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

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Abstract

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

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

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

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

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

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

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

Introduction

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

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

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

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

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

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

Materials and Methods

Cell culture

The human Caco-2 enterocyte cell line (passage 6-40) was obtained from the American Type Culture Collection (ATCC: HTB-37; Middlesex, UK) and grown as monolayers (VWR, Leicestershire, UK) at 37°C in humidified 5% CO_2 in complete medium consisting of Dulbecco's Modified Essential Medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 4 mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 1% non-essential amino acids (Invitrogen Life Technologies, Paisley, UK). For membrane integrity and translocation investigations, trypsinised cells were seeded at a density of 1x 10^6/ml into 12 well plates holding polyethylene terephthalate (3.0µm) cell culture inserts (Transwell inserts, Millipore, Hertfordshire, UK), for immunofluorescence experiments at 5x 10^5/ml into 24-well culture plates (VWR International, Leicestershire, UK) with 13mm glass coverslips, or at 2 x 10^6/ml into 6 well plates for Western blot experiments. Cells were grown until polarization (15-21 days) for translocation studies or until 75% confluent for staining experiments (4-5 days; limiting cell damage during staining

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

Bacterial strains and Parasite

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

In vitro bacterial and parasite infections

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

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

Quantification of transepithelial resistance (TER) and bacterial translocation

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

Fixation of cell monolayers and Immunofluorescence (IF)

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

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

Lactate Dehydrogenase (LDH) Assay

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

Pharmacological therapies

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

Antimicrobial Assay

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

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

Western blotting

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

Cytokine and Chemokine expression assay (Takeda)

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

Data analysis

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

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

248 **Results**

249

250 *Selected pathogens induce barrier dysfunction in Caco2 cell*

251 In preliminary experiments, an appropriate TER (Ω/cm^2) was selected for Caco2 cells to confirm
252 polarity, which correlated with the expression of the bush 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 [29], and regardless of the isolate's original
265 host, colonisation is similar and so not host dependent [30]. 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

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

Barrier dysfunction was associated with reduced claudin-4

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

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

Several molecules were screened for their ability to limit the enteropathogen-induced damage caused to the epithelial barrier integrity (Table 1 and Supplementary table 1). Seven of these agents: zinc, colostrum, human epidermal growth factor (hEGF), trefoil factor-3 (TFF3), resistin-like molecule-beta (RELM-β), 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-β, 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

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

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

TFF3 and Relm-β increase immune-regulation in enterocytes

The disruption of intestinal barrier function and the alteration of the TJ protein claudin-4 following infection, led us to additionally examine the effect of two goblet cell released contra-pathogenic molecules, TFF3 and Relm-β, in regulating the inflammatory response. The effects of TTF3 or Relm-β on cytokine release by enteropathogens in polarised Caco2 cells over 24h was determined by measuring cytokines in supernatants by ELISA. TFF3 and Relm-β modestly increased the induction of interleukin 10 (IL-10) and tumour necrosis factor alpha (TNF-α) by EPEC (Figure 7). Relm-β also increased induction of IL-2 and IL-6 by all three pathogens and induced these two cytokines in the absence of pathogens.

Discussion

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

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

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

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

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

392 resistance to infection[43]. 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[44]. 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 [45].
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-β is
407 exclusively found in goblet cells of the intestines and may play a role during colonisation and
408 infection of the gastrointestinal tract[46]. It plays a role in the production of Th2 cytokines during
409 nematode infections in mice and is essential in helminth expulsion[47]. Relm-β also has been shown
410 to be induced during *C. rodentium* infection, in which it is involved in recruiting CD4+ T-cells[48]. TFF3
411 is also a goblet cell product with well-established protective effects on the small intestinal
412 mucosa[49] working at the cell membrane. ML7 acts on the adenosine triphosphate- binding site of
413 the active centre of MLCK causing inhibition[42] 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-

dependent manner (Figure 6).

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

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

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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

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<0.000.1, *** 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

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

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

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_{10}]. 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 ρ = -
696 0.80 (P<0.0001) for EPEC and ρ = -0.66 (P<0.0001) for *C. rodentium.*
697
698 **Figure 7: Trefoil factor-3 (TFF3) and Relm-β 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-β 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

705 **Supplementary Material**

Therapy	Rationale	Reference
Colostrum	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
Human epidermal growth factor (EGF)	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
Hydrocortisone	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
Leptin	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
Myosin-light chain kinase (MLCK) inhibitor ML-7	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

	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.	
Resistin-like molecule β (RELMβ)	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 *C. rodentium* 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.	46,47,58
Trefoil Factor 3 (TFF3)	Trefoil peptides are also present in breast milk and in goblet cells along the intestinal tract. Goblet cells are depleted during infection with *C. rodentium* and EPEC which results in dramatically reduced TFF3 levels. TFF3 has been shown to down-regulate cytokines (IL-8 & IL-6) and promote the expression of the antimicrobial peptides, beta defensin (hBD2&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.	59
Vitamin A (Retinoic acid)	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 α (RARα) signaling in intestinal epithelial cells (IECs) deregulates epithelial lineage specification, leading to reduced luminal bacterial detection (for *C. rodentium*). Therefore, increases in Vitamin A exposure could reduce bacterial translocation	60
Zinc	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	61,62

	effect in pigs suffering from enterotoxigenic *Escherichia coli* (ETEC) infections. This observation may be extended to enteropathogens-induced epithelial damage.	

Supplementary Table 1: Molecules tested and rationale for selection

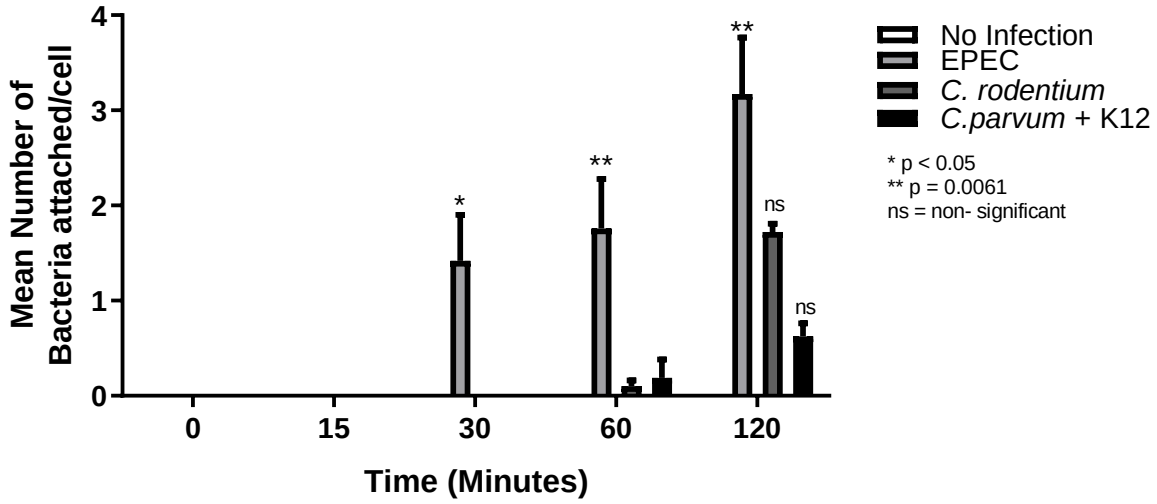
Assessment of Bacterial Adherence

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

Bacterial Invasion Assay

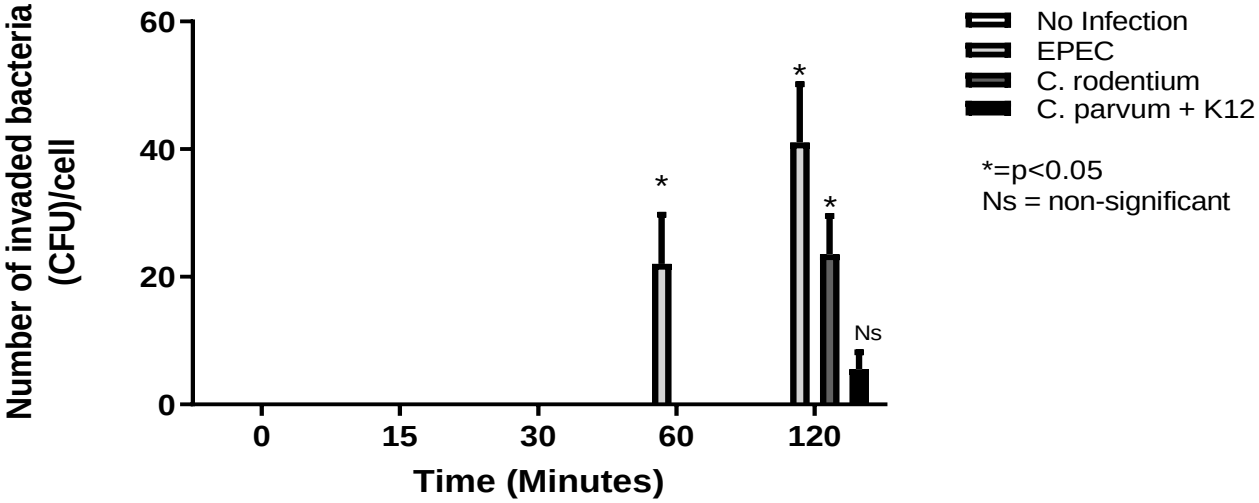
Cells were infected on the apical side with EPEC, *C. rodentium* or *C. parvum* + *E. coli* K12, for 0, 15, 30, 60 or 120min. Cells were washed in culture media and incubated with gentamicin (200ug/ml) for 60min to kill extracellular bacteria. Cells were washed three times and lysed in 10mM Tris-HCL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

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.