild type  
185 LF82 strain (Fig. 3). This indicates that the changes induced by PA were epigenetic and not due to SNPs  
186 or mutations.

#### 187 ***PA supplementation in a murine model promotes colonization and long-term persistence of AIEC***

188 Knowledge of any role for PA in bacterial infection in the intestine is lacking as murine models  
189 are limited by distinct differences in basal levels of SCFAs between the murine and human gut

190 (Supplementary Fig. S2a) [38]. To address this in our *in vivo* infection model, levels of PA in the murine  
191 intestine were increased by supplementing drinking water with 20 mM PA for three days prior to  
192 infection and for the duration of the infection. Caecal SCFA levels post-PA supplementation indicated  
193 that PA levels had increased while no significant changes were seen in either AA or BA levels  
194 (Supplementary Fig. S2b; AA:PA:BA ratios without PA 84.6:7.3:8.1; with PA 78.9:12.6:8.5). PA  
195 supplementation led to significantly greater LF82-PA colonization of both the ileum (Fig. 4a, >4.96 fold;  
196 p-value = 0.044) and colon (Fig. 4b, >5.57 fold; p-value = 0.028) compared to LF82 (Fig. 4). This effect  
197 was PA specific, with no significant difference in colonization observed at either site in the absence of PA  
198 supplementation (Fig. 4e, ileum: >1.99 fold; p-value = 0.550; Fig. 4f, colon: >2.36 fold; p-value = 0.282).  
199 No changes in colonization efficiency for either strain were noted in the caecum under either condition  
200 (With PA: >0.29 fold; p-value = 0.277; Without PA: >0.14 fold; p-value = 0.052; Supplementary Fig.  
201 S3).

202 For analysis of long-term persistence of both LF82 and LF82-PA, mice were again provided PA  
203 supplemented (20 mM) water for three days prior to infection, and for the 21-day duration of the  
204 infection. LF82-PA was again found to persist in significantly greater numbers than LF82 in both the  
205 ileum (Fig. 4c, >54.02 fold; p-value = 0.034) and colon (Fig. 4d, >73.44 fold; p-value = 0.013). Again  
206 this increased persistence was PA dependent, as LF82-PA did not colonize either site to a greater extent in  
207 the absence of PA (Fig. 4g, ileum: >0.16 fold; p-value = 0.237; Fig. 4h, colon: >0.81 fold; p-value =  
208 0.719). No significant difference in the persistence of either strain was observed in the caecum,  
209 irrespective of the presence of PA (Supplementary Fig. S3, >13.05 fold increase; p-value = 0.376).

## 210 **Discussion**

211 Dietary additives, and a mock Western diet, have been demonstrated to contribute to increased  
212 colonization of AIEC in murine models [20,21]. Additionally, recent evidence has implicated the food  
213 additive trehalose as a contributory factor in the emergence and hypervirulence of two epidemic lineages  
214 of *Clostridium difficile* [44]. Here we add to these findings demonstrating that a dietary and common-use



215 antimicrobial can significantly increase the virulence of AIEC. The use of PA as an antimicrobial stems  
216 from its inhibitory effects on bacterial colonization of the host, being both toxic and acting as a signal for  
217 virulence gene repression [5–7]. Given its potent antimicrobial effect, AIEC adapting to and growing on  
218 PA was unexpected (Fig. 1a). PA is of limited nutritional value to most prokaryotes with sustained  
219 growth leading to the production of toxic by-products [45]. However, a small number of bacteria are  
220 capable of growing on PA and it is recognized as being a major virulence determinant for *Mycobacteria*  
221 spp. which can degrade [46] or incorporate PA into membrane surface lipids [40–42]. Here, we saw  
222 similar changes in lipid content post-growth on PA with appearance of novel, and what we speculated to  
223 be branch chain, fatty acid methyl esters (FAMES). Adaptation to PA also manifested in increases in;  
224 adherence to a human colonic intestinal epithelial cell line, biofilm formation and colonization and  
225 persistence in the murine intestine. Importantly, after exposure to PA LF82-PA exhibited increased  
226 resistance to low pH meaning these bacteria are likely to be increasingly resistant to stomach acids and  
227 therefore potentially better able to reach and colonize the human intestine.

228 Mucosa-associated AIEC have been predominantly recovered from both the ileum [30,47,48] and  
229 the colon [25,48–50] of CD patients. In the present study, both LF82 and LF82-PA colonized the ileum,  
230 caecum and colon of male C57BL/6 mice but with no significant difference in colonization levels  
231 between LF82 and LF82-PA in a standard mouse model. However, given PA levels are significantly  
232 lower in the gastrointestinal tract of mice compared to humans (31; Fig. S2a), we employed PA  
233 supplementation to raise PA levels in our *in vivo* mouse model closer to those observed in humans. These  
234 mice were significantly better colonized by LF82-PA in both the ileum and colon (Fig. 4). This suggests  
235 that the prior exposure to PA renders LF82-PA better equipped to colonize an intestine, such as that in  
236 humans, with higher PA levels than those in a normal mouse. The increased intestinal PA also resulted in  
237 greater persistence of LF82-PA in both the ileum and the colon. Again this persistence was LF82-PA  
238 specific with the LF82 strain exhibiting a trend towards reduced persistence in the presence of increased  
239 PA, as previously described for *Campylobacter* and *Salmonella* [5–8].

240 Our findings raise a number of important issues. Firstly, present mouse models, given their  
241 distinct differences in SCFA levels to those of humans, are likely even poorer models for human intestinal  
242 disease than is already known [51]. Secondly, PA reduces colonization and persistence of a wild type  
243 AIEC strain as expected, but repeated exposure to PA creates a readily adapted strain of AIEC (LF82-PA)  
244 that easily colonizes and persists in the mouse where higher PA levels are representative of the human  
245 gut. Given the wide use of PA environmentally and agriculturally it is not inconceivable that bacteria such  
246 as AIEC come into contact with such concentrations of PA with concentrations in animal water, feed and  
247 silage reported to be 20 mM and higher [5,7,8,52]. Such exposure would likely make the PA  
248 concentrations in the human intestine, which increase from 1.5 mM in the ileum to 27 mM in the colon,  
249 easily tolerable to a strain such as the one we indicated here can be adapted [5,38].

250 To elucidate a mechanism behind the increased LF82-PA virulence, genomic and transcriptomic  
251 approaches were undertaken. While no reproducible differences were observed between LF82 and three  
252 independent replicates of LF82-PA at a sequence level, it cannot be ruled out that diverse combinations of  
253 SNPs may converge on the same phenotype. RNA-sequencing did, however, identify significant  
254 transcriptional differences between the strains, with 21 DEGs identified and validated (Fig. 2 and  
255 supplementary Fig. S1). These included DEGs with roles in biofilm formation, stress responses and  
256 metabolism that explained the mechanisms behind LF82-PA enhanced virulence. Biofilm gene regulation  
257 was most dramatically affected with increased expression for a number of known factors involved while  
258 the biofilm repressor, *mcbR*, was decreased greater than 20-fold [53]. Stress and metabolism genes were  
259 also significantly altered with the glyoxylate shunt repressor activated and increased shuttling of 3-carbon  
260 PA through the 2-methylcitrate cycle, as previously reported [54]. A number of DEGs of unknown  
261 function were also identified, requiring further characterization. Interestingly, these results indicate that *E.*  
262 *coli* behaves in stark contrast to *Salmonella* during intestinal infection. While it is reported that  
263 *Salmonella* actively colonize the small intestine [55,56], where PA concentrations are relatively low [38],  
264 the high PA concentration of the caecum and colon have been shown to represses *Salmonella*  
265 pathogenicity island 1 (SPII) which is essential for colonization [5,57,58]. In contrast to these findings,

266 we show that AIEC are not only able to tolerate these higher PA concentrations, but actively use PA as a  
267 stimulus for colonization with genes for such highly upregulated in the presence of PA. However, the  
268 LF82-PA phenotype is reversible (Fig. 3), which ultimately suggests that the adaptation to PA is most  
269 likely the result of an epigenetic trait in a similar manner to the epigenetic memory switch seen in EPEC  
270 [43]. This ability to rapidly adapt to the surrounding environment makes AIEC more likely to cross from  
271 the environment, poultry or FPAs to humans, colonize and persist in the human intestine.

272 In conclusion, the data presented in this study demonstrate that increased exposure to PA results  
273 in a more virulent AIEC strain that is readily adapted to utilize abundant SCFAs such as in the human  
274 gastrointestinal tract. This exposure of AIEC to PA confers no competitive advantage on the bacteria in a  
275 mouse model, unless PA levels are raised in the murine intestine to bring them closer to human intestinal  
276 PA levels. With the AIEC pathotype closely related to APEC and other ExPEC strains, there are  
277 suggestions that AIEC and closely related ExPEC may be environmentally- or avian-derived [28,30–34].  
278 Given the now widespread use of PA as an antimicrobial and the increasing evidence that *E. coli* are  
279 crossing host species boundaries with antimicrobial use a major driver, any role of alternative  
280 antimicrobials in facilitating AIEC spread must be examined [2–4]. The potential for AIEC crossing from  
281 the environment, poultry or FPAs to humans is a greater risk in our opinion as PA is not only an  
282 antimicrobial but also a crucial antibacterial component of the human intestine as well as playing critical  
283 roles in the immune response to infection. The significance of SCFAs to human intestinal immune health  
284 therefore warrants increased scrutiny of their industrial and agricultural use as any effects of their  
285 widespread use on bacterial dissemination need to be better understood.

## 286 **Materials and Methods**

287 ***Bacterial strains and growth conditions.*** Pathogenic AIEC strain LF82 and intestinal commensal *E. coli*  
288 strain F-18 were used in this study and were cultivated on Lysogeny broth or agar. M9 minimal medium  
289 supplemented with 20 mM PA (M9-PA [20% M9 salts (32g Na<sub>2</sub>H<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O (Merck), 12.5 g NaCl  
290 (Merck), 2.5 g NH<sub>4</sub>Cl (Fisher scientific), 7.5g KH<sub>2</sub>PO<sub>4</sub> (Merck) and 400 ml H<sub>2</sub>O], 0.1% Trace metal

291 solution, 0.2 mM MgSO<sub>4</sub> [VWR chemicals], 0.02 mM CaCl<sub>2</sub> [Fisher scientific], 1 mM Thiamine, 0.01 %  
292 5 g/L FeCl<sub>3</sub>, 0.01 % 6.5 g/L EDTA, 0.1% taurocholic acid, 20 mM Sodium propionate and dH<sub>2</sub>O) was  
293 used for growth. Strains were grown in 100 ml of M9-PA at 37°C at 180 rpm, unless stated. Bacterial  
294 growth was measured at optical density 600nm (OD<sub>600nm</sub>). To obtain adapted cells, upon reaching  
295 stationary phase, cultures were back-diluted into fresh M9-PA. Strains for infection were back-diluted  
296 after overnight growth into 10 ml cultures of RPMI-1640 (Sigma) supplemented with 3% foetal calf  
297 serum (FCS) and L-glutamine. These were then grown at 37°C in a shaking incubator at 180 rpm to an  
298 OD<sub>600nm</sub> of 0.6 before further dilution to give a final multiplicity of infection (MOI) of 100. Real-time  
299 PCR was conducted using bacteria grown in No-Carbon-E (NCE) media [59]. Twenty millimolar sodium  
300 propionate (Sigma), 1,2-propanediol (Fisher Scientific) or D-glucose (Sigma) were added with 200 nM  
301 cyano-cobalamin (Sigma) to act as an electron acceptor [60]. Cultures were grown overnight in LB,  
302 washed three times in NCE media with no carbon source added, and inoculated 1:100 into 10 ml NCE  
303 media containing each respective carbon source. Cultures were grown until mid-log phase (OD<sub>600</sub> of 0.6)  
304 and used for RNA-extraction.

305 ***Cell Culture and Maintenance.*** The Caco-2 human intestinal epithelial cell (IEC) line obtained from the  
306 American Type Culture Collection (ATCC) was maintained in Dulbecco's Modified Eagle Medium  
307 (DMEM) medium (Sigma) supplemented with 10% FCS, L-glutamine and penicillin/streptomycin  
308 (Sigma). Cells were maintained at 37°C and 5% CO<sub>2</sub> with regular media changes.

309 ***Biofilm assays.*** Crystal violet static biofilm assays were performed as described previously [61].  
310 Anaerobic culture conditions were achieved using a microaerophilic cabinet. PA was added to a final  
311 concentration of 20 mM.

312 ***Adherence and invasion assays.*** Caco-2 IECs were washed once before infection and bacterial  
313 suspensions were added at an MOI of 100. The infection was allowed to proceed for 2 h at 37°C in 5%  
314 CO<sub>2</sub> atmosphere. Non-adhered bacteria were washed away and the infected cells were lysed with 1%

315 Triton X-100 for 5 min. Bacteria were serially diluted in Luria Bertani (LB) broth and spread onto LB  
316 agar plates. Total bacteria were enumerated by counting colony forming units (CFUs) after overnight  
317 incubation at 37°C. To determine bacterial invasion, cells were infected for 6 h, extracellular bacteria  
318 were then washed away and 50 µg/ml gentamycin sulphate was added for 1 h to kill any remaining cell-  
319 associated bacteria before triton X-100 treatment.

320 ***Acid survival assays.*** Cultures of bacteria were grown overnight at 37°C in LB. The pH of these cultures  
321 was lowered to pH 3 using 1 M HCl. Samples were taken every 20 min for 1 h and serially diluted in LB.  
322 Dilutions were plated in triplicate onto LB agar and incubated overnight at 37°C. Colonies were counted  
323 to determine the number of surviving cells.

324 ***Total RNA extraction and mRNA enrichment.*** Bacterial cultures were grown as above and mixed with  
325 two volumes of RNAProtect reagent (Qiagen, Valencia, CA, USA), before incubating for 5 min at room  
326 temperature. Total RNA was extracted, genomic DNA removed and samples enriched for mRNA as  
327 described previously by Connolly *et al.* (2016). Samples for RNA-sequencing (RNA-seq) analysis were  
328 QC tested for integrity and rRNA depletion using an Agilent Bioanalyzer 2100 (University of Glasgow,  
329 Polyomics Facility).

330 ***Genomic analysis and SNP identification.*** A bacterial lawn generated from single overnight colonies of  
331 LF82 and three independent cultures of LF82-PA were resuspended in a microbank bead tube, inverted  
332 four times and incubated at room temperature for 2 min. The cryopreservative was removed and the  
333 samples sent to MicrobesNG (Birmingham University, UK) for sequencing. Genomic DNA was extracted  
334 using a Illumina Nextera XT DNA sample kit as per manufacturer's protocol (Illumina, San Diego, USA).  
335 Samples were sequenced on the Illumina MiSeq using a 2x250 paired-end protocol, De novo assembled  
336 using SPAdes version 3.5, aligned to the reference genome using BWA-MEM 0.7.5. Variants were called  
337 using samtools 1.2 and VarScan 2.3.9 and annotated using snpEFF 4.2. Subsequent genomic analysis was  
338 performed using a combination of MAUVE, CLC genomics (Version 7.0.1), ExPASy and EMBOSS

339 Needle. The sequence reads in this paper have been deposited in the European Nucleotide Archive under  
340 study (upload in progress).

341 ***RNA-seq transcriptome generation and data analysis.*** cDNA synthesis and sequencing was performed at  
342 the University of Glasgow Polyomics Facility, essentially as described by Connolly *et al.* (2016). Briefly,  
343 sequencing was performed using an Illumina NextSeq 500 platform obtaining 75 bp single end reads.  
344 Samples were prepared and sequenced in triplicate. Raw reads were QC checked using FastQC  
345 (Babraham Bioinformatics, Cambridge, UK) and trimmed accordingly using CLC Genomics Workbench  
346 (CLC Bio, Aarhus, Denmark). Trimmed reads were mapped to the LF82 reference genome (NCBI  
347 accession number: CU651637) allowing for 3 mismatches per read. Analysis of differential expression  
348 was performed using the Empirical analysis of DGE tool, which implements the EdgeR Bioconductor tool  
349 [63]. Differentially expressed genes were identified by absolute fold change (cutoffs  $\log_2$ ) and a P-value  
350 of  $\leq 0.05$ . Volcano plots were generated in CLC Genomics Workbench. The sequence reads in this paper  
351 have been deposited in the European Nucleotide Archive under study (upload in progress).

352 ***Quantitative real-Time PCR (qRT-PCR).*** cDNA was generated from total RNA using an Affinity Script  
353 cDNA Synthesis Kit (Agilent) following the manufacturer's instructions. Levels of transcription were  
354 analyzed by qRT-PCR using PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences). Individual  
355 reactions were performed in triplicate within each of three biological replicates. The 16S rRNA and *rpoS*  
356 genes were used to normalize the results. RT-PCR reactions were carried out using the ECO Real-Time  
357 PCR System (Illumina, San Diego, CA, USA) according to the manufacturer's specifications and the data  
358 were analyzed according to the  $2^{-\Delta\Delta CT}$  method [64]. All primers used are listed in Supplementary Table  
359 S1.

360 **Construction of p16*Slux*.** LF82 and LF82-PA *lux* integrated strains containing the erythromycin  
361 cassette were generated using the protocol of Riedel *et al.* [65]. The bioluminescent properties of these  
362 strains allowed visualization of the establishment of infection but despite bacteria being recovered it was

363 noted that bioluminescent signal was lost. However, upon plating the murine microbiome onto LB agar  
364 containing ampicillin (100 µg/ml), it was observed that several members of the microbiota also harboured  
365 ampicillin resistance. LB supplemented with erythromycin (500 µg/ml) did not support the growth of any  
366 microbiota species; therefore utilizing the erythromycin cassette inserted as part of the *lux* integration  
367 allowed for the selection of LF82 and LF82-PA, and was used in subsequent animal experiments.

368 ***Animal experiments.*** All animal procedures were approved by an internal University of Glasgow ethics  
369 committee and were carried out in accordance with the relevant guidelines and regulations as outlined by  
370 the U.K. Home Office (PPL 70/8584). Male C57BL/6 mice aged between eight and ten weeks were  
371 obtained from The Jackson Laboratory (Envigo). Twenty millimolar sodium propionate was administered  
372 to C57BL/6 mice in drinking water three days prior to infection. Control mice were given only sterile  
373 water. Twenty-four hours prior to infection, mice were treated with an oral dose of 20 mg streptomycin  
374 before oral infection with 0.1 ml PBS (mock-infected) or with approx.  $1 \times 10^9$  colony forming units  
375 (CFU) of LF82 (*lux*) or LF82-PA (*lux*). Mice were euthanized 3 days after infection for colonization  
376 experiments and 21 days after infection for persistence experiments, with caecal contents collected for  
377 SCFA analysis. Ileal, caecal and colonic tissue were weighed and homogenized for enumeration of  
378 bacterial numbers. Bacterial numbers were determined by plating tenfold serial dilutions onto LB agar  
379 containing the appropriate antibiotics. After 24 h of incubation at 37°C, colonies were counted and  
380 expressed as CFU per gram of tissue.

381 ***SCFA analysis by gas chromatography.*** Faecal contents of murine caeca were isolated from PBS treated  
382 mice three dpi. The concentrations of acetate, propionate and butyrate per gram of dry weight were  
383 measured by gas chromatography as previously described [66] and expressed as a ratio for comparison to  
384 the known human acetate:propionate:butyrate SCFA ratio [38]. The effect of PA supplementation in  
385 drinking water on PA levels was measured by extraction of caecal contents from PA treated and untreated  
386 mice and the levels of SCFAs again calculated per gram of dry weight.

387 **Statistical analysis.** Values are represented as means and standard deviation. All statistical tests were  
388 performed with GraphPad Prism software, version 7.0c. All replicates in this study were biological; that  
389 is, repeat experiments were performed with freshly grown bacterial cultures, immortalized cells and  
390 additional mice, as appropriate. Technical replicates of individual biological replicates were also  
391 conducted, and averaged. Significance was determined using t-tests (multiple and individual as indicated  
392 in the figure legends) and one-way ANOVA corrected for multiple comparisons with a Tukey post hoc  
393 test (as indicated in the figure legends). RT-PCR data was log-transformed before statistical analysis.  
394 Values were considered statistically significant when  $P$  values were  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ .

395

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585

## 586 **Acknowledgments**

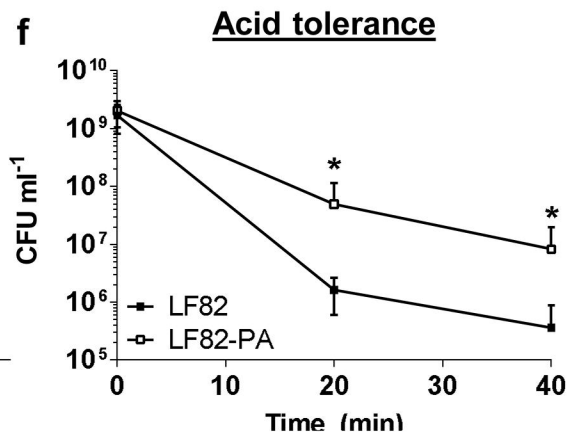
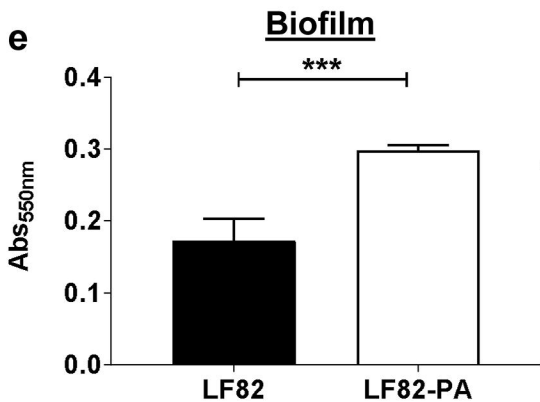
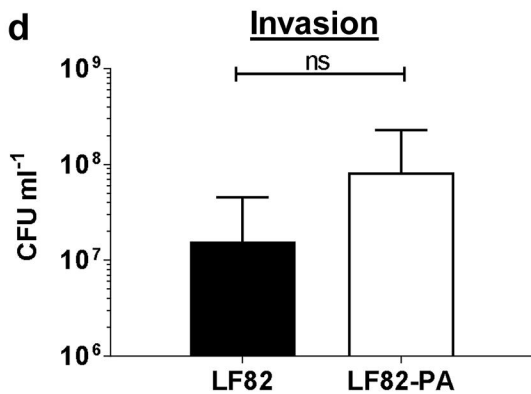
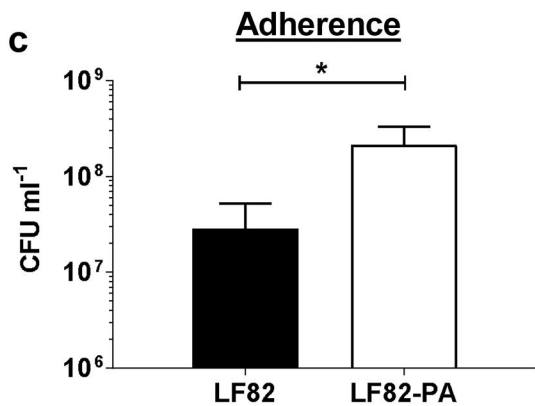
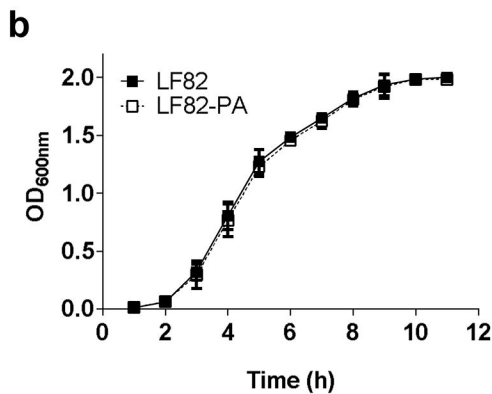
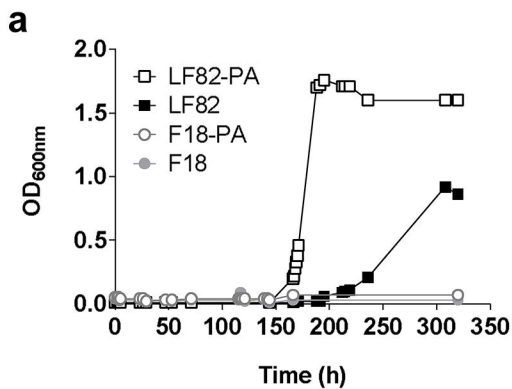
587 **General:** The LF82 strain was a kind gift from Professor Daniel Walker, University of Glasgow.

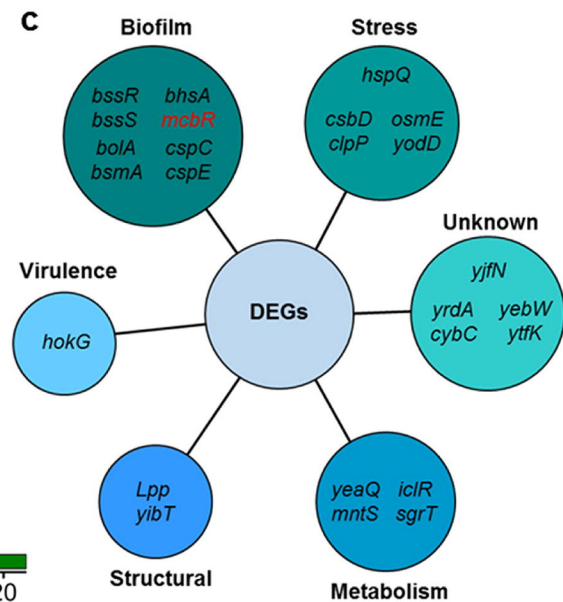
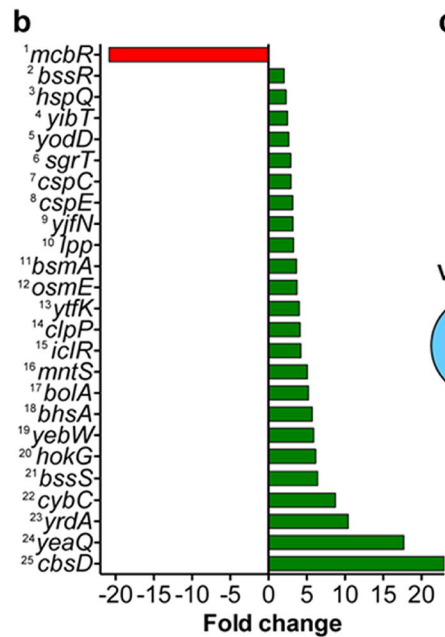
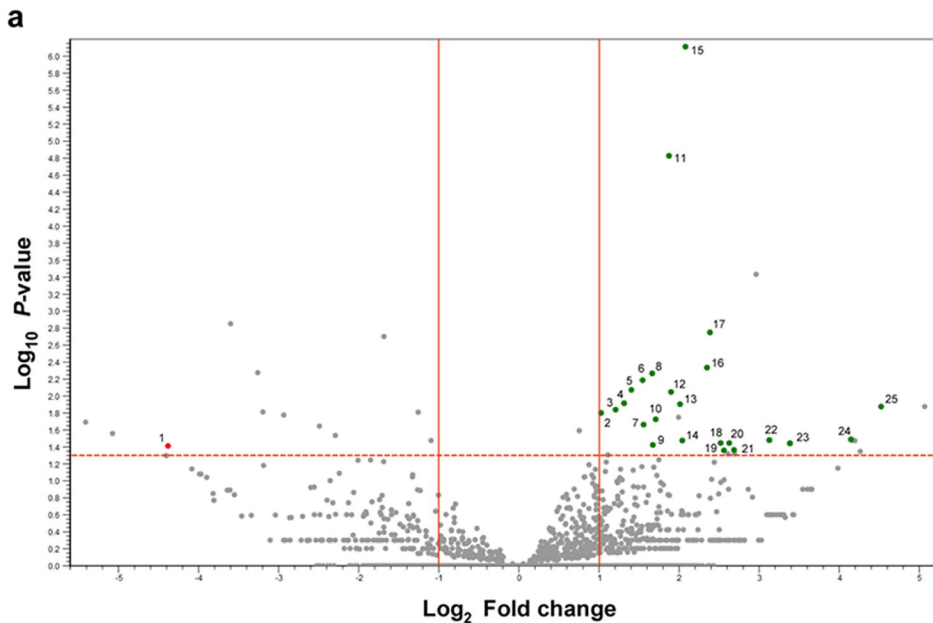
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591 **Author contributions:** MJO designed and performed the experiments, analysed the data and prepared  
592 the manuscript. SAJ assisted in experimental design, animal experiments and RT-PCR. LMM, RJG and  
593 DGES assisted in experimental design. AM provided technical assistance throughout the experiments.  
594 JPRC and AJR advised on and assisted in RNA-seq analysis. CCM generated the primary LF82-PA

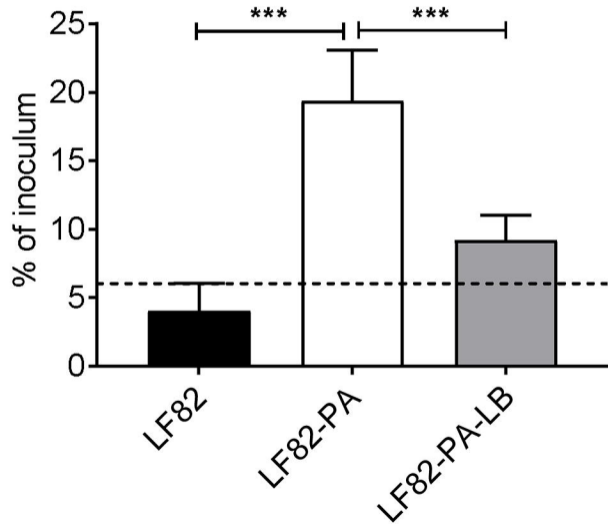
595 strain. HEH and HMW assisted in animal experiments. EF was involved in developing the initial concept  
596 for the study. KG advised on and conducted SCFA experiments. DM conducted GC-IRMS experiments.  
597 DMW was awarded the funding, developed the initial concept, designed and performed experiments and  
598 prepared the manuscript. All authors contributed to editing the manuscript for publication.  
599 **Competing interests:** The authors declare that there are no competing interests.



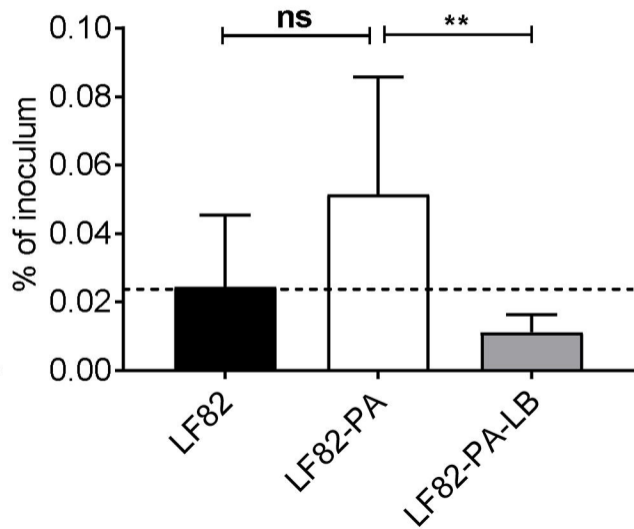




### Adherence



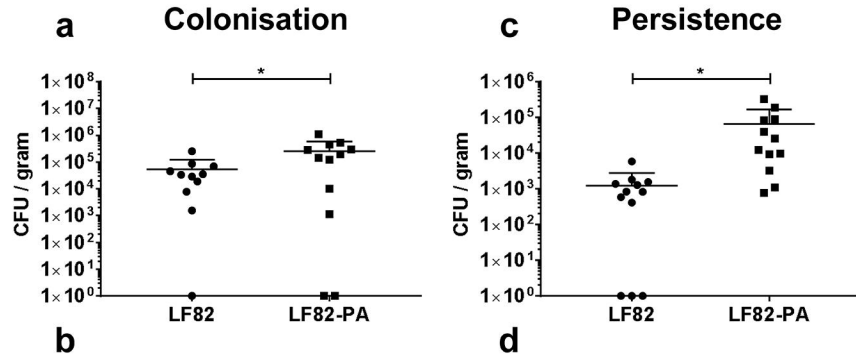
### Invasion



20 mM PA

No PA

Ileum



Colon

