

1 **Reversal of pathogen induced barrier defects in intestinal epithelial cells by contra-**  
2 **pathogenicity agents**

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24

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41

42 **Abstract**

43 **Background:** Environmental enteropathy (EE) is associated with stunting, impairment of  
44 responses to oral vaccines and other adverse health consequences in young children throughout  
45 the developing world. EE is characterised by chronic low-grade intestinal inflammation and  
46 disrupted epithelial-barrier integrity, partly resulting from dysregulation of tight junction  
47 proteins; observed in other enteropathies such as coeliac disease. During EE, this dysregulation  
48 of tight junction expression amplifies translocation of pathogenic bacteria across the intestinal  
49 mucosa.

50 **Aims:** Determine if enteropathogen-mediated epithelial-barrier failure can be ameliorated using  
51 contra-pathogenicity therapies.

52 **Methods:** Intestinal epithelial-barrier damage was assessed in Caco-2 cells incubated with three  
53 important enteropathogens identified in EE patients: Enteropathogenic *Escherichia coli* (EPEC),  
54 *Citrobacter rodentium* (*C. rodentium*) and *Cryptosporidium parvum* (*C. parvum*). Potential  
55 therapeutic molecules were tested to detect effects on transepithelial resistance (TER), bacterial  
56 translocation (BT), claudin-4 expression and regulation of the inflammatory cytokine response.

57 **Results:** All three enteropathogens compared to uninfected cells, reduced TER (EPEC;  $p < 0.0001$ ,  
58 *C. rodentium*;  $p < 0.0001$ , *C. parvum*;  $p < 0.0007$ ), reduced claudin-4 expression, and permitted BT  
59 (EPEC;  $p < 0.0001$ , *C. rodentium*;  $p < 0.0001$ , *C. parvum*;  $p < 0.0003$ ) through the monolayer. Zinc,  
60 colostrum, epidermal growth factor, trefoil factor 3, resistin-like molecule- $\beta$ , hydrocortisone and  
61 the myosin-light chain kinase (MLCK) inhibitor ML7 (Hexahydro-1-[(5-iodo-1-  
62 naphthalenyl)sulfonyl]-1H-1,4-diazepine hydrochloride); ML7) improved TER (up to 70%) and  
63 decreased BT (as much as 96%). Only Zinc demonstrated modest antimicrobial activity.

64 **Conclusion:** The enteropathogens impaired intestinal-epithelial barrier-integrity with  
65 dysregulation of claudin-4 and increased bacterial translocation. Enteropathogen-mediated

66 damage was reduced using contra-pathogenicity agents which mitigated the effects of pathogens  
67 without direct antimicrobial activity.

68

69 **Keywords:** Intestinal-barrier, claudin-4, microbial translocation, Enteropathogenic *Escherichia*  
70 *coli*, *Citrobacter rodentium*, *Cryptosporidium parvum*.

71

## 72 **Introduction**

73 Many disorders of the small intestine are characterised by increased permeability and impaired  
74 barrier function<sup>[1]</sup>. Environmental enteropathy (EE) is emerging as an example of a small intestinal  
75 disorder induced by frequent, often co-existent, sub-clinical intestinal infections <sup>[2,3,4]</sup>, with or  
76 without nutrient deficiencies. It is associated with stunting and poor growth in young children  
77 throughout the developing world<sup>[5]</sup>, with severe impaired responses to oral vaccines <sup>[6]</sup>, and with  
78 reduced net absorption of micronutrients<sup>[7]</sup> and some drugs <sup>[8]</sup>. EE is characterised by chronic  
79 low-grade intestinal inflammation <sup>[9]</sup> such as lymphocytic infiltration of the lamina propria and  
80 increased intraepithelial lymphocytes, increased translocation of bacteria across the intestinal  
81 mucosa<sup>[10]</sup>, reduced epithelial surface area, and immaturity of the absorptive cells of the  
82 intestine. There is also dysregulation of tight junctions (TJ) located at the apical margins of the  
83 lateral membranes of intestinal epithelial cells, which seal the intercellular space and define the  
84 boundary of the host versus the environment in the intestinal lumen <sup>[11]</sup>. As this dysregulation  
85 appears, at least partly, to explain microbial translocation <sup>[10,11]</sup>, characterisation of its  
86 mechanisms is critical for improving health of children in low- and middle-income countries  
87 (LMICs) <sup>[12]</sup>. It may also assist in understanding critical care sepsis, autoimmune and neurological  
88 disorders of the gut.

89

90 Recent major studies, building on decades of observational microbiology, have defined many of  
91 the most important enteropathogens responsible for diarrhoea in children in LMICs <sup>[13,14]</sup>. Our  
92 understanding of the contribution of enteropathogens to EE is less advanced, but emerging  
93 evidence links the severity of enteropathy (and growth failure) to frequent sub-clinical infections  
94 with parasites, viruses and bacteria <sup>[2,15,16]</sup>. Those enteropathogens which are invasive or cause  
95 intestinal epithelial disruption have been associated with more severe inflammation and growth

96 impairment<sup>[17]</sup>. The mechanism through which enteropathogens induce enteropathy is unknown,  
97 but in view of the intense microbial translocation found in children and adults with EE, it seems  
98 that impairment of epithelial barrier function <sup>[1]</sup> is central to pathophysiology. Whether this  
99 impairment is due to impairment of TJ, or to larger defects at sites of epithelial cell shedding, is  
100 unclear. What is known is that shedding of individual intestinal epithelial cells does not lead to  
101 impairment of epithelial barrier function <sup>[18]</sup>, but simultaneous loss of multiple cells does impair  
102 it and may lead to intestinal inflammation.

103  
104 In previous work, our group studied adults with EE in Lusaka, Zambia using several methods  
105 including histology and confocal laser endomicroscopy (CLE). We demonstrated that there was  
106 marked epithelial barrier disruption in these EE patients with corresponding epithelial leakage  
107 images visible by CLE <sup>[10]</sup>. We suggested the epithelial defects were probably sites of bacterial  
108 translocation, and then hypothesised that it might be possible to ameliorate enteropathogen-  
109 mediated damage using therapies targeting the epithelium. Here we describe a series of  
110 experiments to test novel therapeutic approaches for reduction of translocation in EE. These  
111 agents were selected from transcriptomic work (Kelly, unpublished observations) and extensive  
112 literature searches looking at their potential to function as either TJ modifiers or selective  
113 enhancers of epithelial barrier function (full explanations are listed in Supplementary Table 1).

114  
115 We used an *in vitro* modelling system with polarised Caco-2 cells and three different  
116 enteropathogens: 1) Enteropathogenic *Escherichia coli* (EPEC), part of a group of related  
117 pathogens which are an important cause of infant diarrhoea <sup>[19]</sup> and known to disrupt cell-cell  
118 junctions <sup>[20,21]</sup>; 2) *Citrobacter rodentium* (*C. rodentium*), a natural murine intestinal pathogen  
119 which has also been identified in EE cases of adults (human) in Zambia <sup>[22]</sup>, and *Cryptosporidium*

120 *parvum* (*C. parvum*), associated with adverse outcomes in children with malnutrition [14,23]. *C.*  
121 *rodentium* possesses a locus of enterocyte effacement (LEE) pathogenicity island that shares all  
122 41 open reading frames with that of EPEC [24]. Therefore, EPEC and *C. rodentium* share a core set  
123 of virulence factors utilising intimate bacterial attachment to the host cells through attaching and  
124 effacing (A/E) lesions [25,26]. *C. parvum* infects the intestinal epithelium producing a  
125 parasitophorous vacuole in which the parasite resides in an intracellular yet extra-cytoplasmic  
126 manner [23]. Cryptosporidial enteropathy has recently also been shown to occur in an animal  
127 model [27].

128

## 129 **Materials and Methods**

130

### 131 *Cell culture*

132 The human Caco-2 enterocyte cell line (passage 6-40) was obtained from the American Type  
133 Culture Collection (ATCC: HTB-37; Middlesex, UK) and grown as monolayers (VWR, Leicestershire,  
134 UK) at 37°C in humidified 5% CO<sub>2</sub> in complete medium consisting of Dulbecco's Modified Essential  
135 Medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 4 mM L-glutamine,  
136 100U/ml penicillin, 100µg/ml streptomycin and 1% non-essential amino acids (Invitrogen Life  
137 Technologies, Paisley, UK). For membrane integrity and translocation investigations, trypsinised  
138 cells were seeded at a density of 1x 10<sup>6</sup>/ml into 12 well plates holding polyethylene terephthalate  
139 (3.0µm) cell culture inserts (Transwell inserts, Millipore, Hertfordshire, UK), for  
140 immunofluorescence experiments at 5x 10<sup>5</sup>/ml into 24-well culture plates (VWR International,  
141 Leicestershire, UK) with 13mm glass coverslips, or at 2 x 10<sup>6</sup>/ml into 6 well plates for Western  
142 blot experiments. Cells were grown until polarization (15-21 days) for translocation studies or  
143 until 75% confluent for staining experiments (4-5 days; limiting cell damage during staining

144 procedure) before experiments were conducted. In some experiments, FCS was withheld from  
145 the medium to model nutrient depletion in malnutrition. Formation and disruption of polarised  
146 monolayers (membrane integrity) was determined by regular measurement of transepithelial  
147 electrical resistance (TER) (Millicell-ERS; Millipore, Livingston, UK). Caco2 cell monolayers were  
148 considered polarised when TER readings reached 800-1000 ohms/cm<sup>2</sup><sup>[28]</sup> correlating with the up-  
149 regulated expression of the brush border enzyme sucrase isomaltase (data not shown). Migration  
150 of Caco2 cells through the filters was not observed in basolateral media.

151

### 152 *Bacterial strains and Parasite*

153 EPEC strain RDEC-1 serotype O15:H7 (ATCC 49106; rabbit) and *C. rodentium* strain DBS100,  
154 Biotype 4280 (ATCC 51459) were used. A non-pathogenic *Escherichia coli* K12 (*E. coli* K12) isolate  
155 was a generous gift from Dr David Wareham (Blizard Institute, Barts and The London School of  
156 Medicine, UK). Fresh colonies were stored on Luria-Bertani (LB) agar at 4°C and grown up in LB  
157 broth (LB agar and broth; Fisher Scientific, Leicestershire, UK) at 37°C overnight for each  
158 experiment. For immunofluorescence experiments, bacteria were incubated with rhodamine B  
159 isothiocyanate (Sigma-Aldrich, Dorset, UK) at 1mg/ml for 2h in the dark with gentle shaking, then  
160 washed. Oocysts of the *C. parvum* IOWA isolate (Bunch Grass Farm, Deary, ID, USA) were stored  
161 in phosphate buffered saline (PBS) at pH7.2 at 4°C. Prior to use oocysts were surface sterilized in  
162 10% (v/v) sodium hypochlorite (VWR, Leicestershire, UK) and centrifuged at 500xg for 10 min.

163

### 164 *In vitro bacterial and parasite infections*

165 Before infection commenced, monolayers were washed with PBS. Pathogens at 1x10<sup>6</sup> colony  
166 forming units (CFUs)/well (for 12 well plates) were then added to the brush border face of the  
167 monolayer in 1ml complete medium. *C. parvum* infection was initiated using 1x10<sup>6</sup> oocysts in



168 250µl complete medium with 1x10<sup>6</sup> CFU/ml/well of non-pathogenic *E. coli* K12. After 2h at 37°C,  
169 cells were washed to remove oocyst debris and 1ml complete medium with 1x10<sup>6</sup> CFU/ml/well  
170 of *E. coli* K12 introduced. Both bacterial and parasite infections developed for 24h without  
171 antibiotics at any stage. For studies using 6- and 24-well plates the numbers of bacteria, parasites  
172 and volumes of medium were scaled up or down accordingly.

173

#### 174 *Quantification of transepithelial resistance (TER) and bacterial translocation*

175 Electrical resistance across the stratified epithelium was measured using a Millicell-ERS-2  
176 instrument (Millipore, Bedford, Mass) with tweezer-like electrodes. Before measurement, the  
177 electrodes were equilibrated and sterilized according to the manufacturer's recommendations.  
178 The value obtained from a blank insert (with culture medium) was subtracted to give the net  
179 sample resistance, which was then multiplied by the membrane area to give the resistance in  
180 area-corrected units ( $\Omega/\text{cm}^2$ ). TER was measured before and after 24h infections. To observe  
181 bacterial translocation (BT), the medium from the basolateral compartment of the Transwell  
182 insert after infection was cultured for colony quantification. The apicomplexan parasite, *C.*  
183 *parvum*, was co-cultured with the non-invasive bacteria *Escherichia coli* K12 (K12) in the *in vitro*  
184 model, Therefore, K12 was used as a marker of BT during *C. parvum* infection. Whereas, for EPEC  
185 and *C. rodentium* experiments, each bacterium was used as the BT marker

186

#### 187 *Fixation of cell monolayers and Immunofluorescence (IF)*

188 Confluent monolayers were fixed for 20min in 4% paraformaldehyde (Room temperature; RT),  
189 washed in PBS, then heated at 95°C for 10min with antigen retrieval buffer (10mM Tris Base,  
190 1mM EDTA, 0.05% Tween, pH 9.5) and washed again in PBS. *C. parvum* infected cells were  
191 permeabilized with 0.2% Triton X-100 at RT for 3 min then washed in PBS. Cells were blocked

192 with 20% goat serum (Abcam, Cambridge, UK) for 30min, incubated with 1:100 rabbit polyclonal  
193 anti-claudin 4 (antibodies from Abcam, Cambridge, UK) in 20% goat serum for 2h at RT, then  
194 washed in PBS and incubated with conjugated secondary antibody (anti-rabbit IgG H & L -  
195 AlexaFluor) for 1h at RT. Cells were mounted in Vectorshield including DAPI (Vector Laboratories,  
196 Peterborough, UK). For *C. parvum* staining, 1 $\mu$ g/ml<sup>-1</sup> VVL (lectin of *Vicia villosa*, Vector  
197 laboratories, Peterborough, UK) in PBS with 1% Bovine serum albumin (BSA) was added to cells  
198 for 1h at RT, washed and incubated with 1 $\mu$ g/ml conjugated streptavidin CY3. After 10min, cells  
199 were mounted with vectorshield and DAPI. A Leica DM5000 upright epifluorescence microscope  
200 was used for images at 400x magnification. A Zeiss LSM710 point scanning confocal microscopy  
201 was used for representative confocal images at 400x magnification.

202

### 203 *Lactate Dehydrogenase (LDH) Assay*

204 To evaluate the extent of intestinal epithelial cell damage after enteropathogen infection, the  
205 LDH assay (Promega, Southampton, UK) was performed on culture medium from cell monolayers  
206  $\pm$  infection (24h), and analysed according to the manufacturer's instructions.

207

### 208 *Pharmacological therapies*

209 The potential therapeutic agents optimised and tested are listed in Table 1, and the rationale for  
210 their selection is tabulated in Supplementary Table 1.

211

### 212 *Antimicrobial Assay*

213 To establish the antimicrobial activity of each molecule on the bacteria, EPEC, *C. rodentium* and  
214 *E. coli* K12 was grown overnight in LB broth, washed and 1x10<sup>6</sup> CFU/ml incubated with a molecule  
215 at optimal testing concentrations for 24h at 37°C. *E. coli* K12 was co-incubated with *C. parvum*.

216 After the incubation period, the samples were cultured on LB agar and bacterial colonies  
217 enumerated.

218

#### 219 *Western blotting*

220 Proteins for western blotting were extracted using lysis buffer (Cell RIPA lysis plus protease  
221 inhibitor cocktail, Sigma-Aldrich, Dorset, UK) and sonicated (100W, 10s; Microson, New York,  
222 USA), then centrifuged (13000xg, 10min, 4°C). Total protein (20µg) was boiled at 95-100°C for 5  
223 min, cooled on ice before electrophoresis on 12% SDS-Tris-Glycine gels and then transferred to a  
224 Polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, Bucks, UK). After overnight  
225 rabbit polyclonal claudin-4 Ab (1:1000) incubation (4°C), the membrane was washed repeatedly  
226 in tris-buffer saline (TBS) and then incubated for 1h with horseradish peroxidase (HRP; 1:10;  
227 Supersignal West Pico rabbit kit (Pierce Fast; Thermo Scientific, Loughborough). Recombinant  
228 human claudin-4 was used as a positive control. To confirm equal protein loading, the membrane  
229 was stripped and Re-Blot plus strong solution (Millipore, Livingston, UK) was added for 20min.  
230 After washes in TBS, the membrane was incubated at 4°C with anti-human GAPDH (1:5000  
231 dilution, Sigma-Aldrich, Dorset, UK) for 1hr and re-developed.

232

#### 233 *Cytokine and Chemokine expression assay (Takeda)*

234 Cell supernatants were assessed for cytokine and chemokine expression using the V-PLEX pro-  
235 inflammatory panel 1 human assay (Meso Scale Discovery MD, USA). The assay was performed  
236 according to the manufacturer's instruction.

237

#### 238 *Data analysis*

239 TER is presented without transformation whilst bacterial translocation is presented as log colony-

240 forming units/ml. TER % change was calculated by using the mean decrease due to each  
241 enteropathogen. The Wilcoxon signed-rank test was used to test the effect of the intervention  
242 on paired data from each experiment conducted on adjacent wells, with or without the therapy.  
243 One-way ANOVA with Kruskal-Wallis/Tukey's post hoc was used for antimicrobial activity, LDH,  
244 bacterial invasion and adherence experiments and cytokine analysis where appropriate.  
245 Statistical significance was established at  $p < 0.05$ .

246

247

248 **Results**

249

250 *Selected pathogens induce barrier dysfunction in Caco2 cell*

251 In preliminary experiments, an appropriate TER ( $\Omega/\text{cm}^2$ ) was selected for Caco2 cells to confirm  
252 polarity, which correlated with the expression of the brush border enzyme sucrase isomaltase  
253 (data not shown). All three enteropathogens reduced TER irrespective of nutrient rich or nutrient  
254 deplete conditions (Figure 1A). Both bacterial pathogens elicited a greater decline in TER than *C.*  
255 *parvum* whilst non-pathogenic *E. coli* K12 alone did not modify the TER readings. EPEC and *C.*  
256 *rodentium* added to the apical sides of the intestinal monolayer were isolated and cultured from  
257 the basal Transwell chamber signifying bacterial translocation (BT) had occurred across the  
258 epithelium (Figure 1B). Consistent with observations of TER, there was no BT during incubation  
259 of *E. coli* K12 alone in the *in vitro* model, but this was induced by co-culture with *C. parvum*.

260

261 To confirm that human Caco2 cells were infected by *C. rodentium* (murine; DBS100), EPEC (rabbit;  
262 RDEC-1) and *C. parvum* + *E. coli* K12 (human; K12), adherence and invasion (gentamicin) assays  
263 were performed (Supplementary Figure 1A and B). Previous studies have reported that both  
264 animal and human EPEC strains are clonally related <sup>[29]</sup>, and regardless of the isolate's original  
265 host, colonisation is similar and so not host dependent <sup>[30]</sup>. This study has confirmed those key  
266 observations by demonstrating the ability of EPEC (RDEC-1) to attach to the human intestinal  
267 epithelial cells from 30min onwards ( $P < 0.05$ ; Supplementary Figure 1A) and were isolated from  
268 inside Caco2 cells after 60min ( $P < 0.05$ ; Supplementary Figure 1B). The murine pathogen *C.*  
269 *rodentium*, also infected Caco2 cells but took longer to attach (60min) and were isolated from  
270 within the cell monolayer at 120min. *E. coli* K12 when co-cultured with *C. parvum*, attached and  
271 were isolated from within Caco2 cells over a similar timescale to *C. rodentium* but in much lower

272 numbers. The results confirmed the pathogenicity of the three enteropathogens in Caco2 cells  
273 and indicated that *C. parvum* may induce paracellular translocation of *E. coli* K12 whilst EPEC and  
274 *C. rodentium* appeared to demonstrate partial transcellular movement.

275

#### 276 *Barrier dysfunction was associated with reduced claudin-4*

277 Our group has previously shown dysregulation of claudin-4 in biopsies from adults and children  
278 with EE <sup>[10,31]</sup>. To establish if enteropathogen-induced barrier disruption was also accompanied  
279 by impairment of claudin-4 expression, immunofluorescence and Western blotting of the protein  
280 was performed. In monolayers infected with either of the three enteropathogens, the expression  
281 of claudin-4 (green) was reduced (Figure 2A and B), and this was confirmed by Western blotting  
282 (Figure 2C) and densitometry (Figure 2D). Interestingly, unlike EPEC and *C. parvum* infections, at  
283 the site of *C. rodentium* infection the claudin-4 protein appeared to aggregate around the  
284 bacterium, with reduced expression over the remaining cells in the monolayer (Figure 2A).  
285 Although, the LDH assay showed a small increase in Caco2 cell lysis after enteropathogen-  
286 infection this was not statistically significant compared to non-infected controls and therefore is  
287 unlikely to explain the loss of claudin-4 expression (Figure 2D). Consistent with TER findings  
288 (Figure 1A), the results show bacterial infections elicited greater reductions of claudin-4 than *C.*  
289 *parvum* infection.

290

#### 291 *Partial reversal of pathogen-induced barrier dysfunction using contra-pathogenicity agents*

292 Several molecules were screened for their ability to limit the enteropathogen-induced damage  
293 caused to the epithelial barrier integrity (Table 1 and Supplementary table 1). Seven of these  
294 agents: zinc, colostrum, human epidermal growth factor (hEGF), trefoil factor-3 (TFF3), resistin-  
295 like molecule-beta (RELM- $\beta$ ), hydrocortisone and the myosin-like chain kinase (MLCK) inhibitor

296 ML7 (Hexahydro-1-[(5-iodo-1-naphthalenyl)sulfonyl]-1H-1,4-diazepine-hydrochloride); ML7),  
297 impeded the pathogen-induced decrease in TER (Figure 3A) and they also reduced BT (Figure 3B),  
298 suggesting a relationship between these two processes. Vitamin A and leptin had minimal effect  
299 on pathogen-induced damage. Interestingly, to determine the dependence of the molecules on  
300 nutrients, Caco2 cells were starved of fetal calf serum (FCS) which crudely mimics clinical  
301 malnutrition. Nutrient-rich conditions improved TER readings in cells treated with, TFF3,  
302 hydrocortisone, hEGF, zinc and ML7. Colostrum, ML7 (in *C. parvum* only) and Relm- $\beta$ , had a  
303 greater effect in nutrient-deplete conditions. To determine whether the nine molecules acted on  
304 the enteropathogens in an antimicrobial manner we incubated these molecules directly with the  
305 pathogens, and no antimicrobial activity was observed except with zinc (Figure 4). Tissue inhibitor  
306 of metalloproteinases (TIMP) 1 and 2 were tested along with low minimum inhibitory  
307 concentrations (MICs) of tetracycline and doxycycline (known to also function as  
308 metalloproteinase inhibitors). However, data are not included due to the strong antimicrobial  
309 activity observed with these widely used antibiotics.

310

311 Two of the most effective contra-pathogenic agents TFF3 and ML7, were further evaluated by  
312 investigating their effect on expression of claudin-4. Both molecules ameliorated the pathogen-  
313 induced reduction of claudin-4 protein expression (Figure 5A and C). The Western blot results  
314 were confirmed by densitometry (Figure 5B and D), correlating with observations that both  
315 molecules decreased LDH release from cells induced by enteropathogens infections (Figure 5E).  
316 Although the LDH readings after infection from either of the three enteropathogens was already  
317 low (Figure 2E and Figure 5E), ML7 appeared to block LDH release completely from the three  
318 enteropathogen-infected Caco2 cells ( $p < 0.0001$ ). However, TFF3 appeared slightly more effective  
319 in reducing LDH levels in EPEC-infected cells ( $p < 0.0001$ ) compared to *C. rodentium* ( $P < 0.007$ ) and

320 *C. parvum* ( $p < 0.007$ ) – infected monolayers.

321

322 A dose-response relationship was apparent for TFF3 (Figure 6), which was also dependent on  
323 nutrient availability. TFF3 increased TER and reduced BT in both nutrient-rich and nutrient-  
324 depleted conditions for both EPEC (Figure 6A and B,) and *C. rodentium* (Figure 6C and D). The  
325 concentration-dependence of TFF3 was clearer in *C. rodentium* infection, and translocation was  
326 reduced further in the presence of FCS (Fig 6A and C) than in its absence (Fig 6B and D). These  
327 dose-response experiments also demonstrated the marked inverse correlation between TER and  
328 BT (EPEC:  $\rho = -0.80$ ,  $P < 0.0001$ ; *C. rodentium*:  $\rho = -0.66$ ,  $P < 0.0001$ ).

329

330 *TFF3 and Relm- $\beta$  increase immune-regulation in enterocytes*

331 The disruption of intestinal barrier function and the alteration of the TJ protein claudin-4  
332 following infection, led us to additionally examine the effect of two goblet cell released contra-  
333 pathogenic molecules, TFF3 and Relm- $\beta$ , in regulating the inflammatory response. The effects of  
334 TFF3 or Relm- $\beta$  on cytokine release by enteropathogens in polarised Caco2 cells over 24h was  
335 determined by measuring cytokines in supernatants by ELISA. TFF3 and Relm- $\beta$  modestly  
336 increased the induction of interleukin 10 (IL-10) and tumour necrosis factor alpha (TNF- $\alpha$ ) by EPEC  
337 (Figure 7). Relm- $\beta$  also increased induction of IL-2 and IL-6 by all three pathogens and induced  
338 these two cytokines in the absence of pathogens.

339

## 340 **Discussion**

341

342 Several investigators have shown that EPEC<sup>[32]</sup>, *C. rodentium*<sup>[33]</sup> and *C. parvum*<sup>[34]</sup> infections of  
343 intestinal epithelial monolayers alter barrier function. Using a similar *in vitro* model, we have tried



344 to model the epithelial barrier dysfunction in environmental enteropathy (EE) utilising these  
345 important enteropathogens. We have demonstrated that all three pathogens reduced intestinal  
346 epithelial integrity by decreasing TER associated with downregulation of claudin-4. This barrier  
347 defect could be prevented by agents that mitigated pathogenic effects including the reduction of  
348 bacterial translocation. We refer to this mechanism as a contra-pathogenicity effect as it occurs  
349 without demonstrable antimicrobial activity (except for zinc) and therefore appears to alter the  
350 balance between host and pathogen. There was a clear inverse correlation between membrane  
351 integrity (TER) and BT although details of the mechanism(s) involved require further work. The  
352 contra-pathogenicity effect seems to entail protection against cytopathic effects and tight junction  
353 disruption and reduced ionic conductance.

354  
355 The rabbit EPEC strain, RDEC-1, has extensively been used over the years to study the human EPEC  
356 infection because it contains a homolog of the human EPEC 35kb LEE locus (locus of enterocyte  
357 effacement), which encodes all necessary determinants for the (A/E) phenotype<sup>[35]</sup>. Previous  
358 studies have shown human and rabbit EPEC strains cause A/E lesions in infant pigs indicating EPEC  
359 colonisation is not host-dependent<sup>[30]</sup> and supports our data. Sharing a genetically similar LEE  
360 pathogenicity island is the pathogen *C. rodentium*; the causative agent of transmissible murine  
361 colonic hyperplasia, a naturally occurring disease in laboratory mice <sup>[36]</sup>. The protozoan parasite *C.*  
362 *parvum* causes cryptosporidiosis<sup>[37]</sup> and has been listed as one of the four major enteric pathogens  
363 causing life-threatening diarrhoea in infants globally<sup>[3,13]</sup>. Importantly, for our investigation all three  
364 enteropathogens have been isolated in EE patients (both adults and children) in Zambia <sup>[22,23,24]</sup> and  
365 when used in our *in vitro* model have been shown to infect Caco2 cells (See supplementary Figure  
366 1).

367

368 Previous *in vitro* studies have observed that EPEC-induced barrier dysfunction is attributed in part  
369 to the secretion of the effector protein, EspF<sup>[38]</sup> within the first 24hr of infection and not at later  
370 time-points<sup>[21]</sup>. The mechanism by which EspF expression disrupts the TJ barrier has been correlated  
371 with the redistribution of occludin<sup>[38]</sup>. Our study now demonstrates the TJ protein, claudin-4, is also  
372 affected within the first 24hr of EPEC infection. Other effector molecules such as EspG1 and EspG2  
373 (involved in microtubule destruction) cause TJ perturbation but claudin-4 localisation has not been  
374 shown to be affected<sup>[39]</sup>. Unsurprising, *C. rodentium* also increased intestinal epithelial permeability  
375 in our *in vitro* model which correlated with claudin-4 disruption. It has been previously reported  
376 that claudin-4 expression was downregulated during *C. rodentium* infection of the murine cell line  
377 CMT-93, due to the Rho-Rho kinase (ROCK) signalling pathway<sup>[33]</sup>. However, this mechanism has  
378 not been determined in our model. In agreement with our investigation, the third enteropathogen  
379 tested, *C. parvum* has been shown to decrease protein levels of claudin-4 in Caco2 cells, mouse  
380 enteroid-derived monolayers and the ileal and jejunal mucosa of mice post- 24hr infection<sup>[37]</sup>.  
381 Kumar *et al.*, (2018) observed that in Caco2 cells the parasite had no effect on mRNA levels of  
382 claudin-4, whereas both mRNA and protein levels were decreased *in vivo* (murine and enteroids)  
383 models. This suggested posttranslational mechanisms, play a role in *C. parvum*-induced modulation  
384 of claudin-4. Cell death would also reduce claudin-4 expression and increase bacterial translocation  
385 although our lactate dehydrogenase assay findings (Figure 2E) demonstrated this was not a  
386 significant factor.

387  
388 Two of the agents we selected for testing (Figure 3) are micronutrients (Vitamin A and Zinc) which  
389 are well known to have protective or therapeutic effects in reducing diarrhoea incidence or  
390 severity<sup>[40,41]</sup>. ML7 is a small molecule inhibitor of Myosin Light Chain Kinase (MLCK) which has been  
391 implicated in TJ remodelling<sup>[42]</sup>. Leptin is a hormone postulated to have effects in intestinal

392 resistance to infection<sup>[43]</sup>. Whilst, the remaining five are paracrine mediators of mucosal healing, or  
393 nutrient solutions such as colostrum which are likely to contain them. Vitamin A and leptin had very  
394 little effect at the concentrations and in the preparations used. Among the other active agents,  
395 there was considerable variation in nutrient dependence: hydrocortisone seems to have greater  
396 efficacy under nutrient-deplete conditions, whereas colostrum, hEGF and TFF3 seem to be more  
397 effective in nutrient replete states. Zinc deficiency is known to result in epithelial barrier leak in the  
398 intestines and alter TJ composition<sup>[44]</sup>. We found zinc improved TER readings and decreased BT. Its  
399 effect on translocation was proportionately greater in *C. parvum* infection than in bacterial  
400 infections (EPEC and *C. rodentium*). Interestingly, this effect was nutrient-independent. These  
401 findings are in line with previous studies which showed zinc had a protective effect in pigs suffering  
402 from enterotoxigenic *Escherichia coli* (ETEC) infections as zinc supplementation prevented or  
403 alleviated diarrhoea in this model <sup>[45]</sup>.

404  
405 It appeared that the contra-pathogenicity agents tested varied in effect and this is likely to reflect  
406 variation in cellular receptors or targets. Retinoids act through nuclear receptors. Whilst, Relm- $\beta$  is  
407 exclusively found in goblet cells of the intestines and may play a role during colonisation and  
408 infection of the gastrointestinal tract<sup>[46]</sup>. It plays a role in the production of Th2 cytokines during  
409 nematode infections in mice and is essential in helminth expulsion<sup>[47]</sup>. Relm- $\beta$  also has been shown  
410 to be induced during *C. rodentium* infection, in which it is involved in recruiting CD4+ T-cells<sup>[48]</sup>. TFF3  
411 is also a goblet cell product with well-established protective effects on the small intestinal  
412 mucosa<sup>[49]</sup> working at the cell membrane. ML7 acts on the adenosine triphosphate- binding site of  
413 the active centre of MLCK causing inhibition<sup>[42]</sup> and therefore will perform throughout the cell. The  
414 agents which had the greatest effect on translocation, such as TFF3 and ML7, also reduced the  
415 effect of pathogens on claudin-4 expression (Figure 5). With TFF3 demonstrating its action in a dose-

416 dependent manner (Figure 6).

417

418 Despite the absence of lymphoid cells in this model we were still able to detect low levels of  
419 cytokine and chemokine secretion, except for IL-8 which was in the range reported by Sansonetti  
420 *et al.*, (1999). The significance of these immunological results is unclear at present. It is possible that  
421 the enteropathogens disrupt claudin-4 distribution and inhibit its restoration and so the contra-  
422 pathogenicity molecules may act by modulating the inflammatory response preventing complete  
423 claudin-4 obliteration. Further investigations are needed to understand the cytokine results and to  
424 establish if these therapies involve dual actions, working on both the pathogen and by controlling  
425 the release of the inflammatory mediators which if in excess could be detrimental to host health.

426

427 Given the emergence of antimicrobial resistance as a global phenomenon, development of  
428 strategies to reduce the impact of pathogens on the host intestinal mucosa could be helpful. Some  
429 of the agents we studied are in clinical use, could be easily applied to clinical studies or inserted  
430 into clinical trials.

431

432

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618

619 *Pharmacological therapies.*

Therapy	Concentration(s) tested	Incubation Time	Reference
Colostrum	160mg/ml	Added to Caco2 cells at same time as infection	51
Human epidermal growth factor (EGF)	100 & 200 ng/ml		52,53
Hydrocortisone	20,30 & 50µg/ml		54
Resistin-like molecule β (RELMβ)	100nM		58 preliminary unpublished data
Trefoil Factor 3 (TFF3)	10ng/well		59
Colostrum + hEGF +TFF3	Colostrum – 160mg/ml hEGF – 200ng/ml TFF3 – 10ng		51,54,55
Vitamin A (Retinoic acid)	1, 10 & 100 µmol		60
Leptin	10µM	Pre-treated Caco2 cells 30mins prior infection	55,56
Myosin-light chain kinase (MLCK) inhibitor ML-7	50µM	Pre-treated Caco2 cells 2h prior infection	32, 57
Zinc	50 & 100µM	Pre-treated Caco2 cells 24h or 7days prior infection	61,62

620 **Table 1:** Therapeutic molecules tested including concentrations used and incubation conditions.

621 **Figure Legends**

622 **Figure 1: Selected enteropathogens modulate barrier integrity leading to bacterial**  
623 **translocation *in vitro*.** Polarised Caco2 cells grown on Transwell inserts were infected with  
624 Enteropathogenic *Escherichia coli* (EPEC), *Citrobacter rodentium* (*C. rodentium*), *Cryptosporidium*  
625 *parvum* (*C. parvum*) or *Escherichia coli* K12 for 24h, with or without fetal calf serum (FCS) to model  
626 nutrient-rich or nutrient-deplete conditions respectively. During the *C. parvum* incubations,  
627 addition of *Escherichia coli* K12 (K12) was used as a marker of passive translocation. **(A)** Trans-  
628 epithelial resistance (TER) was measured to establish membrane integrity; n=50. One-way  
629 ANOVA with the Kruskal-Wallis test was performed separately for each stimulus with or without  
630 (w/o) FCS. Non-infected Caco2 cells (no infection) was used as the control and compared to each  
631 pathogen dataset: \*\*\*\*p<0.0001, \*\*\* p=0.0007 and ns= non-significant. **(B)** Basal media from  
632 beneath the monolayer of Caco2 cells (Transwell inserts) were cultured to measure bacterial  
633 translocation (CFU/ml) from the *in vitro* model; n=50. One-way ANOVA with the Kruskal-Wallis  
634 test was performed separately on samples with or without (w/o) FCS. Caco2 cells with no  
635 infection were used as the control and compared to the datasets of each pathogen: \*\*\*  
636 p=0.0003, \*\*\*\*p<0.0001, , ns = non-significant.

637

638 **Figure 2: Enteropathogens dysregulate the expression of the tight junction protein, Claudin-4.**

639 **(A)** Enteropathogenic *Escherichia coli* (EPEC), *Citrobacter rodentium* (*C. rodentium*) or  
640 *Cryptosporidium parvum* (*C. parvum*) were added to non-polarised Caco2 cells grown on  
641 coverslips for 24h. Cells were then fixed, and immunofluorescence staining for claudin-4 (green)  
642 performed. Bacteria (red) were labelled with rhodamine B isothiocyanate (588nm) whilst *C.*  
643 *parvum* was identified using the lectin VVL-biotin and CY3-streptavidin (red; 565nm). Cell nuclei  
644 (blue) were stained using DAPI (405nm). Composite images are shown, and scale bars represent

645 100µm. **(B)** A representative confocal image of EPEC infection in Caco2 cells; solid arrows indicate  
646 bacterial colonies and the dotted arrow highlights interruption of Claudin-4 expression. **(C)**  
647 Western blotting of Claudin-4 protein expression (22kDa) in cells infected for 24h. A positive  
648 control of recombinant claudin-4 (49kDa) was used with levels of GAPDH (36kDa) used to confirm  
649 equal protein loading. **(D)** densitometry confirmed the Western blotting \*p<0.005 E) LDH assay  
650 on supernatants of infected monolayers confirmed non-significant (ns) cell lysis compared to the  
651 control (non-infected Caco2 cells). n=24.

652  
653 **Figure 3: Selected pharmacological agents impede enteropathogen-induced barrier damage**  
654 **and subsequent bacterial translocation. (A)** Transepithelial resistance (TER) of polarised Caco2  
655 cells was measured after 24h infection with Enteropathogenic *Escherichia coli* (EPEC), *Citrobacter*  
656 *rodentium* (*C. rodentium*) and *Cryptosporidium parvum* (*C. parvum*) together with *Escherichia coli*  
657 K12, in the presence or absence of one of a range of potential therapeutic molecules. 24h prior  
658 to infection Caco2 cells were incubated with or without fetal calf serum (FCS) to model nutrient-  
659 rich or nutrient-deplete conditions respectively. The TER results are presented as a percentage  
660 change from Caco2 cells infected with each specific enteropathogen and no molecule. The Mann-  
661 Whitney U-test was performed to compare differences between nutrient deplete and nutrient  
662 replete conditions; n=6, \*p<0.05, \*\*p<0.005 **(B)** Bacterial isolation (CFU/ml) from the basal  
663 medium beneath Caco2 cell monolayers (Transwell inserts) was measured to demonstrate  
664 bacterial translocation. CFU results are presented as a percentage change from Caco2 cells  
665 infected with the pathogen alone and without a molecule. Differences between nutrient deplete  
666 and nutrient replete conditions were tested using the Mann-Whitney U- test.; n=6, \*p<0.05,  
667 \*\*p<0.005

668

669 **Figure 4: The contra-pathogenic molecules have limited antimicrobial activity.** The  
670 enteropathogens A) EPEC, B) *C. rodentium* and C) *C. parvum* + *E. coli* K12 were incubated with  
671 each potential therapeutic molecule for 24h and cultured, to establish direct killing effects of the  
672 molecules on the bacteria and presented as CFU/ml [Log<sub>10</sub>]. The Mann-Whitney U-test was  
673 performed to compare each dataset with each pathogen(s) incubated in medium alone (control).  
674 n= 4; \*p<0.02.

675  
676 **Figure 5: Trefoil factor-3 (TFF3) and the MLCK inhibitor ML-7, ameliorate dysregulation of**  
677 **Claudin-4 expression after enteropathogen infection. (A) TFF3 and (C) ML-7** were added to  
678 polarised Caco2 cells prior to infection with Enteropathogenic *Escherichia coli* (EPEC), *Citrobacter*  
679 *rodentium* (*C. rodentium*) or *Cryptosporidium parvum* (*C. parvum*). After 24h, cells were lysed to  
680 measure protein levels of Claudin-4 (22kDa) by Western blotting, with recombinant claudin-4  
681 (49kDa) used as a positive control. Levels of GAPDH (36kDa) were also determined to confirm  
682 equal protein loading. B) TFF3 and D) ML7, represent densitometry of the Western blots \*p<0.05,  
683 \*\*P<0.005, \*\*\*\*P<0.0001. E) supernatants of infected monolayers co-stimulated with TFF3 or  
684 ML7 were tested for cell cytotoxicity using the LDH assay and compared with controls (infection  
685 + no molecule) using One-way ANOVA with the Kruskal-Wallis test. n=12; \*p<0.05, \*\*p<0.005,  
686 \*\*\*p<0.005.

687  
688 **Figure 6: TFF3 improves transepithelial resistance and reduces bacterial translocation in a dose-**  
689 **dependent manner, demonstrating the reciprocal relationship between them.** Transepithelial  
690 resistance (TER) and bacterial translocation (BT; CFU/ml) were measured in experiments with  
691 varying concentrations of TFF3 during Enteropathogenic *Escherichia coli* (EPEC; A and B) and  
692 *Citrobacter rodentium* (CR; C and D) infections for 24h. The experiments were conducted in the



693 presence (A and C) or absence (B and D) of fetal calf serum (FCS) to model nutrient-rich or  
694 nutrient-deplete conditions respectively. TFF3 concentrations were 0ng/ml (red), 5ng/ml (green),  
695 10ng/ml (blue) or 20ng/ml (brown). Spearman's rank correlation coefficient demonstrates  $\rho = -$   
696 0.80 ( $P < 0.0001$ ) for EPEC and  $\rho = -0.66$  ( $P < 0.0001$ ) for *C. rodentium*.

697

698 **Figure 7: Trefoil factor-3 (TFF3) and Relm- $\beta$  increase both pro- and anti-inflammatory cytokine**  
699 **production.** Polarised Caco2 cells were infected with: Enteropathogenic *Escherichia coli* (EPEC),  
700 *Citrobacter rodentium* (CR) and *Cryptosporidium parvum* (CP) and co-stimulated with TFF3 or  
701 Relm- $\beta$  for 24h in nutrient (FCS) deprived conditions. The supernatant from the cells were used  
702 to measure a range of cytokines and chemokines. n=6. One-way ANOVA with Tukey's post hoc  
703 was used for analysis. \* $p = 0.01$ , \*\* $p = 0.002$ , \*\*\* $p < 0.001$ . LLOD= Lowest level of detection.

704

## Supplementary Material

Therapy	Rationale	Reference
<b>Colostrum</b>	The first milk produced after birth, rich in bioactive molecules including growth factors. Colostral fractions or individual peptides present in colostrum have been shown to be beneficial in the treatment of gastrointestinal conditions such as inflammatory bowel disease and so could limit enteropathogen-induced epithelial barrier damage.	51
<b>Human epidermal growth factor (EGF)</b>	A component of human colostrum (200µg/L) and milk (30-50µg/L). EGF receptors are present on the basolateral membrane of enterocytes. Luminal EGF may gain access to the basolateral receptors in the immature neonatal gut due to increased permeability, or in any enteropathy characterised by increased permeability to macromolecules.	52,53
<b>Hydrocortisone</b>	The glucocorticoid hydrocortisone is present in breast milk. It has anti-inflammatory properties in both rat and human studies, has been shown to regulate the expression of genes involved in development of cell polarity, tight junction formation and interactions with extracellular matrices and so may assist in maintaining epithelial cell integrity after enteropathogen-induced epithelial barrier damage	54
<b>Leptin</b>	Leptin is an adipose-tissue derived hormone that functions in maintaining homeostatic control of adipose tissue mass and so its levels fall during starvation. Leptin exerts pleiotropic effects on the intestinal epithelium including nutrient absorption, epithelial growth, inflammation and injury. It contributes to the defence against intestinal amoebiasis. Therefore, leptin could protect the intestinal epithelium against pathogens directly or indirectly.	55,56
<b>Myosin-light chain kinase (MLCK) inhibitor ML-7</b>	MLCK induces contraction of the peri-junctional actomyosin ring through myosin II regulatory light chain phosphorylation which regulates tight junction permeability. EPEC infection has been shown to induce phosphorylation of the 20 kilodalton myosin light chain (MLC) in the intestinal epithelium, which causes changes in	32,57

	<p>intestinal barrier function, contributing to the diarrhoea associated with the infection. However, Inhibition of the MLC20 phosphorylation prevented EPEC induced changes to the epithelium. Therefore, ML-7 could be a class of targeted therapeutic agents that restore barrier function in intestinal disease states.</p>	
<p><b>Resistin-like molecule <math>\beta</math> (RELM<math>\beta</math>)</b></p>	<p>Resistin-like molecule (RELM) beta is secreted by goblets cells in the intestine and are essential for normal spontaneous expulsion and IL-4-induced expulsion of parasitic helminths, which live in the intestinal lumen but not in IECs. It is strongly induced within goblet cells during <i>C. rodentium</i> infection. It appears also to act as a CD4+T cell chemoattractant in the colon, in part via the induction of increased IEC proliferation. Therefore, the molecule could induce an immunomodulatory response towards the enteropathogens, maintaining epithelial barrier integrity.</p>	46,47,58
<p><b>Trefoil Factor 3 (TFF3)</b></p>	<p>Trefoil peptides are also present in breast milk and in goblet cells along the intestinal tract. Goblet cells are depleted during infection with <i>C. rodentium</i> and EPEC which results in dramatically reduced TFF3 levels. TFF3 has been shown to down-regulate cytokines (IL-8 &amp; IL-6) and promote the expression of the antimicrobial peptides, beta defensin (hBD2&amp;4) in enterocytes. They have been shown to be activated in intestinal cells through protease activated receptors 2 (PAR-2) and so increased TFF3 levels during enteropathogen infections may improve the intestinal epithelium.</p>	59
<p><b>Vitamin A (Retinoic acid)</b></p>	<p>Vitamin A and its analogs are essential for normal development. Vitamin A deficiency (VAD) is associated with decreased immune response and increased bacterial translocation and mortality e.g. the retinoic acid (RA) receptor <math>\alpha</math> (RAR<math>\alpha</math>) signaling in intestinal epithelial cells (IECs) deregulates epithelial lineage specification, leading to reduced luminal bacterial detection (for <i>C. rodentium</i>). Therefore, increases in Vitamin A exposure could reduce bacterial translocation</p>	60
<p><b>Zinc</b></p>	<p>Zinc is an essential nutrient. Its deficiency is known to result in epithelial barrier leak in the intestines and alter TJ composition. Zinc oxide (ZnO) has a protective</p>	61,62

	effect in pigs suffering from enterotoxigenic <i>Escherichia coli</i> (ETEC) infections. This observation may be extended to enteropathogens-induced epithelial damage.	
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706 **Supplementary Table 1: Molecules tested and rationale for selection**

707

708 *Assessment of Bacterial Adherence*

709 Cells grown on coverslips were infected with EPEC, *C. rodentium* or *C. parvum* + *E. coli* K12 for 0,  
710 15, 30, 60 and 120min. Medium was then removed, cells washed with PBS and fixed in methanol  
711 (VWR, Leicestershire, UK) for 15min followed by overnight Giemsa (VWR, Leicestershire, UK)  
712 staining. Numbers of cells and bacteria were counted at x200 magnification in 20 randomly  
713 selected fields under a Leica DM500 upright Epi-fluorescent Dual Camera.

714

715 *Bacterial Invasion Assay*

716 Cells were infected on the apical side with EPEC, *C. rodentium* or *C. parvum* + *E. coli* K12, for 0,  
717 15, 30, 60 or 120min. Cells were washed in culture media and incubated with gentamicin  
718 (200ug/ml) for 60min to kill extracellular bacteria. Cells were washed three times and lysed in  
719 10mM Tris-HCL pH7.5, 1mM EDTA, and 1% Triton-X (Sigma-Aldrich Company Ltd, Dorset, UK)  
720 prior to culture.

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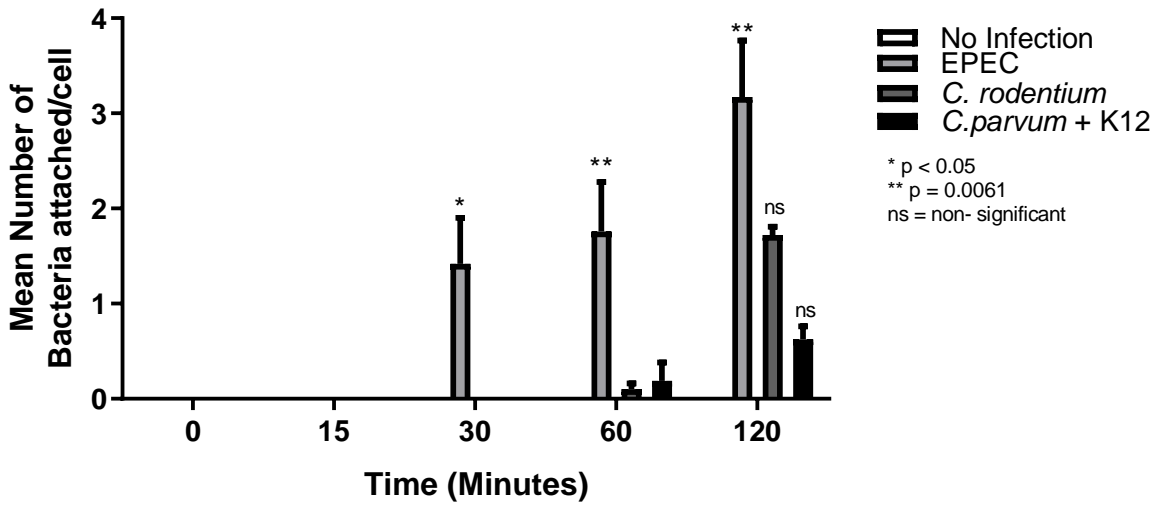
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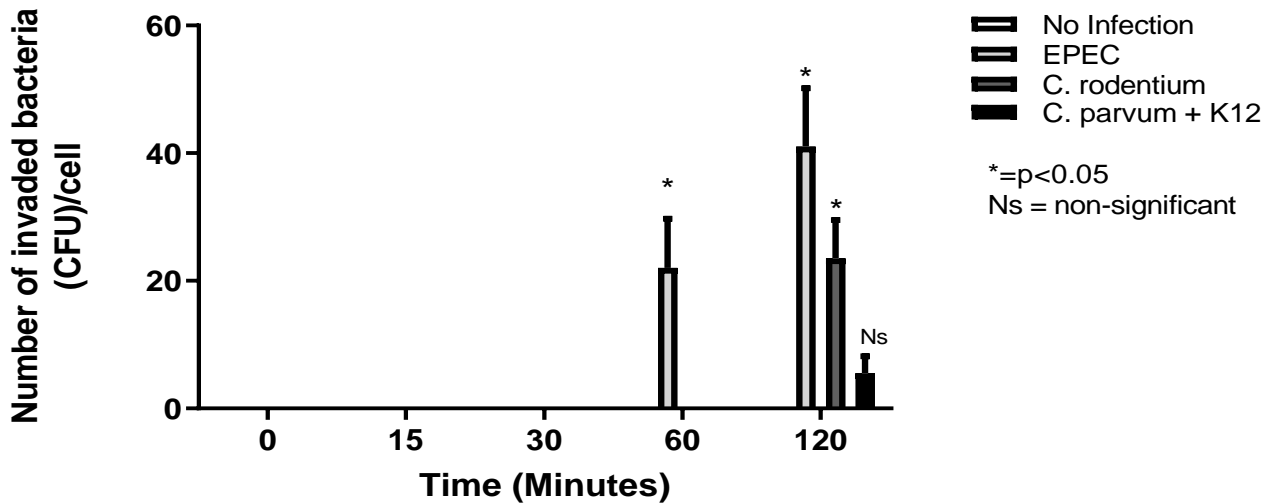
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728 a)



729

730 b)



731

732 **Supplementary Figure 1: EPEC (rabbit), *C. rodentium* (murine) and *C. parvum* + *E. coli* K12**

733 **(human) attach and infect Caco2 cells.** Enteropathogenic *Escherichia coli* (EPEC), *Citrobacter*

734 *rodentium* (*C. rodentium*) or *Cryptosporidium parvum* (*C. parvum*) together with *Escherichia coli*

735 K12 (K12) were incubated with Caco2 cell monolayers for 0, 15, 30, 60 and 120min. Cells were

736 washed and underwent Giemsa staining **a)** The number of bacteria attached per cell was then

737 enumerated in 20 fields for the adhesion assay **b)** infected cells were treated with gentamicin for

738 1h to kill only the extracellular pathogens. The Caco2 cells counted then lysed and cultured on

739 Luria-Bertani (LB) agar plates to enumerate the number of viable intracellular bacteria per Caco2  
740 cell over the time course. Statistical testing using One-way ANOVA with the Kruskal-Wallis test  
741 was used to compare the infections with the no-infection control at each time point; \*P<0.05,  
742 \*\*p=0.006.