

# Commensal-derived OMVs elicit a mild proinflammatory response in intestinal epithelial cells

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29 **Abstract**

30 Under normal physiological conditions, the intestinal immunity remains largely  
31 hyporesponsive to the commensal microbiota, yet also retains the inherent ability to rapidly  
32 respond to pathogenic antigens. However, immunomodulatory activities of extracellular  
33 products from commensal bacteria have been little studied, with previous investigations  
34 generally utilising the live bacterium to study microbiota-epithelial interactions. In this study,  
35 we demonstrate that extracellular products of a commensal bacterium, *Escherichia coli* C25,  
36 elicit a moderate release of proinflammatory IL-8 and stimulate transcriptional up-regulation  
37 of Toll-like receptors (TLRs) in intestinal epithelial cell lines, HT29-19A and Caco-2.  
38 Additionally, we show that removal of outer membrane vesicles (OMVs) reduces the  
39 proinflammatory effect of secreted products from *E. coli* C25. Furthermore, we show that  
40 isolated OMVs have a dose-dependent proinflammatory effect on IECs. Interestingly, a  
41 relatively high concentration (40 µg/ml protein) of OMVs had no significant regulatory  
42 effects on TLR mRNA expression in both cell lines. Finally, we also demonstrate a that pre-  
43 incubation with *E. coli* C25-derived OMVs subsequently inhibited the internalisation of the  
44 bacterium itself in both cell lines. Taken together, our results suggest that commensal-derived  
45 extracellular products, in particular OMVs, could significantly contribute to intestinal  
46 homeostasis. We also demonstrate a unique interaction between commensal-derived OMVs  
47 and host cells.

48

49

## 50 **Introduction**

51 The intestinal commensal microbiota, consisting of  $\sim 10^{14}$  bacteria [1], is considered one of  
52 the densest and most diverse microbial communities on the planet [2]; consequently, our  
53 knowledge of the highly dynamic role the microbiota plays in host immunity is still very  
54 basic. Nevertheless, advances in technology have allowed some compositional  
55 characterisation of the commensal microbiota via metagenomic analyses [1,3]. For example,  
56 in early neonatal life, *E. coli* are among the first bacteria to colonise the human intestine [4]  
57 and these early commensal pioneers offer a preliminary defence against enteropathogens, due  
58 to physical and nutritional competition [5]. An early example of a commensal bacterium is *E.*  
59 *coli* C25 which was originally isolated from the faeces of a healthy individual in the mid-  
60 1950s [6] and was subsequently demonstrated to have antagonistic activities against the  
61 enteric pathogen, *Shigella flexneri* [7,8]. Also, C25 lacks the traditional virulence genes  
62 found in pathogenic strains of *E. coli*, such as extraintestinal pathogenic (ExPEC),  
63 enterohaemorrhagic (EHEC) and enteropathogenic (EPEC) [9,10]; moreover, it is a poor  
64 recipient of plasmid transfer [11], so is unlikely to acquire such genes from other bacteria.  
65 Nevertheless, studies utilising live C25 have demonstrated its ability to translocate through  
66 the intestinal epithelial barrier [10,12,13] and to initiate a proinflammatory response in  
67 intestinal epithelial cell lines [9,10,12,14]. Yet, the immunoregulatory ability of the  
68 extracellular products from C25 have only been briefly considered previously [15].

69 Gram negative bacteria, and *E. coli* in particular, are well characterised in their  
70 production of outer membrane vesicles (OMVs) [16-20], which are small (50-250 nm  
71 diameter), spherical, bilayered membranous structures naturally secreted into the bacterium's  
72 immediate surroundings [21]. OMVs have been isolated from a diverse range of  
73 environments, from liquid and solid lab cultures to river beds and waste water pipes [22], and  
74 even from the human body [23,24]. The composition, conformation and surface chemistry of

75 OMVs is representative of the intact outer membrane (OM) of Gram-negative bacteria, with  
76 lipopolysaccharides (LPSs), outer membrane proteins (OMPs), phospholipids and  
77 periplasmic proteins all present [17,21]. Therefore, it is unsurprising that OMVs from  
78 pathogens, such as *Pseudomonas aeruginosa*, *Helicobacter pylori* and *Vibrio cholera* have  
79 been suggested to contribute to the pathology of chronic inflammatory diseases, as they  
80 exhibit the ability to elicit IL-8 from gastric [25], bronchial [26] and intestinal epithelial cells  
81 [27,28], respectively. However, more recent studies have focussed on OMVs derived from  
82 probiotic bacteria, such as *E. coli* Nissle 1917 [18,29] and commensal bacteria, such as  
83 *Bacteroides fragilis* [30], *Bacteroides thetaiotaomicron* [31] and *E. coli* strain ECOR12 [29].  
84 Nevertheless, with the recent exception of Fábrega *et al.*, who showed that the two strains of  
85 *E. coli* studied were able to stimulate cytokine release from explanted colonic tissue [29], the  
86 direct interactions of OMVs from non-pathogenic bacteria with the host intestinal epithelium  
87 have been little studied [32]. Therefore, the current study aimed to investigate the direct  
88 inflammatory potential of OMVs derived from *E. coli* C25 on the intestinal epithelial cell  
89 lines, HT29-19A and Caco-2.  
90

## 91 **Materials and Methods**

### 92 **Cell culture**

93 HT29-19A and Caco-2 cell lines were kindly donated by Prof. G. Warhurst (Royal NHS  
94 Foundation Trust and University of Salford, UK). Both cell lines were cultured in a standard  
95 media of high glucose (4500mg/l) Dulbecco's Modified Eagles Media (DMEM), 10 % foetal  
96 bovine serum (FBS), 4 mM glutamine and a mixture of 50 IU/ml penicillin and 50 µg/ml  
97 streptomycin (PenStrep). Additionally, HT29-19A cells were supplemented with 20 mM  
98 HEPES and Caco-2 cells had 0.1 mM MEM NEAA (non-essential amino acids) added. Both  
99 cell lines were seeded at a density of  $0.5 \times 10^5$  cells/cm<sup>2</sup> and cultured to confluence (~7 days)  
100 in 35 mm x 10 mm cell culture dishes. After culturing the cells to confluence on tissue culture  
101 plastic over 7 days, both cell lines became semi-polarised; indeed, Caco-2 cells were  
102 observed to undergo dome formation, which is indicative of unidirectional water transport  
103 and polarisation.

104

### 105 **Bacterial products**

106 *E. coli* C25 was a kind gift from Prof. G. Warhurst and was cultured on tryptone soy agar  
107 (TSA) at 37 °C. DMEM, supplemented with 4 mM glutamine was inoculated with *E. coli*  
108 C25 and incubated overnight (~15 h), until the culture reached the stationary phase of growth  
109 (Supplementary Fig 1;  $\sim 1 \times 10^9$  CFU/ml). Subsequent to incubation, the culture was  
110 centrifuged at 6000 x g for 10 min to pellet out the bacteria. The supernatant was removed,  
111 had its pH adjusted to 7.4 and was subsequently filtered using 0.45 µm syringe-driven filters  
112 (Millex<sup>®</sup>, Millipore UK Ltd.). The cell-free supernatant was diluted 1:10 in cell culture  
113 medium and used in cell challenge experiments.

114 10 ml aliquots of overnight *E. coli* C25 cultures in DMEM with 4 mM glutamine  
115 (containing  $\sim 1 \times 10^9$  CFU/ml) were sonicated, using a Vibracell VCX 130 (Sonics and

116 Materials Inc.) at 85 % amplitude for a 5 x 6 s pulse program. Cultures were sonicated on ice  
117 and with a 24 s cooling step between pulses, in order to minimise denaturation of bacterial  
118 products. Resultant solutions were filtered through a 0.45 µm syringe-driven filter, diluted  
119 1:10 in cell culture medium and subsequently used in cell challenge experiments.

120 Flagellin isolated from *Salmonella typhimurium* strain 14028 was purchased from  
121 Enzo Life Sciences Ltd.

122

### 123 **Cytokine stimulation and analysis**

124 Cells were challenged with the bacterial stimuli for 24 h, at 37 °C, 5 % CO<sub>2</sub> and constant  
125 humidity. Supernatants were collected and frozen at -80 °C until assayed for IL-8 by enzyme-  
126 linked immunosorbant assay (ELISA) analysis (IL-8 Human Antibody Pairs, Invitrogen).  
127 ELISA analysis was carried out according to the manufacturer's instructions.

128

### 129 **qPCR**

130 Epithelial cells were challenged with the bacterial stimuli for 24 h. The cells were  
131 subsequently lysed and the total RNA was extracted using the RNeasy<sup>®</sup> Mini Kit and RNase-  
132 free DNase Set (Qiagen). RNA was quantified spectrophotometrically using the absorbance  
133 at 260 nm ( $A_{260}$ ) x 44 µg/ml x dilution factor and the purity was measured using  $A_{260}/A_{280}$ .  
134 cDNA was synthesised from 2 µg of total RNA by the iScript<sup>™</sup> cDNA Synthesis Kit (Bio-  
135 Rad Laboratories Ltd.). cDNA synthesis was carried out to the manufacturer's instructions.

136 PCR primers (Table 1) were purchased from Eurofins MWG Operon. Universal  
137 ProbeLibrary probes and Lightcycler<sup>®</sup> Taqman<sup>®</sup> Master Mix were purchased from Roche  
138 Diagnostics Ltd. Amplification was carried out in 20 µl reaction volume containing 1.5 µl  
139 cDNA, 0.5 µl F-primer and R-primer (0.4 µM), 0.5 µl Universal probe, 4 µl 5x Mastermix  
140 and 13 µl DNase/RNase-free water. The following program was used: 95 °C for 10 min

141 followed by 45 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 1 s. Target gene  
142 expression was normalised to the housekeeping gene GAPDH, and the fold difference of  
143 expression from the control was calculating using the  $2^{-\Delta\Delta Ct}$  method [33].

144

#### 145 **OMV isolation**

146 Outer membrane vesicle (OMV) isolation was achieved by a well-established method first  
147 described by Kadurugamuwa and Beveridge [34] and more recently modified by Vanaja *et al.*  
148 [35] and Fabrega *et al.* [29]. Briefly, 250 ml overnight (15 h) cultures of *E. coli* C25 in  
149 tryptone soy broth (TSB) were centrifuged at 6000 x g for 10 min to pellet out the bacteria.  
150 The supernatant was sequentially filtered through 0.80 and 0.45 µm pore size vacuum-driven  
151 bottle top filters. A sample of the filtrate was transferred to TSA plates and incubated at 37  
152 °C to ensure there was no contaminating bacteria were present. The filtrates were  
153 ultracentrifuged at 150,000 x g for 1.5 h, at 5 °C, to pellet out the OMVs. The supernatant  
154 was removed and the pellet was resuspended in 50 mM HEPES buffer (pH 6.8) and  
155 ultracentrifuged again for 30 min at 120,000 x g, 5 °C. The supernatant was again removed  
156 and the pellet was resuspended in 50 mM HEPES buffer (pH 6.8), filtered through a 0.45 µm  
157 syringe filter and stored at 4 °C. Approximately 400 µg OMVs by protein concentration were  
158 isolated from 250 ml culture supernatant, measured by the modified Lowry assay as per the  
159 manufacturer's instructions (DC™ Protein Assay; Bio-Rad). Subsequently, OMVs were  
160 serially diluted (to give 4-100 µg/ml protein) in cell culture medium and utilised in cell  
161 challenge experiments.

162

#### 163 **Outer membrane isolation**

164 *E. coli* C25 outer membrane (OM) was isolated using a slightly modified protocol from that  
165 previously described by Zhou *et al.* [36]. Briefly, 250 ml overnight (15 h) cultures of *E. coli*



166 C25 grown in TSB were centrifuged at 10,000 x g for 10 min and the resultant pellet was  
167 washed twice in PBS. The bacterial pellet was then resuspended in 10ml PBS with 0.01 M  
168 EDTA, incubated at room temperature for 30 min and sonicated for 10 s at 85% amplitude.  
169 The mixture was then centrifuged again at 10,000 x g for 10 min at 4°C and the supernatant  
170 was collected, with the pellet being discarded. The supernatant was subsequently centrifuged at  
171 80,000 x g for 2 h at 4°C. The translucent yellow pellet was resuspended in sterile water and  
172 was centrifuged again at 80,000 x g for 2 h at 4°C. The final pellet was resuspended in sterile  
173 water and frozen at -80°C [36].

174

#### 175 **Transmission electron microscopy**

176 OMVs were isolated and resuspended at approximately 1 mg/ml protein content in 50 mM  
177 HEPES buffer (pH 6.8). Vesicles were placed on Carbon Films on 400 Copper Mesh Grids  
178 (Agar Scientific) for 1 min. Grids were then negatively stained with 1% aqueous uranyl acetate  
179 for 1 min and visualized on a LoJeol 1200EX TEM.

180

#### 181 **SDS-PAGE**

182 Protein concentrations of isolated OMVs and OMs were measured using the modified Lowry  
183 assay as per the manufacturer's instructions (DC<sup>TM</sup> Protein Assay; Bio-Rad), with BSA used  
184 as a protein standard (Sigma-Aldrich). 30 µg of samples were heated at 70°C for 10 minutes  
185 and subsequently resolved on a 4-12% NuPAGE Novex Bis-Tris precast protein gel  
186 (Invitrogen) in 1x MES buffer (50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH  
187 7.0). The gel was then stained using the Pierce<sup>TM</sup> Silver Stain Kit (Thermo Scientific) to the  
188 manufacturer's instructions and viewed using the InGenius gel viewing cabinet (Syngene)  
189 and GeneSnap software (Syngene).

190

191 **Bacterial internalisation assay**

192 The method for this assay was developed from the technique described by Macutkiewicz *et*  
193 *al.* [10]. Epithelial cells were cultured to confluence (~7 days) and treated with fresh medium  
194 24 h in advance of the assay. 10 ml sterile tryptone soy broth (TSB) was inoculated with *E.*  
195 *coli* C25 and incubated at 37 °C overnight (15 h), giving a culture entering the stationary  
196 phase of growth (Supplementary Fig 1) and at a density of  $\sim 1 \times 10^9$  CFU/ml (serial dilutions  
197 and plating out on to tryptone soy agar (TSA) gave exact numbers retrospectively). The  
198 culture was centrifuged at 10,000 x *g* for 10 min and the bacterial pellet was resuspended in  
199 10 ml HBSS supplemented with 10 mM sodium bicarbonate and 180 mg/dl glucose (Trans-  
200 HBSS) in order to reduce bacterial growth during the assay [11]. Epithelial cells were washed  
201 twice in sterile Trans-HBSS, had  $\sim 1 \times 10^9$  CFU C25 in 2 ml Trans-HBSS added and were  
202 incubated at 37 °C for 4 h. After incubation epithelial cells were washed twice with Trans-  
203 HBSS, thus removing any non-adherent/non-internalised bacteria. 1 ml Trans-HBSS/50  
204  $\mu$ g/ml gentamicin was added to the cells and incubated at room temperature for 15 min,  
205 killing all but the internalised bacteria. The supernatant was removed and plated out neat, in  
206 TSA, to confirm that the antibiotic had killed the extracellular bacteria. The epithelial cells  
207 were lysed by osmotic pressure with the addition of 1 ml sterile deionised water, and  
208 subsequently by sheer force with repeated pipetting, thus releasing the bacteria contained  
209 within the cells. A serial dilution of the resultant lysates was performed to give  $10^{-3}$  and  $10^{-4}$   
210 dilutions, which were subsequently plated out on TSA, using the agar pour plate method. All  
211 resultant plates were incubated overnight at 37°C and bacterial colonies were manually  
212 counted.

213

214 **Statistical Analysis**

215 Results are expressed as median  $\pm$  interquartile range (IQR) for the stated number of  
216 experimental repeats ( $n$ ). Statistical significance was calculated using Mann Whitney  $U$ -test  
217 or Kruskal-Wallis test with Dunn's *post hoc* analysis and a  $p$  value  $\leq 0.05$  was considered  
218 significant. All statistical analyses were undertaken using Prism<sup>®</sup> 6 (GraphPad Software Inc.).  
219

## 220 **Results**

### 221 **Extracellular products derived from *E. coli* C25 elicit a moderate proinflammatory** 222 **response from IECs**

223 Earlier studies have shown that live *E. coli* C25 can induce secretion of proinflammatory  
224 cytokines from intestinal epithelial cells [9,14]; however, the extracellular products from this  
225 bacterium have only briefly been considered before [15]. To investigate the inflammatory  
226 potential of *E. coli* C25-derived extracellular products, HT29-19A and Caco-2 intestinal  
227 epithelial cells lines were challenged with the cell-free supernatant from cultures of *E. coli*  
228 C25 and sonicated samples of *E. coli* C25 cultures for 24 h. Sonicated samples were used to  
229 give maximal levels of antigenic material and mimic cells lysed by antimicrobial peptides  
230 (AMPs) *in vivo*. Additionally, the responses to both cell-free supernatant and sonicates were  
231 compared to the pathogenic antigen, flagellin, which is known to be a major contributing  
232 antigen in the pathology of inflammatory bowel disease [37] and therefore represents a  
233 relevant positive control. Moreover, significant IL-8 release in response to flagellin has  
234 previously been described in both cell lines utilised in this study [38,39].

235 *E. coli* C25 cell-free supernatant was shown to elicit a significant (~5-fold;  $p \leq 0.005$ )  
236 increase in IL-8 release in HT29-19A cells (Fig. 1a). The 1.5-fold increase in IL-8 release in  
237 Caco-2 cells was much more modest (Fig. 1b), but still statistically significant ( $p \leq 0.05$ ). The  
238 increased IL-8 release in both cell lines was also reflected at the transcriptional level, with  
239 up-regulation of IL-8 mRNA in both HT29-19A (~9-fold increase; Fig 1c) and Caco-2 (~5-  
240 fold increase; Fig. 1d) cells, but neither was statistically significant. Additionally, both HT29-  
241 19A and Caco-2 cells exhibited an increased release (~7-fold;  $p \leq 0.01$  and ~2-fold;  $p \leq 0.05$ ,  
242 respectively) of IL-8 when challenged with the sonicated samples of *E. coli* C25, with levels  
243 comparable to those seen for the cell-free supernatant challenges (Figs. 1a and 1b).  
244 Furthermore, as with cell-free supernatants, challenging with sonicates increased IL-8 mRNA

245 expression in both cell lines, with HT29-19A cells exhibiting ~20-fold increase (Fig. 1c) and  
246 Caco-2 cells demonstrating a ~4-fold increase (Fig 1 d), although neither of these trends were  
247 calculated to be statistically significant.

248 Although the release and transcriptional up-regulation of IL-8 was potentiated in  
249 response to the extracellular products of *E. coli* C25 in both cell lines, when compared to that  
250 observed in response to the pathogenic positive control, 100 ng/ml final concentration  
251 flagellin, the increase is relatively modest (Fig 1); on addition of flagellin, IL-8 release was  
252 ~20-fold ( $p \leq 0.005$ ) higher in HT29-19A (Fig 1a) and ~40-fold ( $p \leq 0.005$ ) in Caco-2 cells  
253 (Fig. 1b), compared to control. mRNA expression was up-regulated ~250-fold ( $p \leq 0.01$ ) and  
254 ~35-fold ( $p \leq 0.01$ ) in HT29-19A (Fig. 1(c)) and Caco-2 cells (Fig. 1d), respectively. Despite  
255 this, upon direct comparison of cells treated with C25 extracellular products and those treated  
256 with flagellin, none of the trends were calculated to be statistically significant.

257

### 258 **Removal of OMVs from *E. coli* C25 extracellular products reduces their** 259 **proinflammatory effect on IECs**

260 Gram negative bacteria are well characterised in their production of outer membrane vesicles  
261 (OMVs) [21,40], which, when derived from either pathogenic or commensal bacteria, have  
262 previously shown immunomodulatory activity on different intestinal cell types [27,29,30].  
263 Therefore, to elucidate the contribution of OMVs to the proinflammatory profile of *E. coli*  
264 C25-derived extracellular products, we removed them from the C25 cell-free supernatant via  
265 ultracentrifugation. HT29-19A and Caco-2 cells were subsequently challenged with OMV-  
266 free cell-free supernatant (1:10 dilution in cell culture media) for 24 h and the resultant  
267 release of IL-8 was quantified.

268 In both cell lines, the release of IL-8 in response to the OMV-free cell-free  
269 supernatant was significantly ( $p \leq 0.001$ ) increased from the control (Fig. 1a, 1b and 2). Once

270 the OMVs had been removed from the supernatant, the level of IL-8 expressed from both cell  
271 lines in response to OMV-free cell-free supernatant appeared to be reduced in comparison to  
272 untampered cell-free supernatant (Fig. 2); nevertheless, it was only statistically significant ( $p$   
273  $\leq 0.01$ ) in Caco-2 cells (Fig. 2b) and not HT29-19A (Fig. 2a). Despite appearing to be  
274 increased compared to the control cells, the IL-8 production in cells challenged with the  
275 OMV-free cell-free supernatant was not calculated to be significantly different from these  
276 controls (Fig. 2).

277

278 ***E. coli* C25-derived OMVs elicit a dose-dependent proinflammatory response from**  
279 **IECs, but have no regulatory effects on TLR mRNA expression.**

280 To investigate the immunomodulatory potential of isolated *E. coli* C25 outer membrane  
281 vesicles (OMVs), HT29-19A and Caco-2 cells were challenged with a 4-100  $\mu\text{g/ml}$  protein  
282 concentration range of OMVs for 24 h. Release and expression of proinflammatory IL-8 was  
283 investigated by ELISA and qPCR analysis.

284 In the HT29-19A cell line, an increased level of IL-8 was observed at a 4  $\mu\text{g/ml}$   
285 concentration of OMVs and increased in a dose-dependent manner; nevertheless, the increase  
286 was only calculated to be statistically significant ( $p \leq 0.05$ ) from 20  $\mu\text{g/ml}$  OMVs (Fig. 3a).  
287 Similarly, in Caco-2 cells, 20  $\mu\text{g/ml}$  OMVs was the minimum concentration required to elicit  
288 a statistically significant ( $p \leq 0.05$ ) increase in IL-8 secretion (Fig. 3b). A ~6-fold increase in  
289 IL-8 mRNA was produced by 40  $\mu\text{g/ml}$  OMVs in HT29-19A cells ( $p \leq 0.001$ ), in contrast, no  
290 change was observed in Caco-2 cells (Fig. 3c). This distinct difference in the responsiveness  
291 to OMVs was again indicative of the phenotypic variance between the two cell lines. OMVs,  
292 which were shown to measure 50-100 nm (Fig 3d), had their protein content compared to that  
293 of the outer membrane (OM) via SDS-PAGE (Fig 3e). The two had very similar protein  
294 compositions, with only subtle differences in band intensity evident; therefore, we can

295 speculate that the surface protein composition of the OMVs is representative of antigens  
296 present on the whole parent bacterium.

297 Previous studies have reported that agonist binding results in the up-regulation of their  
298 cognate TLR receptors [41-43]; we confirmed this phenomenon in the current study by  
299 measuring the regulation of TLR-5 mRNA expression in both cell lines, in response to a 24 h  
300 challenge with 100 ng/ml flagellin. Significant ( $p \leq 0.001$ ) up-regulation of TLR-5 mRNA  
301 was observed in HT29-19A cells (~110-fold increase; Supplementary Fig. 2) and Caco-2  
302 cells (~26-fold increase; Supplementary Fig. 2). To investigate whether this was also true in  
303 reaction to commensal-derived antigenic material, we monitored transcriptional expression of  
304 the TLRs most relevant to bacterial antigens (TLRs-1, -2, -4, -5 and -9) in response to *E. coli*  
305 C25 cell-free supernatant. In HT29-19A cells, we observed a significant ( $p \leq 0.05$ ) increase  
306 in all the TLRs tested in response to challenge with cell-free supernatant (Fig 4a). Similarly,  
307 the Caco-2 cell line showed up-regulation in mRNA expression of TLR-1, -4, -5 and -9,  
308 although only the data for TLRs-1 and -9 was considered statistically significant ( $p \leq 0.05$ ;  
309 Fig. 4b). Interestingly, cell-free supernatant-challenged Caco-2 cells did not exhibit any  
310 regulation in TLR-2 mRNA expression, as the levels remained comparable to the control.  
311 This was in complete contrast to HT29-19A cells, which showed the largest increase in TLR-  
312 2 mRNA expression (~17-fold increase (Fig. 4a)). This contradiction in reaction is likely to  
313 arise from the distinct phenotypic differences between the two cell lines, as Caco-2 cells  
314 exhibit a significant ( $p \leq 0.001$ ; ~540-fold) increased constitutive expression in TLR-2  
315 mRNA, when compared to HT29-19A cells (data not shown).

316 Surprisingly, given the fact that the OMVs possess all the surface antigens of the  
317 parent bacterium (Fig. 3e) when HT29-19A cells were challenged with a 40 µg/ml OMVs no  
318 significant differences were observed in TLR mRNA expression (Fig. 4a), despite TLRs-2, -4  
319 and -5 appearing to be slightly up-regulated and TLR-9 was completely undetectable in the

320 presence of cell-free supernatant, when compared to the control. Indeed, a number of TLRs  
321 appeared to be down-regulated in Caco-2 cells; however, none of these were statistically  
322 significant (Fig. 4b).

323

324 **Pre-incubation with *E. coli* C25-derived OMVs inhibits the internalisation of the parent**  
325 **bacterium**

326 As mentioned previously, past studies have utilised *E. coli* C25 as a model strain for bacterial  
327 translocation across the intestinal epithelium [10,12]; therefore, we sought to investigate the  
328 regulatory ability of OMVs on this process. To explore this, we performed a bacterial  
329 internalisation assay in both HT29-19A and Caco-2 cells. Interestingly, we observed a  
330 reduction in the number of bacteria internalised in both cell lines which had been pre-treated  
331 with 40 µg/ml OMVs (Fig. 5); however, only the decrease seen in Caco-2 cells (~3.5-fold)  
332 was statistically significant ( $p \leq 0.05$ ).



## 333 **Discussion**

334 We have previously described the potential of specific extracellular products derived from  
335 commensal enteric bacteria to modulate the low-level inflammation which exists in intestinal  
336 homeostasis [44,45]; however, there is still a paucity of research in this field. In the present  
337 study, we aimed to explore the inflammatory profile of extracellular products secreted by the  
338 commensal enteric bacterium *E. coli* C25 on two immortalised intestinal epithelial cell lines,  
339 HT29-19A and Caco-2. Here, we principally demonstrate that the extracellular products of *E.*  
340 *coli* C25 (both naturally secreted and after the artificial enhancement of their production via  
341 bacterial sonication) elicit a moderate proinflammatory response, via secretion of the potent  
342 neutrophil chemoattractant, IL-8, from the intestinal cell lines, HT29-19A and Caco-2. In this  
343 study, the cells were cultured on tissue culture plastic, which allowed them to become semi-  
344 polarised; however, in future studies it may be more physiologically accurate to culture the  
345 cell lines to complete polarisation on Transwell inserts and examine the effects of *E. coli*  
346 C25-derived factors on both the apical and basolateral surfaces.

347 In corroboration with previous studies on these cell lines [46], it is evident that the  
348 two possess a marked difference in constitutive secretion of IL-8 and that their  
349 responsiveness to antigenic material is relatively dissimilar. It has previously been speculated  
350 that HT29 and Caco-2 cell lines were isolated from different cell type populations within the  
351 epithelial layer. HT29 cell lines are thought to originate from hyper-responsive intestinal  
352 epithelial crypt cells [47,48], whereas Caco-2 cells were derived from the more  
353 immunotolerant villus enterocytes [48-50]. Nevertheless, despite the distinct phenotypic  
354 differences between HT29-19A and Caco-2 cells, we were able to confirm a mild  
355 proinflammatory response in both cells lines during challenges with extracellular products  
356 derived from *E. coli* C25. Also, we show that the naturally secreted products present in cell-  
357 free supernatant from cultures of *E. coli* C25 can induce a modest up-regulation of the major

358 TLRs associated with recognition of bacterial antigens. As mentioned previously, agonist  
359 binding of TLRs results in the up-regulation of their cognate receptor [41-43] and we  
360 confirmed this phenomenon occurs in IECs in response to flagellin; therefore, from the  
361 mRNA up-regulation of multiple TLRs observed in this study, we can speculate that C25  
362 cell-free supernatant contains multiple TLR ligands. One such secretory product which we  
363 hypothesised to contribute to this was outer membrane vesicles (OMVs).

364 OMVs isolated from Gram-negative bacteria are receiving increasing interest in  
365 microbiological research [40,51]; yet, despite the vast number of Gram-negative bacteria  
366 present within the intestinal microbiota, there is a lack of studies considering the  
367 immunoregulatory activity of OMVs derived from this population [32]. Also, the limited  
368 studies performed to date are divided in their opinion of the potential role of OMVs in the  
369 intestinal niche. It has recently been suggested that macrophage-induced immune responses  
370 to OMVs from the commensal bacterium *B. thetaiotaomicron* could drive colitis in  
371 genetically susceptible hosts [31]; however, this is contradicted by an elegant study  
372 previously undertaken by Shen *et al.*, which suggests a more beneficial role for commensal-  
373 derived OMVs. In their study, they demonstrated that capsular polysaccharide (PSA)-  
374 containing OMVs, isolated from *B. fragilis*, can protect against inflammation in the 2,4,6-  
375 trinitrobenzenesulfonic acid (TNBS) experimental model of colitis in mice via the production  
376 of anti-inflammatory cytokines by DCs, which subsequently enhanced the protective  
377 regulatory T cell response [30]. Additionally, Fábrega *et al.* have recently shown that OMVs  
378 from both probiotic and commensal strains of *E. coli* stimulate a more anti-inflammatory  
379 cytokine profile from explanted colonic tissue, despite a moderate increase in  
380 proinflammatory cytokines, such as IL-6 and IL-8 [29]. In the current study, we corroborate  
381 the findings of Fábrega *et al.*, as we show that the naturally secreted OMVs of an enteric  
382 commensal bacterium have a direct proinflammatory effect on the intestinal epithelial cell

383 lines, HT29-19A and Caco-2. However, we suggest that, should this proinflammatory effect  
384 also be observable *in vivo*, then it is moderate enough to be beneficial to the host by  
385 contributing to the homeostatic low-level inflammatory environment which is characteristic  
386 of the normal intestine.

387         Previously, it has been shown that OMVs are able to directly interact with host cells  
388 via TLRs [52-55]; nevertheless, we have demonstrated that a relatively high concentration  
389 (40 µg/ml) of C25-derived OMVs does not elicit an up-regulation of TLR mRNA expression,  
390 as was observed in response to the cell-free supernatant from cultures of the parent  
391 bacterium. It is well established that activation of TLRs by their agonists significantly  
392 enhances the internalisation of bacteria in both professional immune cells, such as  
393 macrophages [56,57], and non-professional immune cells, such as intestinal epithelial cells  
394 [58]. In addition to this, *E. coli* C25 has been used as a model strain for bacterial translocation  
395 through the intestinal epithelial barrier [10,12,13]; therefore, we decided to explore the  
396 regulatory effects of OMVs on this process. Consequently, we demonstrate that pre-treatment  
397 with C25 OMVs was able to reduce the subsequent internalisation of the C25 bacterium in  
398 intestinal epithelial cells.

399         Therefore, we propose that, through limiting the up-regulation of TLRs by other  
400 secretory products, OMVs can reduce the number of their parent bacterium which translocate  
401 the intestinal epithelial layer. It has been suggested that indigenous bacteria constitutively  
402 translocate transcellularly from the intestinal lumen of healthy, immunocompetent  
403 individuals, but are subsequently killed en route or *in situ* by professional immune cells once  
404 they reach the lymphoid organs [59]. Furthermore, Lichtman *et al.* suggested that bacterial  
405 translocation is required to generate immunocompetent cells within the gut-associated  
406 lymphoid tissue (GALT) [60]; however, prolonged and excessive immune reaction to the  
407 microflora leads to the chronic inflammation of the intestinal mucosa classically associated

408 with inflammatory bowel disease (IBD) [61]. Consequently, in order to maintain the fine  
409 balance of intestinal homeostasis, it is necessary to allow low numbers of commensal bacteria  
410 to translocate the intestinal epithelium; however, it is evident that this process must be  
411 stringently regulated. Here, we propose that, through the production of OMVs, the  
412 commensal microbiota themselves are able to directly contribute to the regulation of their  
413 own translocation, thus maintaining the mutually beneficial symbiosis with a healthy host and  
414 avoiding the pathogenesis of IBD.

415 In summary, these data demonstrate that, *in vitro*, extracellular products derived from  
416 a commensal bacterium have a mild proinflammatory effect on host intestinal epithelial cells  
417 and stimulate a moderate up-regulation of TLRs. We hypothesise these effects could be  
418 beneficial *in vivo* by priming the intestine and subsequently allowing a rapid, but more  
419 controlled, response to pathogenic bacteria and their associated antigens. Also, we show that  
420 OMVs are key contributors to the proinflammatory effect of the *E. coli* C25-derived  
421 extracellular products. Furthermore, we demonstrate a novel interaction between the  
422 commensal microbiota and host cells; through the inhibition of TLR up-regulation,  
423 membrane vesicles derived from a commensal bacterium are able limit the internalisation of  
424 the parent bacterium into intestinal epithelial cells. Finally, we hypothesise that, were the  
425 results presented here to be representative of the *in vivo* environment, then the products  
426 secreted into the intestinal milieu by the commensal microbiota, and OMVs in particular,  
427 could play a key role in the induction of the homeostatic low-level inflammatory response  
428 that is highly characteristic of the healthy intestine.

429

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432

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436

437 **Conflicts of interest**

438 The authors have no conflicts of interest to declare.

439

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616 472.
- 617
- 618
- 619

620 **Table 1 – qPCR primers and probes**

<b>Gene</b>	<b>Primer Sequence</b>		<b>Universal Probe No.</b>
<b>GAPDH</b>	F – gctctctgctcctctggtc	R – acgaccaaatccggtgactc	#60
<b>IL-8</b>	F – agacagcagagcacacaagc	R – aggaaggctgccaagagag	#72
<b>TLR-1</b>	F – aaacaacattgaaacaactggaa	R – cacgttgaaattgagaaatacca	# 65
<b>TLR-2</b>	F – ctctcggtgctcggaatgctc	R – aggatcagcaggaacagagc	#56
<b>TLR-4</b>	F – gaaggtcccagaaaagaatgtt	R – cctgattgccttttcttgaatg	# 75
<b>TLR-5</b>	F – ctccacagtcaccaaacag	R – cctgtgtattgatgggcaaa	# 72
<b>TLR-9</b>	F – tgtgaagcatccttcctgta	R – gagagacagcgggtgcag	#56

621

622

623 **Figure Legends**

624 **Figure 1 – *E. coli* C25-derived extracellular products elicit IL-8 release expression in**  
625 **IECs.** HT29-19A and Caco-2 cells were challenged with *E. coli* C25 cell-free supernatant  
626 (1:10 dilution) and sonicated bacteria (1:10 dilution) for 24 h; 100 ng/ml of flagellin was used  
627 a positive pathogenic control. **(a)** IL-8 release from HT29-19A cells ( $n = 3-12$ ). **(b)** IL-8  
628 release from Caco-2 cells ( $n = 3-12$ ). **(c)** mRNA expression of IL-8 in HT29-19A cells ( $n =$   
629  $3-6$ ). **(d)** mRNA expression of IL-8 in HT29-19A cells ( $n = 3-6$ ). Results are median  $\pm$  IQR.  
630 \*, \*\* and \*\*\* indicate significance from the control, where  $p \leq 0.05$ , 0.01 and 0.005,  
631 respectively.

632 **Figure 2 – Removal of OMVs decreases the proinflammatory activity of *E. coli* C25-**  
633 **derived extracellular products.** *E. coli* C25 cell-free supernatant was ultracentrifuged to  
634 remove OMVs. The resultant OMV-free cell-free supernatant was diluted 1:10 in cell culture  
635 media and **(a)** HT29-19A and **(b)** Caco-2 cells were challenged for 24 h and IL-8 release was  
636 measured via ELISA ( $n = 6-12$ ). Results are median  $\pm$  IQR. \*\* and \*\*\*\* indicate statistical  
637 significance, where  $p \leq 0.01$  and 0.001, respectively.

638 **Figure 3 – OMVs isolated from *E. coli* C25 mediate a dose dependent release of IL-8**  
639 **from IECs.** **(a)** HT29-19A and **(b)** Caco-2 cells were challenged with serial dilutions (4-100  
640  $\mu\text{g/ml}$ ) of *E. coli* C25 OMVs for 24 h and IL-8 expression was measured ( $n = 6$ ). **(c)** mRNA  
641 expression of IL-8 in cells challenged with 40  $\mu\text{g/ml}$  OMVs for 24 h ( $n = 3$ ). Results are  
642 median  $\pm$  IQR. \*, \*\* and \*\*\* indicate significance from the control, where  $p \leq 0.05$ , 0.01 and  
643 0.005, respectively. **(d)** TEM micrograph of C25 OMVs. **(e)** Comparison of protein content of  
644 *E. coli* C25 outer membrane vesicles (OMV) and outer membrane (OM) preparations by  
645 SDS-PAGE.

646 **Figure 4 – *E. coli* C25-derived OMVs have no regulatory effects on TLR mRNA**  
647 **expression.** HT29-19A (a) and Caco-2 (b) cells were challenged with 1:10 dilutions of *E. coli*

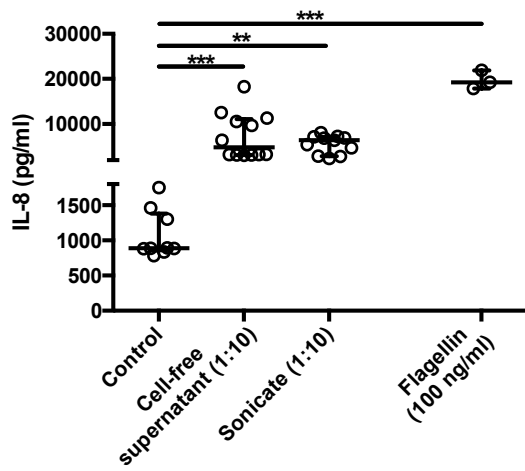
648 C25 cell-free supernatant or 40 µg/ml OMVs culture concentration of *E. coli* C25 outer  
649 membrane vesicles (C25 OMVs) for 24 h and qPCR was utilised to study TLR mRNA  
650 expression. Results are median ± IQR,  $n = 3$ . \* and \*\* indicate significance from the control,  
651 where  $p \leq 0.05$  and 0.01, respectively.

652 **Figure 5 – Commensal- derived OMVs block internalisation of their parent bacterium.**

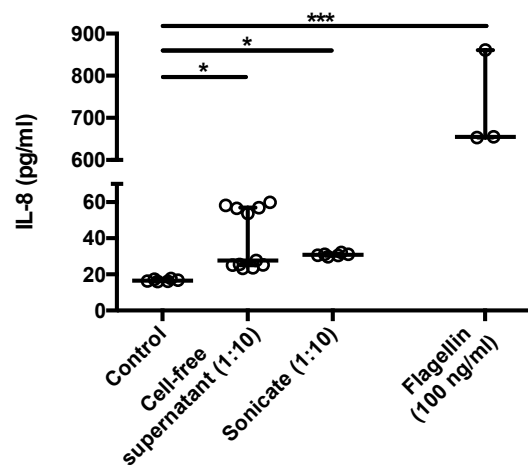
653 HT29-19A (a) and Caco-2 (b) cells were challenged with 40 µg/ml *E. coli* C25 OMVs for 24  
654 h. Subsequently, the supernatants were removed and cell layers were co-cultured with  $\sim 1 \times$   
655  $10^9$  CFU of *E. coli* C25 for 4 h. Non-internalised bacteria were killed and epithelial cells were  
656 lysed, releasing internalised bacteria. Lysates were serially diluted, plated out and incubated  
657 for 24h. Resultant colonies were counted and expressed as a % of the original inoculum.  
658 Results are median ± IQR,  $n = 4-6$ . \* indicates significance from the control, where  $p \leq 0.05$ .

659

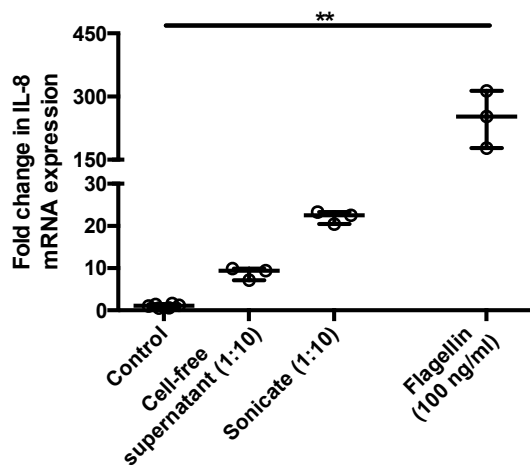
(a) HT29-19A



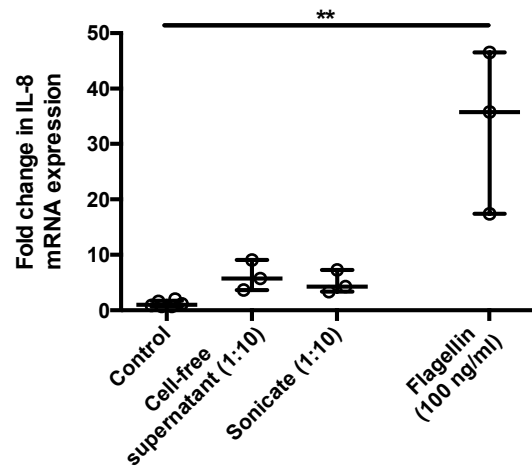
(b) Caco-2



(c) HT29-19A



(d) Caco-2





663 **Figure 2**

664

**(a) HT29-19A**

665

666

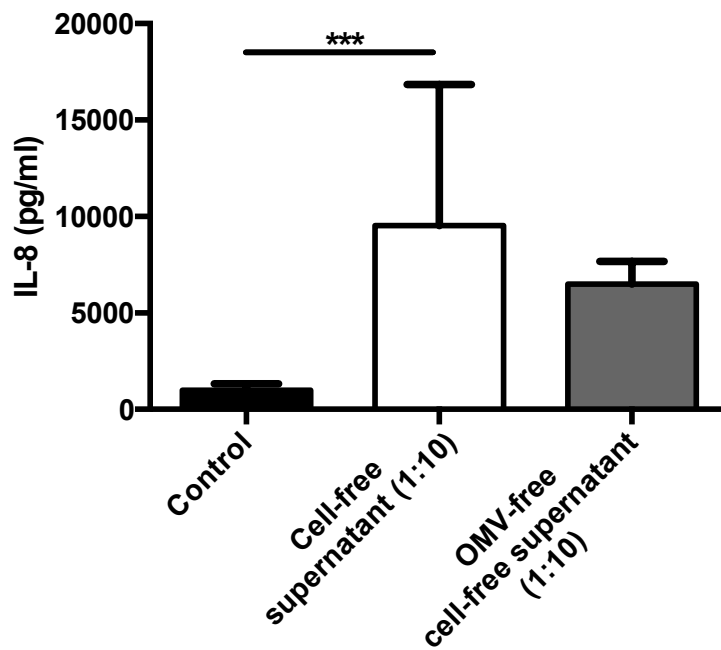
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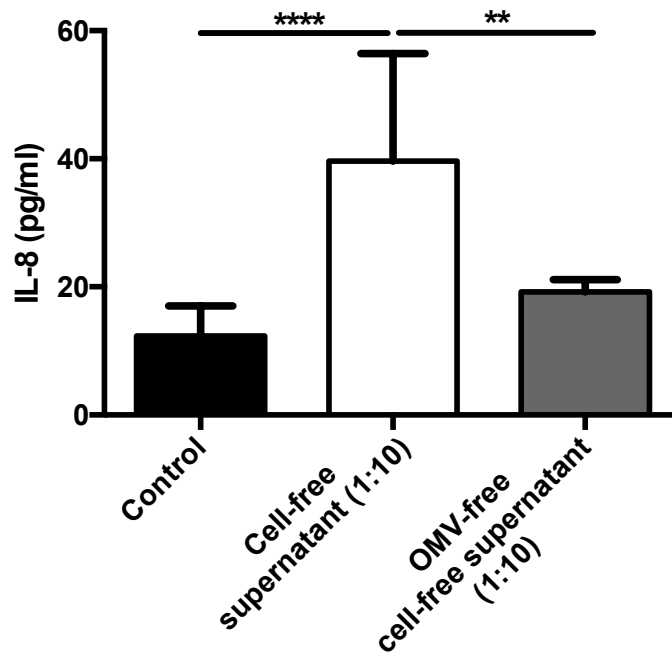
**(b) Caco-2**

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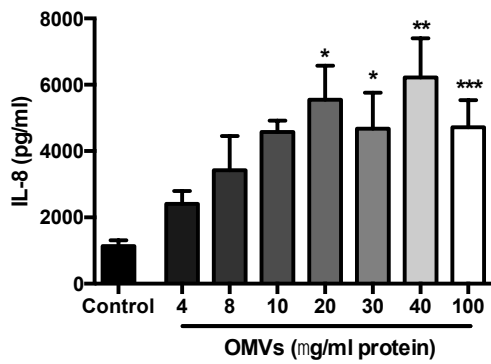
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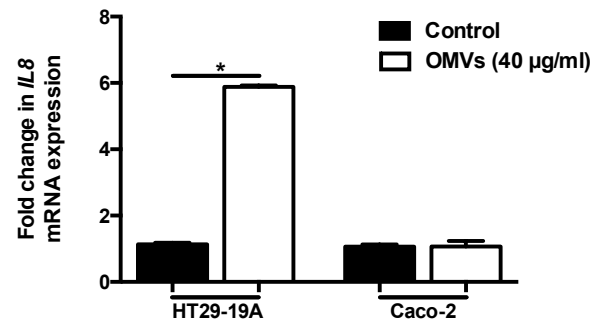
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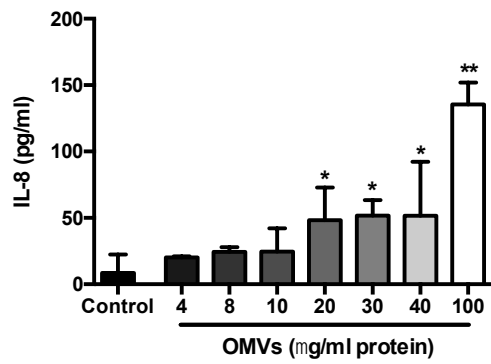
**(a) HT29-19A**



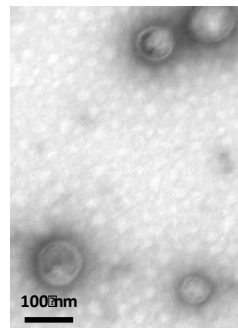
**(c)**



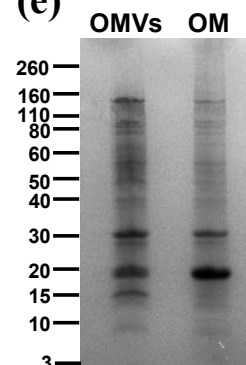
**(b) Caco-2**



**(d)**



**(e)**



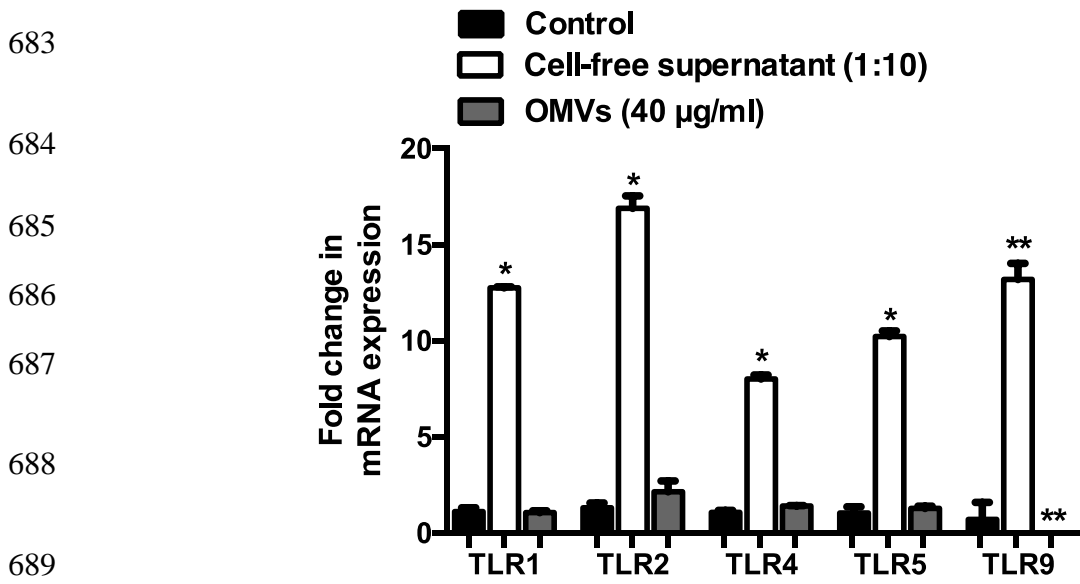
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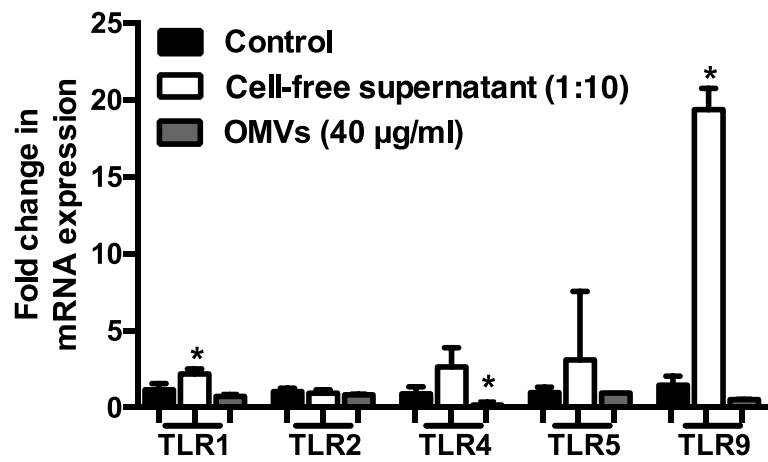
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681 **Figure 4**

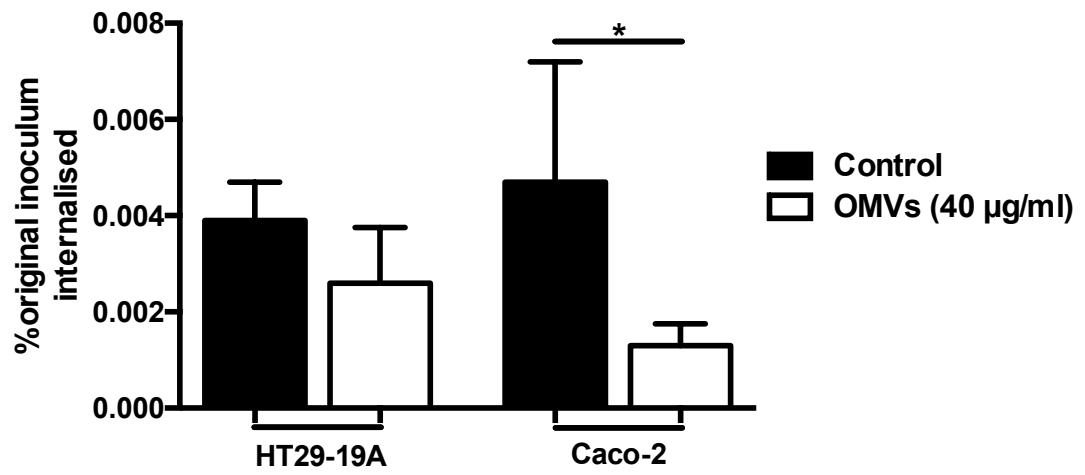
682 **(a) HT29-19A**



**(b) Caco-2**



690 **Figure 5**



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695 **Supplementary Figure 1**

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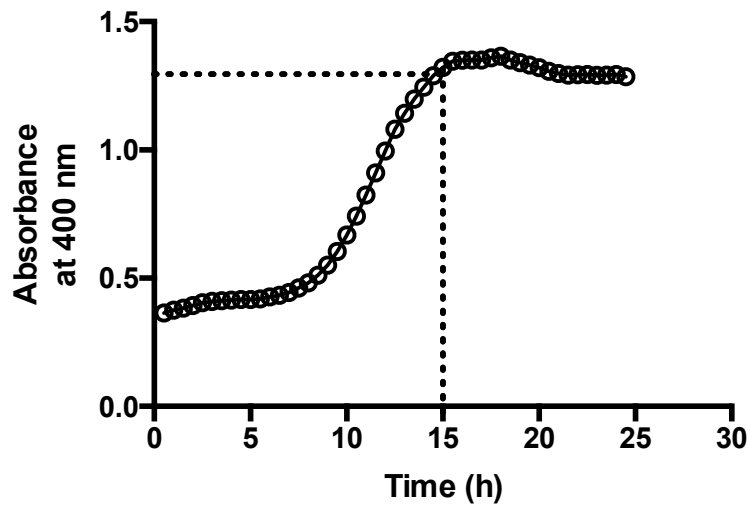
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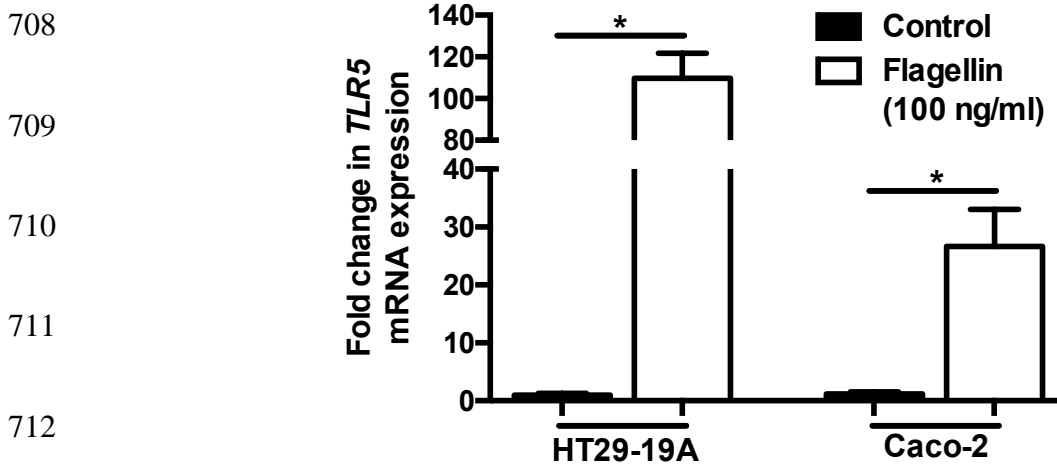
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**Supplementary Figure 1 – *E. coli* C25 growth curve. *E. coli* C25 was cultured in TSB broth for 24 h, with the absorbance at 400 nm measured every 30 min. The 15 h culture time utilised in the experiments in this study is indicated by the dotted line.**

707 **Supplementary Figure 2**



713 **Supplementary Figure 2 – Flagellin up-regulates TLR5 mRNA expression in IECs.**

714 **HT29-19A and Caco-2 cells were challenged with 100 ng/ml final concentration of flagellin**

715 **for 24 h and TLR5 mRNA expression was measured. Results are median ± IQR, n = 4-6. \***

716 **indicates significance from the control, where  $p \leq 0.05$ .**

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