Investigating the influence of commensal *Limosilactobacillus reuteri* and *Ruminococcus gnavus* on infection with enteropathogenic *Escherichia coli* in a microaerobic human intestinal cell model.

Conor McGrath, MBiolSci (Hons)

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Abstract

The gut microbiota has an important role in maintaining intestinal health and protecting against enteric infections (colonisation resistance). Nevertheless, most of these interactions haven't been explored, largely due to a lack of experimental model systems that can culture oxygen-sensitive commensals alongside intestinal cells. In this project, we have established a novel *in vitro* model system of the human intestinal epithelium (Vertical Diffusion Chamber, VDC) which supports growth of strictly anaerobic bacteria. We have applied this system to investigate the interactions of gut commensals *Ruminococcus gnavus* and *Limosilactobacillus reuteri* with a functioning mucus-producing epithelium, established using T84 and goblet-like LS174T cell lines, and their effect on infection with enteric pathogen enteropathogenic *E. coli* (EPEC).

Previously published work identified a culture medium that supports commensal and EPEC growth whilst maintaining epithelial integrity and barrier function. This was achieved by establishing bacterial growth curves in different media and assessing epithelial barrier function by transepithelial electrical resistance and immunofluorescence staining (IFS) of tight-junction protein occludin. Further IFS demonstrated that introduction of LS174T cells to the epithelium caused mucin secretion and facilitates colonisation by commensals. Co-culture of EPEC with commensals reduces numbers of viable and adherent EPEC, as well secretion of pro-inflammatory cytokine interleukin-8 by the infected epithelia. For *R. gnavus*, reduced EPEC viability and adherence is only observed when LS174T are present.

Here, we build on existing work showing potential colonisation resistance activities which would not be possible to study using traditional cell culture models. As the resident bacterial of the human gut are predominantly oxygen-sensitive, this system can be used to study a plethora of host-pathogen-commensal interactions and aid development of probiotic therapies.

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List of abbreviations

AAD	Antibiotic-associated diarrhoea
A/E	Attaching and effacing
AIEC	Adherent invasive *E. coli*
AM	Anti-microbial
AP-1	Activator protein 1
APS	Ammonium persulfate
ARP 2/3	Actin related protein 2/3
ATP	Adenosine triphosphate
BFP	Bundle-forming pilus
BHI	Brain heart infusion
BHI-YH	BHI-yeast extract, haemin
BSA	Bovine serum albumin
CBM	Carbohydrate binding module
CDC42	Cell division control protein 42
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
CFS	Cell-free supernatant
CFU	Colony forming units
Cif	Cycle-inhibiting factor
CK	Cysteine knot
CpG	Cytosine-guanidine dinucleotide
Cps	Capsular polysaccharide
Cx43	Connexin 43
D3	von Willebrand domain 3
DAEC	Diffusely adherent *E. coli*
DAPI	4'-6-diamidino-2-phenylindole

DC	Dendritic cell
DEC	Diarrheagenic *E. coli*
dH_2O	Distilled H_2O
Dithiothreitol	DTT
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DRA	Downregulated in adenoma
DSS	Dextran sulphate sodium
EAEC	Enteroaggregative *E. coli*
EAF	EPEC adherence factor
ECP	*E. coli* common pilus
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *E. coli*
ERK	Extracellular signal related kinase
Esp	*E. coli* secreted protein
ETEC	Enterotoxigenic *E. coli*
EV	Extracellular vesicle
ExPEC	Extraintestinal *E. coli*
F-12	Ham's F-12 nutrient
FAS	Fluorescent actin staining
FITC	Fluorescein isothiocyanate
FMT	Faecal matter transplant
GEMS	Global Enteric Multicenter Study
GH	Glycoside hydrolase
GIT	Gastrointestinal tract

GPCR	G-protein coupled receptor
GuMi	Gut Microbiome physiomimetic platform
hIO	Human intestinal organoid
HM	Host membrane
HMI	Host-Microbiota
HoxBan	Human oxygen-Bacteria anaerobic
HuMiX	Human-Microbe Crosstalk
ID	Infectious diarrhoea
IEC	Intestinal epithelial cells
IFN	Interferon
IFS	Immunofluorescence staining
IL	Interleukin
IM	Inner membrane
IVOC	*In vitro* organ culture
LB	Lysogeny broth
LEE	Locus of enterocyte effacement
LMIC	Low- and middle-income country
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
M cell	Microfold cells
MAMP	Microbe associated molecular pattern
Map	Mitochondrial associated protein
MAPK	Mitogen-associated protein kinase
MDR	Multi-drug resistance
MOI	Multiplicity of infection
MRS	de Man, Rogosa, Sharpe

MUB	Mucus binding protein?
MUC	Mucin
NAC	N-acetyl cysteine
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHE3	N^+/H^+ exchanger 3
NI	Non-infected
Nle	Non-LEE effector
NLR	NOD-like receptor
N-WASP	Neural Wiskott- Aldrich syndrome protein
ONC	Overnight culture
ORT	Oral rehydration therapy
P/S	Penicillin/streptomycin
PBS	Phosphate buffered saline
PG	Peptidoglycan
PGM	Porcine gastric mucin
pIVOC	Polarised IVOC
pO_2	Oxygen pressure
PP	Peyer's patch
PRR	Pattern recognition receptor
PSA	Polysaccharide A
PTS	Proline, Threone, Serine
RCT	Randomised control trial
REPEC	Rabbit EPEC
RGB	Running gel buffer
rRNA	Ribosomal RNA
RSB	Reducing sample buffer

RT	Room temperature
SCFA	Short chain fatty acid
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS Polyacrylamide gel electrophoresis
SERT	Serotonin transporter
SGB	Stacking gel buffer
SGLT1	Sodium glucose transporter 1
SHIME	Simulator of the Human Intestinal Microbial Ecosystem
SIgA	Secretory IgA
Slp	Surface layer protein
SRRP	Serine-rich repeat protein
STEC	Shiga toxin-producing *E. coli*
T3SS	Type III secretion system
T84/LS	T84 + LS174T (9:1)
TA	Teichoic acid
TBS	Tris-buffered saline
TEER	Transepithelial electrical resistance
T_h	T helper
Tir	Translocated intimin receptor
TJ	Tight junction
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	T regulatory
VDC	Vertical Diffusion Chamber
WHO	World Health Organisation
WT	Wild type

YCFA	Yeast casitone fatty acids
ZO	Zonular occluden

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Chapter 1: Introduction

1.1. Enteropathogenic *Escherichia coli*

1.1.1 History and diversity of *Escherichia coli*

Escherichia coli is a Gram-negative, rod-shaped bacterium discovered in 1885 by the German bacteriologist Theodor Escherich (article re-printed in English in 1988) (Escherich 1988). Originally isolated from the human colon and part of the intestinal microbiota, *E. coli* has been detected in environments outside the host. This allows transition, often through sewage systems and contaminated food products, of strains between and within mammalian species. Since its discovery, a considerable amount of knowledge has been accumulated on *E. coli*, largely due to its use as the single most common model organism for scientific experiments involving bacteria. From two laboratory strains, K-12 and B strain, an array of sub-strains has been cultivated, each with distinct features for useful applications in research (Schneider *et al.* 2002). The widescale use of *E. coli* as a model organism for discovering fundamental processes in molecular biology is a result of multiple factors, including rapid replication time and ability to grow in a variety of conditions (Cronan 2014). Not only is *E. coli* a facultative anaerobe, capable of aerobic respiration as well as fermentation, but it can also grow in minimal medium. Laboratory strains aside, wild-type *E. coli* show an even greater level of diversity ranging from fatal human pathogens, such as the much-maligned O157:H7 strain, to health-promoting members of the microbiota, such as the Nissle 1917 strain (Sonnenborn and Schulze 2009; Wells *et al.* 1983).

Historically, *E. coli* strains were classified by serotyping, which categorises bacteria based on the specificity of antibodies for their surface antigens. Effective classification requires serotyping based on three antigens: lipopolysaccharide (O); capsule (K) and flagella (H). Using this method of classification has yielded nearly 200 *E. coli* serotypes, demonstrating the vast diversity of the species (Fratamico *et al.* 2016). Serotyping has now been widely replaced by multi-locus sequence typing, which categorises strains based on the sequence similarity between fragments of several conserved housekeeping genes (Maiden *et al.* 1998). Whole genome sequencing of 61 strains identified a pan-genome (total genes identified for all strains) of 16,000 genes but a core genome (conserved in all strains) of only 1,000 genes, accounting for ~20% of the average genome size for a given strain (Lukjancenko, Wassenaar, and Ussery 2010). Further analysis narrowed down the variable gene content to various 'islands' within the genome, and hypothesised these regions were subject to transfer of DNA through horizontal gene transfer (HGT) and phage insertion. With regards to HGT, *E. coli*'s apparent promiscuity is likely to be the driving factor for its adaptation to a range of environments and evolution of strains with a diverse set of phenotypes.

1.1.2 Human pathogenic *E. coli*

E. coli can cause disease in all mammals and at various sites in the body. Consequently, *E. coli* features in a range of diseases including diarrhoea, cystitis, pneumonia, neonatal meningitis and life-threatening sepsis (Johnson and Russo 2002; Kaper, Nataro, and Mobley 2004). Human pathogenic *E. coli* are a diverse group and have been categorised into pathovars based on their associated disease symptoms and mechanism of pathogenesis. To date, 9 different *E. coli* pathovars have been identified, seven of which are diarrhoeagenic (DEC) and two extraintestinal *E. coli* (ExPEC) (Croxen and Finlay 2010). Enteric pathovars include adherent invasive *E. coli* (AIEC); diffusely adherent *E. coli* (DAEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC). Classification criteria are based on pathogenic phenotypes, such as characteristic adherence patterns or presence of specific toxins. EPEC is classified by its ability to colonise the small intestine (SI) and form distinctive attaching and effacing (A/E) lesions but lacks the Shiga toxin which is found in A/E lesion forming STEC. Adherence assays with Hep-2 cells and fluorescent actin staining (FAS) tests to visualise A/E lesions have been developed to identify strains belonging to the EPEC pathovar (Cravioto *et al.* 1979; Knutton *et al.* 1989). A/E lesions are the result of intimate attachment of the bacteria to the epithelium and subsequent effacement of the microvilli (Shaw *et al.* 2005). Nevertheless, other pathovars/organisms can also form A/E lesions (STEC, Rabbit Diarrhoeagenic *E. coli*, *Citrobacter rodentium* and *Escherichia albertii*), limiting the selectivity of FAS testing for diagnosing EPEC. This is not an isolated case. Cross-over of characteristics, originally thought to be unique to a given pathovar, have previously raised issues with identifying aetiological agent of diarrhoea caused by *E. coli*. The most famous example is a particularly virulent *E. coli* O104:H4 strain which caused 900 hospitalisations and 54 deaths in Germany in 2011 and exhibited genotypic characteristics of EAEC and STEC pathotypes (Bielaszewska *et al.* 2011; Frank *et al.* 2011; Scheutz *et al.* 2011).

Similar issues have impacted classification between strains belonging to EPEC. In 1995, during the Second International Symposium on EPEC in São Paulo, there was a consensus to split EPEC into 2 distinct groups based on the presence of the EPEC adherence factor plasmid (EAF) (Kaper 1996). Typical EPEC strains harboured this plasmid and demonstrated a localised adherence pattern on Hep-2 epithelial cells, typified by the formation of microcolonies held together by bundle-forming pili encoded on the EAF plasmid (Scaletsky, Silva, and Trabulsi 1984). Atypical EPEC strains, however, do not possess this plasmid and exhibit more diffuse adherence patterns on Hep-2 cells (Scaletsky *et al.* 2001; Beutin *et al.* 2003). A systematic review comparing the epidemiology of typical and atypical EPEC identified a much greater contribution of the latter to infantile diarrhoea (Ochoa *et al.* 2008).

Furthermore, studies have shown that patients infected with atypical EPEC exhibit longer duration of diarrhoea compared with typical EPEC (Afset *et al.* 2004; Nguyen *et al.* 2006). The exact causes of enhanced prevalence of atypical EPEC are not known; however there is mounting evidence to suggest a greater frequency of strains harbouring genes belonging to other DEC pathovars (Hernandes *et al.* 2009). These include haemolysin from STEC and serine-protease autotransporters, including the *pic* gene characterised from EAEC and *Shigella flexneri* (Abreu *et al.* 2016; Gomes *et al.* 2004; Schwidder, Heinisch, and Schmidt 2019). The *pic* gene from atypical EPEC strain BA589 was essential to elicit colonisation of both mice and human intestinal biopsies, which may correspond to its mucolytic activity (Abreu *et al.* 2016). In line with these observations, further investigations into atypical EPEC highlighted their heterogeneity with regards to serotyping and genotyping, with some strains demonstrating greater genomic homology with other *E. coli* pathovars (Bando *et al.* 2009). Despite differences at the genetic, phenotypic and clinical level, atypical EPEC still fall under the EPEC pathovar as they form A/E lesions and lack genes encoding Shiga toxin. This highlights a fundamental issue with classifying a diverse and genetically dynamic group such as pathogenic *E. coli* which necessitates frequent amendments to reflect novel discoveries. In the case of EPEC, much of our understanding on pathogenicity, clinical significance and epidemiology has been obtained using typical EPEC.

1.1.3 EPEC Pathogenesis

1.1.3.1 Initial Adherence

EPEC pathogenesis has been well defined and is highly reliant on intimate adherence to the epithelium of the small intestine and secretion of effector proteins into the host intestinal epithelial cells (IECs). For typical EPEC strains, initial adherence is mediated by bundle-forming pili (BFP) encoded by the EAF plasmid (Donnenberg *et al.* 1992). The type IV pilus interacts with the membrane lipids phosphatidylethanolamine and glycoprotein N-acetyllactosamine, which are expressed on host cell surfaces (Hyland *et al.* 2008; Khursigara *et al.* 2001). In addition to host cell interactions, BFP filaments can bind to other surrounding EPEC bacteria, tethering them together to form a tight collection of bacteria known as a microcolony (Giron, Ho, and Schoolnik 1991). Microcolony formation is responsible for the localised adherence phenotype associated with typical EPEC infection (Scaletsky, Silva, and Trabulsi 1984). In addition to BFP, the *E. coli* common pilus (ECP) acts as an accessory mediator of adherence to the intestinal epithelium. Other surface proteins, such as EspA filaments and flagella have also been implicated in initial adherence of EPEC (Cleary *et al.* 2004; Giron *et al.*

2002). In these studies, adherence is affected differently depending on the combination of mutants used, suggesting BFP, ECP and EspA may act in concert to mediate effective initial adherence.

1.1.3.2 Structure and function of the T3SS

In addition to *bfp*, the EAF plasmid also encodes the perABC operon consisting of a collection of transcriptional regulators. These regulators promote expression of both *bfp* and the locus of enterocyte effacement (LEE) (Tobe *et al.* 1999). The LEE encodes the apparatus for a type III secretion system, which EPEC utilises to inject effector proteins into the host cell (see Fig 1.1). Whilst some of these effectors are encoded alongside the T3SS on a pathogenicity island known as the locus of enterocyte effacement (LEE), the majority, known as non-LEE effectors (Nle), are present at different loci (McDaniel *et al.* 1995; Jarvis *et al.* 1995).

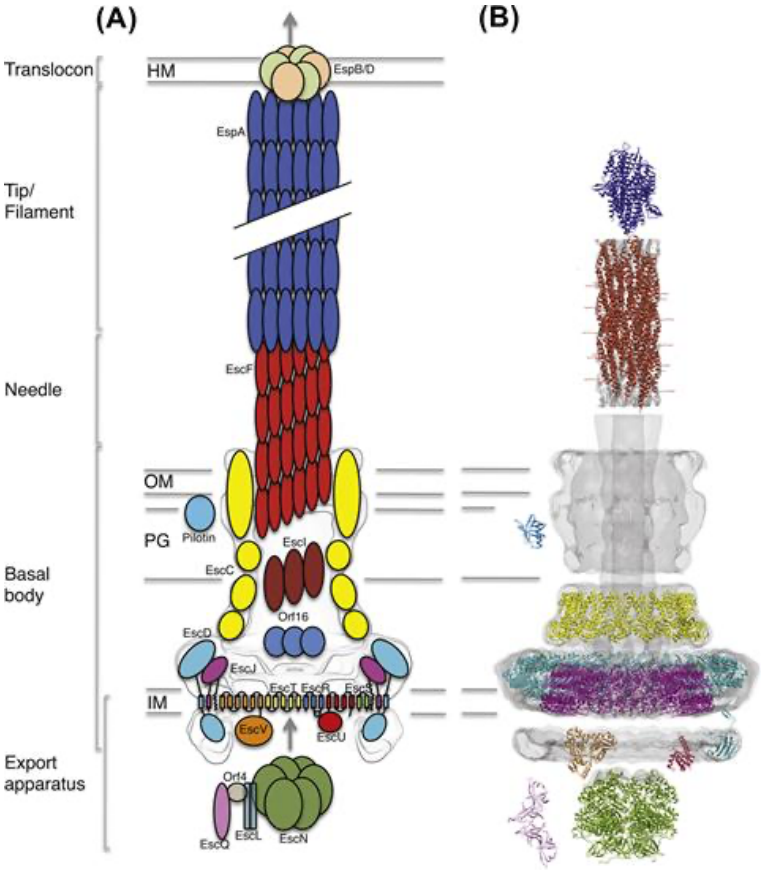


Figure 1.1 Schematic representation of the T3SS from EPEC. (A) Schematic diagram constructed using structural data collected from other species with homologous systems (B). The T3SS consists of cytoplasmic export apparatus, a basal body spanning the periplasm, EscF polymeric needle, an extended EspA tip (crucial to access the host membrane) and translocon pore. HM = host membrane, OM = outer membrane, PG = peptidoglycan and IM = inner membrane. Figure sourced from (Worrall, Bergeron, and Strynadka 2013).

Much of the work identifying the structure of the T3SS and function of each domain has been conducted in *Salmonella*, although the core architectural subunits were analysed across several species (Gaytán *et al.* 2016). There are 4 main domains for the T3SS, each with distinct function produced by the concerted activity of numerous proteins (see **Fig 1.1**). The basal body spans the inner membrane, periplasm and outer membrane of EPEC. EscD and EscJ form a ring across the inner membrane, and EscC forms the neck of the basal body, which traverses the periplasm and outer membrane (Marlovits *et al.* 2004). Within EscC is an inner rod known as EscI, which is believed to facilitate effector transport across the bacterial surface (Marlovits *et al.* 2004; Zhong *et al.* 2012). The next fundamental T3SS domain is the export apparatus. Five highly conserved inner membrane proteins (EscRSTUV) make up the inner membrane machinery, which is crucial for efficient assembly of the basal body and activity of the T3SS (Wagner *et al.* 2010). Although the activity of most of these components is yet to be elucidated, EscU and EscV have cytoplasmic domains that may interact with proteins and are thought to directly regulate effector translocation (Abrusci *et al.* 2013; Zarivach *et al.* 2008). The cytoplasmic ATPase EscN, which unfolds proteins for export, completes the export apparatus domain. EscN mutants are unable to translocate effectors, indicating the importance of protein unfolding for export (Akeda and Galan 2005; Cepeda-Molero *et al.* 2017). The third domain, the needle, protrudes from the basal body and can be tens of nanometres in length. The protein EscF forms the needle, creating a pore which was hypothesised to function as the translocation tube for effector proteins (Loquet *et al.* 2012). Although the hypothesis that this needle structure functioned as the effector translocation tube was proposed over 20 years ago, direct evidence of unfolded proteins found within the needle was only confirmed recently (Dohlich *et al.* 2014; Kubori *et al.* 1998; Radics, Konigsmaier, and Marlovits 2014). The needle tip and translocation pore form the fourth and final domain of the T3SS. In most species, the needle tip is a short pentameric cap that interacts with the host cytoplasm to form the conduit between bacteria and host. In EPEC, however, the needle tip is formed by long polymers of EspA, which contain a pore of similar size to the EscF needle (Daniell *et al.* 2003; Knutton *et al.* 1998a). The extension of needle by the EspA filament has been suggested to allow penetration of the glycocalyx, which implies that direct enterocyte interaction is not a prerequisite for effector translocation; however, evidence supporting this hypothesis is lacking (Mueller, Broz, and Cornelis 2008). EspB and D form the translocon pore in the host membrane, resulting in a complete T3SS capable of translocating effectors from the bacterial to the host cytoplasm.

A defined characteristic of the T3SS is that effector proteins are secreted in a defined hierarchy and sequence. After translation, proteins are targeted towards the T3SS by a terminal amino acid sequence that is recognised by proximal chaperones (Stebbins and Galan 2001). The effector-chaperone forms

a complex with the ATPase EscN, which uses ATP to drive unfolding of the bound protein which can then be fed through the translocon needle (Akeda and Galan 2005). The specificity of the inner membrane machinery component EscU is thought to drive the transition from structural to effector proteins. In addition to the EscU molecular switch, there may be a role for gatekeeper proteins SepL and SepD in ordering protein secretion (Deng *et al.* 2004; O'Connell *et al.* 2004). Further work is required to characterise these proteins and their role in organising protein secretion by T3SS in EPEC.

1.1.3.3 Intimate adherence and A/E lesion formation

There is evidence that the translocated intimin receptor (Tir) is the first non-structural effector protein secreted into the host following T3SS assembly. Translocation dynamics, determined using beta-lactamase reporter fusion proteins for all LEE effector proteins, indicated Tir accumulation in HeLa cells ~30 mins post-infection, 30 minutes earlier than any other effector protein (Mills *et al.* 2013). Nevertheless, use of a fusion protein may interfere with protein re-folding in the host cytoplasm following translocation, limiting the application of these results to native proteins. Following translocation, Tir inserts itself into the host membrane via a transmembrane loop rich in hydrophobic residues, with intracellular domains exposed to the host cytoplasm (Kenny *et al.* 1997; Race, Lakey, and Banfield 2006). Subsequent binding of the EPEC outer membrane protein intimin to Tir mediates intimate adherence, allowing close proximity to the epithelial surface (~10 nm) (Nougayrède, Fernandes, and Donnenberg 2003; Moon *et al.* 1983). Furthermore, interaction with intimin results in bundling of Tir at the host plasma membrane. Clustering of Tir intracellular domains triggers a chain of events in the cell by hijacking host proteins: host kinases phosphorylate bundled Tir; phosphorylated Tir recruits Nck to the site of attachment; Nck activates neural Wiskott-Aldrich syndrome protein (N-WASP) and the actin-related protein 2/3 (ARP2/3) complex which ultimately leads to remodelling of host actin and pedestal formation at the site of attachment (see **Fig. 1.2**) (Bommarius *et al.* 2007; Gruenheid *et al.* 2001; Kalman *et al.* 1999; Phillips, Hayward, and Koronakis 2004; Swimm *et al.* 2004). The production of actin pedestals underneath microcolonies of EPEC, combined with effacement of the host microvilli, result in the formation of an A/E lesion. These pedestals were first observed by electron microscopy of pig and rabbit intestines infected with EPEC, indicating loss of microvilli and “cup-like” projections beneath the site of attachment (Moon *et al.* 1983). Following this, a new technique using fluorescent actin staining (FAS) of EPEC-infected IECs showed that these projections were high in filamentous actin, providing a quicker and simpler method of visualising A/E lesions (Knutton *et al.* 1989). Interestingly, transfer of the LEE to non-pathogenic *E. coli* granted the ability to form A/E lesions on human embryonic IECs, indicating that intimate

adherence is exclusively dependent on the T3SS (Cepeda-Molero *et al.* 2017; McDaniel and Kaper 1997). Although originally thought to be a pre-requisite to A/E lesion formation, actin polymerisation by Nck is not always necessary. EPEC O125:H6 strains were unable to polymerise actin in HeLa cells, however scanning electron microscopy confirmed they could form A/E lesions on human biopsy tissue (Bai et al 2020). This suggests that alternative pathways for A/E lesion exist. Indeed, the protein T3SS effector protein TccP can interact directly with N-WASP at a site distinct from Nck binding, resulting in activation and recruitment of ARP2/3 (Garmendia *et al.* 2005; Whale *et al.* 2006).

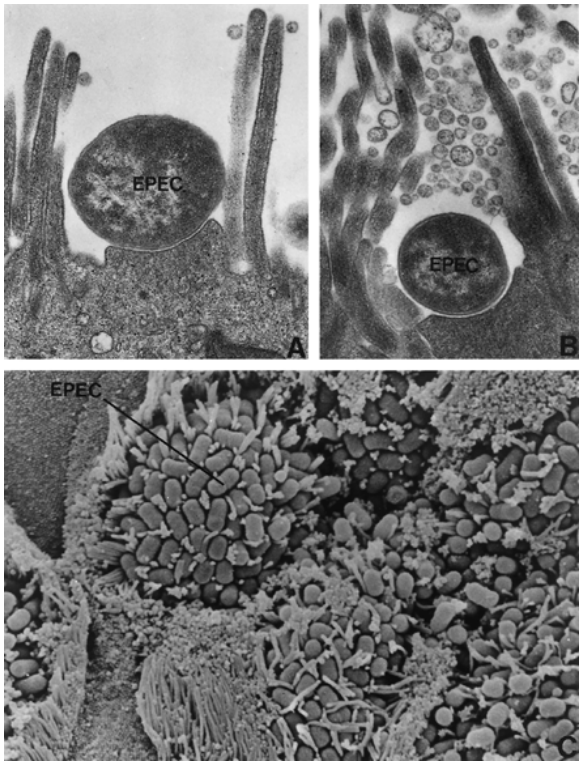


Figure 1.2 A/E lesion formation by EPEC on intestinal epithelial cells. Electron micrographs illustrating actin pedestal formation beneath adherent EPEC (**A**), localised destruction of the microvilli (**B**) and aggregation into microcolonies to form the characteristic A/E lesion (**C**). Figure sourced from (Frankel *et al.* 1998).

Although the canonical molecular pathway of A/E lesion formation has been well defined in cultured cell lines, the sequence of events is different in intestinal tissue. Using *in vitro* organ culture (IVOC) of human intestinal biopsies, Tir phosphorylation has been shown to be dispensable for formation of actin pedestals (Schuller *et al.* 2007). In addition, recent studies have demonstrated that Tir and intimin are sufficient for EPEC A/E lesion formation in cell lines, whereas additional Nle proteins are required *ex vivo* (Cepeda-Molero *et al.* 2017). This work demonstrates the importance of using model systems that more accurately represent the gut environment when investigating EPEC pathogenesis.

1.1.3.3 Stimulation of acute diarrhoea

Acute diarrhoea is the most defining symptom of EPEC infection. To this end, several EPEC effector proteins have been associated with increased water levels in the gut lumen (see **Fig. 1.3**). The mechanisms by which effectors promote development of diarrhoea include modulation of ion transport across the epithelium, interference with IEC membrane proteins directly involved in water absorption (aquaporins) and disruption of tight junctions (TJs) between IECs of the epithelium. These mechanisms cause secretory diarrhoea, which is the result of decreased net water absorption by the epithelium, either through elevated water secretion and/or inefficient re-absorption (Sweetser 2012). This is distinct from osmotic diarrhoea, which is characterised by an inability of the intestinal epithelium to absorb ingested substances (such as sorbitol or mannitol) that result in water loss across an osmotic gradient.

Ion transport has been shown to be modulated by the effectors EspG and EspG2 which reduce the activity of the Cl^-/OH^- ion exchanger Down-regulated in adenoma (DRA) by increasing internalisation of the transporter into the host cytoplasm (Gill *et al.* 2007). In addition, EspF decreases expression of the Na^+/H^+ exchanger 3 (NHE3) (Hodges *et al.* 2008). Furthermore, Tir, mitochondrial associated protein (Map) and EspF synergistically downregulate activity of the sodium-glucose co-transporter SGLT1 that is responsible for the uptake for ~4 litres of water per day in the small intestine (Dean *et al.* 2006; Meinild *et al.* 1998).

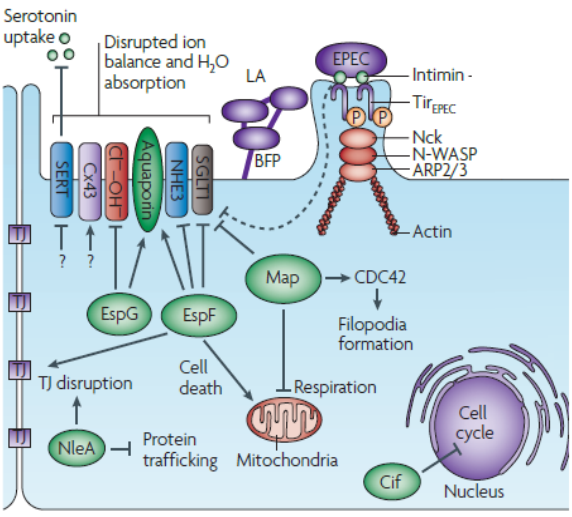


Figure 1.3 EPEC secreted effectors involved in intimate adherence and interference with host cell functions. Pointed arrows indicate activation whereas blunt arrows indicate inhibition. CDC42, cell division control protein 42; Cx43, connexin 43: Cif, cycle-inhibiting factor; Map, mitochondrial-associated protein; NHE3, Na^+–H^+ exchanger 3; NleA, non-LEE-encoded effector A; SERT, serotonin transporter; TJ, tight junctions. Figure sourced from Croxen and Finlay 2010.

In addition to affecting ion transport, EPEC can directly interfere with water uptake by targeting transmembrane aquaporins (Verkman 2013). EspF and EspG have been shown to cause mis-

localisation of aquaporins AQP2 and AQP3 following infection of mice with the EPEC-related pathogen *C. rodentium* (Guttman *et al.* 2007).

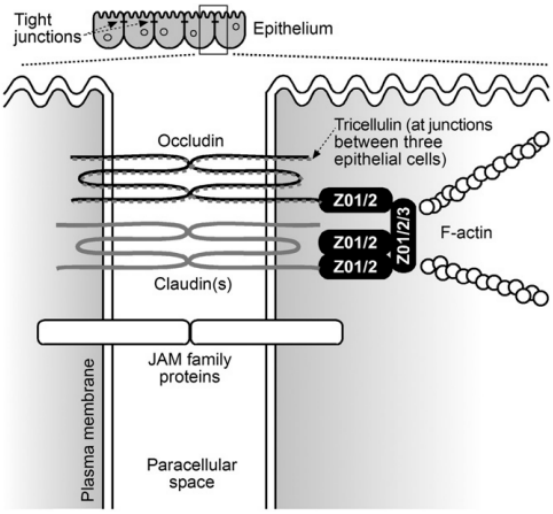


Figure 1.4 Structure of TJs at the intestinal epithelium. Schematic representation of the protein complexes that comprise TJs between intestinal epithelial cells. Figure sourced from Ulluwishewa *et al.* 2011.

Another mechanism contributing to diarrhoea is mediated by TJs which are formed in the paracellular space between IECs. Several protein complexes span the space between cells, composed primarily of the claudin and JAM family proteins, as well as occludin (**Fig. 1.4**) (Ulluwishewa *et al.* 2011). These complexes can be anchored via the cytoplasmic zonular occluden (ZO) protein family which connect them to cytoskeletal components such as F-actin. TJs function as a seal between adjacent epithelial cells and restrict paracellular flow (Farquhar and Palade 1963). This is crucial in the intestine, as interaction between the microbe-dense lumen and underlying lamina propria, inhabited by an array of lymphocytes, could result in considerable inflammation and tissue damage. Indeed, breakdown of TJs and increased paracellular transport has been associated with several diseases, including chronic inflammatory disorders and infectious diarrhoea (Hollander and Kaunitz 2020). Intriguingly, virulence proteins targeting TJs have been characterised from several diarrheagenic pathogens, leading to the hypothesis that paracellular leakage of water may contribute to the severity of diarrhoea (Eichner *et al.* 2017). EPEC infection has been associated with TJ disruption in cell lines and mice, often through the activity of T3SS effector proteins (Canil *et al.* 1993; Shifflett *et al.* 2005; Spitz *et al.* 1995; Weflen, Alto, and Hecht 2009). Infection of polarised Caco-2 cells with WT EPEC resulted in loss of occludin between cells and reduced transepithelial electrical resistance (TEER), which was abolished in Δ*espF* and Δ*map* EPEC strains (Dean and Kenny 2004a). The requirement of EspF in TJ disruption was also observed *in C. rodentium*-infected mice. which was associated with internalisation of claudin-1 and -3 (Guttman *et al.* 2006). In addition to EspF, EspG1/2 has been shown to enhance paracellular flux of small molecules (< 4 kDa) with a particular size, without affecting TJ integrity. (Matsuzawa, Kuwae, and Abe 2005). In spite of these studies highlighting TJ disruption by several EPEC effector proteins,

data directly linking this to increased luminal water content is required and warrants further investigation.

1.1.3.3 Host immune response

1.1.3.3.1 Inflammation

At the small intestinal mucosa, pathogens must breach the mucus layer and attach to the intestinal epithelium to avoid being flushed away by strong peristaltic waves. The epithelium responds by mounting an immune response to clear the infection, re-build the mucus layer and retain homeostasis. The small intestinal epithelium is home to microfold-cells (M cells), goblet cells and protrusions of dendritic cells (DCs), all of which are capable of delivering microbial antigens to lymphocytes in the underlying lamina propria (Chieppa *et al.* 2006; McDole *et al.* 2012; Neutra, Frey, and Kraehenbuhl 1996). Furthermore, IECs express pattern recognition receptors (PRRs) which detect bacterial signatures known as microbe-associated molecular patterns (MAMPs), activating intracellular signalling pathways and producing pro-inflammatory cytokines. These receptors are often expressed on the cell surface but can also be found in the endosomal membrane, where they detect MAMPs or products of microbial degradation following internalisation (El-Zayat, Sibaii, and Mannaa 2019; Swanson and Hoppe 2004). Toll-like receptors (TLR) are a well-studied group of PRRs, capable of recognising common MAMPs such as lipopolysaccharide (LPS), peptidoglycan (PG) and flagella (El-Zayat, Sibaii, and Mannaa 2019). Surface TLRs activate the MyD88 signalling pathway upon ligand binding, resulting in nuclear translocation of transcriptional regulators AP-1 and NF-κB (El-Zayat, Sibaii, and Mannaa 2019; Sugiyama *et al.* 2016). Once in the nucleus, these factors stimulate the expression of a large array of genes, including those encoding pro-inflammatory cytokines such as TNF-α, IL-1β and IL-8 (Liu *et al.* 2017). These cytokines have a wide array of functions, including enhancing leukocyte activity and triggering programmed cell death (apoptosis).

EPEC infection stimulates inflammatory signalling pathways, including mitogen-associated protein kinase (MAPK) and NF-κB pathways, in IECs resulting in the secretion of pro-inflammatory cytokines. EPEC infection of T84 cells results in activation of extracellular signal-regulated kinase (ERK1/2) leading to degradation of NF-κB inhibitor IκB (Savkovic *et al.* 2001). ERK1/2 also activates c-Fos, which interacts with c-Jun to form AP-1 (Roskoski 2012). Furthermore, EPEC can induce pro-inflammatory cytokine secretion via activation of the MAPK p38 pathway (Khan *et al.* 2008). Of the pro-inflammatory cytokines released as a result of activating these pathways, interleukin 8 (IL-8) secretion has been most commonly associated with EPEC infection. IL-8 acts as a chemoattractant for polymorphonuclear leukocytes and initiates the innate immune response (Savkovic, Koutsouris, and Hecht 1996). Flagellin

(FliC), the structural subunit of EPEC flagella has been identified as a main stimulator of IL-8 secretion from IECs (Zhou *et al.* 2003; Schuller *et al.* 2009). EPEC *fliC* mutants exhibited abrogated induction of IL-8 secretion from T84 colon carcinoma cells and purified FliC demonstrated potent stimulatory effects on IL-8 secretion. Bacterial flagellin activates TLR5, triggering an intracellular signalling cascade resulting in activation of NF-κB (Hayashi *et al.* 2001). There is some debate over the immunostimulatory activity of flagellin during EPEC infection, as expression of the LEE and flagellin secretory apparatus may be negatively correlated. GrlA, a global transcriptional regulator present in both EPEC and STEC, is capable of inducing the expression of *ler* (Iyoda *et al.* 2006; Jiménez *et al.* 2010). Interestingly, Iyoda *et al.* revealed repression of the flagella operon by GrlA in STEC, suggesting that flagella secretion is minimised during close contact with host cells. If this translates to EPEC infection, the role of alternative immunostimulatory MAMPs could be emphasised. Correspondingly, stimulation of IL-8 secretion can also occur independent of flagellin as *fliC* mutants exhibited significant induction of IL-8 secretion from HT-29 cells (Khan *et al.* 2008; Sharma *et al.* 2006). A 50 kDa, heat-sensitive protein isolated from EPEC cell-free supernatants (CFS) appeared to be the cause, but its identity was not elucidated.

In addition to IL-8, increased secretion of several pro-inflammatory cytokines (IL-6, IL-1β and IFN-γ) was observed in the colon of mice infected with EPEC. IL-6 has a wide-range of functions in both acute and chronic inflammation. During the acute phase, IL-6 promotes influx and prolonged survival of neutrophils which can target pathogens through release of secretor anti-microbials and phagocytosis (Asensi *et al.* 2004; Fielding *et al.* 2008). Similarly, IL-1β further aids the recruitment of leukocytes at the site of infection through the release of chemokines and the expression of adhesins on endothelial cell (Gabay, Lamacchia, and Palmer 2010). IFN-γ appears to be directly involved in augmenting the anti-microbial activity of macrophages by stimulating inducible nitric oxide synthase, increasing levels nitric oxide (Kopitar-Jerala 2017). Furthermore, IFN-γ can trigger pyroptosis, a form of apoptosis associated with inflammation, in infected cells, limiting further deleterious effects of the pathogen on the host cell (Kader *et al.* 2021). Interestingly, IFN-γ has been detected from the faecal matter of children with symptomatic EPEC infection, suggesting a role in the human immune response to EPEC (Long *et al.* 2010).

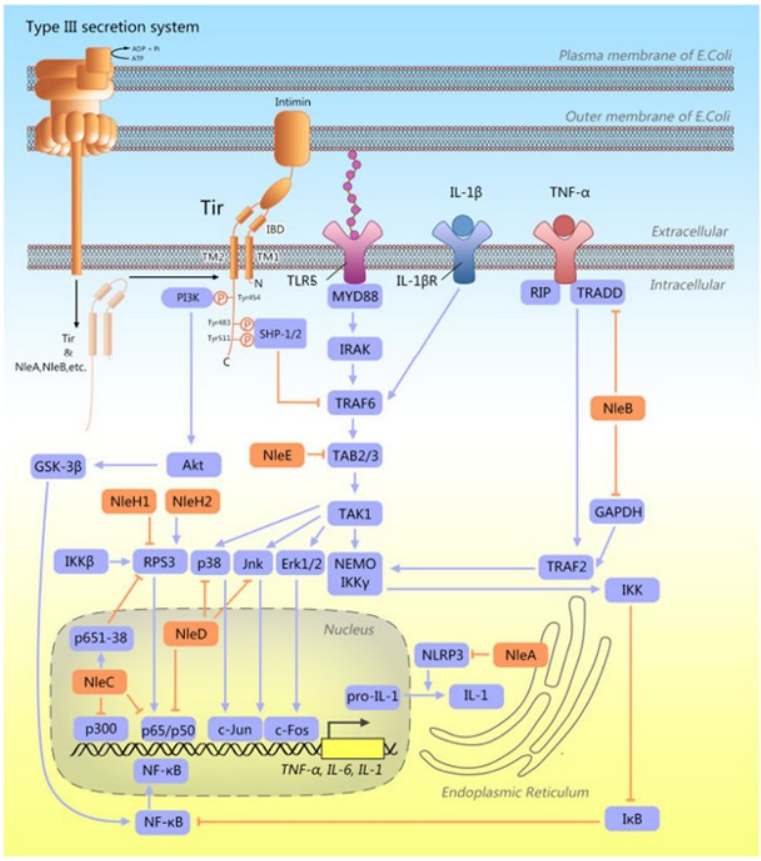


Figure 1.5 Overview of the host cell targets for EPEC-derived pro-inflammatory and anti-inflammatory mediators. Flagellin is recognised by TLR5 which activates NF-κB and MAPK pathways in the host cell, resulting in expression of pro-inflammatory cytokines (including IL-8). Outline of pro-inflammatory host cell signalling pathway components disrupted by non-LEE effectors (Nle, shown in orange). Figure modified from Zhuang *et al.* 2017.

In spite of the numerous mechanisms by which EPEC induces inflammation, the inflammatory response during EPEC infection is milder relative to other enteric pathogens such as *Shigella* (Ruchaud-Sparagano, Maresca, and Kenny 2007). Interestingly, induction of pro-inflammatory cytokines is weaker in response to EPEC-containing cultures compared to CFS, suggesting a cell-dependent suppression of inflammation (Sharma *et al.* 2006). Further investigations revealed a role for the T3SS, as a Δ*escN* strain incapable of translocating proteins via the T3SS stimulated ~4x more IL-8 than the WT. Indeed, many of the Nle proteins are associated with suppression of NF-κB and MAPK pathways (see **Fig. 1.5**). In particular, NleC and NleE repress transcription of IL-8 by acting on the NF-κB transcription factor subunit p65. NleC has been shown to directly cleave p65, whereas NleE inhibits translocation of p65 into the host nucleus where it would otherwise induce genes encoding pro-inflammatory cytokines (Baruch *et al.* 2011; Newton *et al.* 2010). Further direct inhibition of NF-KB activity is provided by NleH, which inhibits ubiquitination and subsequent degradation of IκB (Royan *et al.* 2010). Activation of NF-κB by TNF-a is blocked by NleB, which interferes with TRAF2, a crucial adapter protein linking TNF receptor to downstream signalling pathways (Gao *et al.* 2013). In addition to NF-KB, AP-1 is also a target of Nle proteins. NleD cleaves and inactivates the MAPKs c-Jun amino-terminal kinase and p38, resulting in attenuated IL-8 and IL-6 secretion (Baruch *et al.* 2011). These

effectors likely act in concert, producing a robust anti-inflammatory effect that promotes EPEC survival during infection.

1.1.4 Epidemiology & clinical significance

Infantile diarrhoea is a global issue causing millions of deaths per annum, particularly in sub-Saharan Africa and South-East Asia (**Fig. 1.6**). Poor sanitation and a warmer climate likely promote growth and transmission of diarrhoeagenic agents in these areas. *Bacterium coli* var. *neapolitanum*, later named EPEC, was initially discovered as the causative agent of the infant summer diarrhoea in the UK during World War II (Bray 1945; Bray and Beavan 1948). Symptoms included fever, vomiting, loss of appetite, mucus in stool and abdominal cramps. However, the most clinically significant symptom was acute diarrhoea. Indeed, severe dehydration and malnutrition caused by acute diarrhoea are the major contributors to mortality from EPEC infection. For typical EPEC, zoonotic infections appear rare; however, several animal reservoirs have been identified for atypical EPEC, including household pets and livestock (Blanco Crivelli *et al.* 2021; Gioia-Di Chiacchio *et al.* 2018; Sanches *et al.* 2017; Watson *et al.* 2017). Additionally, ingestion of contaminated water, weaning fluids or food, and host-host contact may cause rapid transmission of EPEC (Croxen *et al.* 2013; Levine and Edelman 1984a). Children under 5 are particularly susceptible to infection, likely due to the presence of EPEC receptors that are lost in adulthood, decreased immunity, and under-developed gut microbiota (Croxen *et al.* 2013).

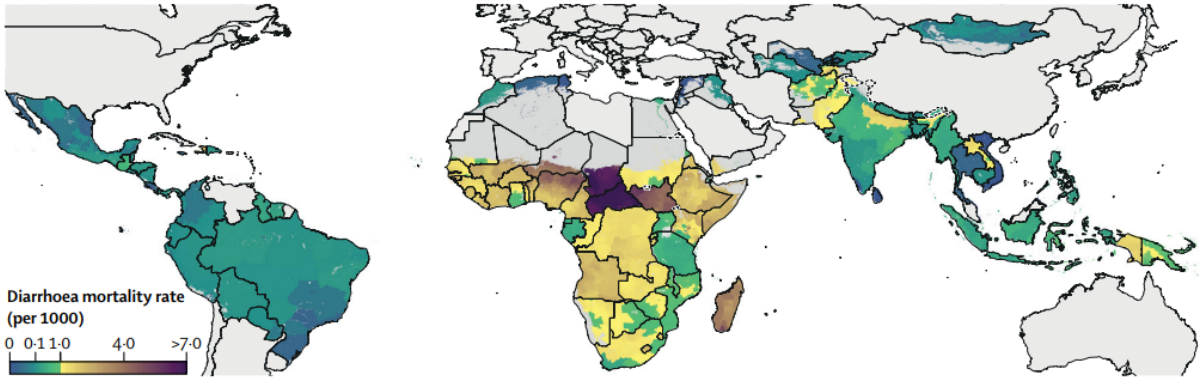


Figure 1.6: Global mortality due to infant diarrhoea. Rate of deaths (per 1000 people) in infants under 5 years of age caused by diarrhoea according to analysis performed for the Global Burden of Disease Study 2017. Countries coloured purple, such as those in sub-Saharan Africa, have a particularly high mortality rate Reiner *et al.* 2020.

Large scale studies determining the causative agents of diarrheal disease in infants are scarce, providing a significant challenge to determining the contribution of EPEC. Nevertheless, the recent Global Enteric Multicenter Study (GEMS), investigated the aetiology of diarrheal disease in 10,000 children across 7 different regions (localised to sub-Saharan Africa and south Asia) and three age groups (Kotloff *et al.* 2013). Despite the considerable variation in prevalence of diarrhoea-causing agents across locations and age groups, DEC was identified in almost all demographics. Although the GEMS data showed that ETEC was the most common cause of DEC-induced diarrheal disease in infants, particular areas such as Kenya were severely affected by EPEC. This finding agrees with previous smaller-scale epidemiological studies, which often show distinct variation based on location, age group, diagnostic criteria and detection methods used (Ochoa *et al.* 2008). As a result, more large scale, multi-site studies are required to assess the prevalence of EPEC-induced diarrhoea.

In addition to epidemiological studies, recent systematic reviews concluded that the contribution of EPEC to infantile diarrhoea is far greater than that suggested by GEMS. Two separate reviews have implicated EPEC in around 123,000 deaths of children under 5 years of age in 2010 and 79,000 deaths in 2011, both ranking it the second most fatal diarrhoeal agent identified (Kirk *et al.* 2015; Lanata *et al.* 2013).

1.1.5 Current Treatments for EPEC Diarrhoea

Epidemiological studies show that EPEC is a considerable burden on low- and middle-income countries (LMICs) and existing treatments are not suitable. Currently, the World Health Organization (WHO) recommends administration of oral rehydration therapy (ORT) to those affected with acute diarrhoea, including EPEC infection. ORT involves ingestion of water, glucose and salts combined in proportions that are most effective for rehydration (Victora *et al.* 2000). Although ORT is effective in 97% of cases of acute diarrhoea, EPEC-induced diarrhoea is particularly resilient to rehydration-based treatments. EPEC infection results in downregulation of the SGLT transporter required for water uptake in the small intestine (Nataro and Kaper 1998). Consequently, this could impact the effectiveness of ORT as a treatment for EPEC-induced diarrhoea.

In addition to acute disease, infectious diarrhoea can last longer than 14 days (persistent diarrhoea). Persistent diarrhoea is being increasingly recognised as a significant contributor to mortality, with experts estimating that 50% of diarrheal deaths are now due to persistent infections (Abba *et al.* 2009). A systematic review discovered that DEC strains, including EPEC, were isolated from up to 41% of children with persistent diarrhoea (Abba *et al.* 2009). Interestingly, studies also highlight that EPEC

is more likely to produce cases of persistent diarrhoea than other common enteric pathogens, such as rotavirus, *Campylobacter* and *Salmonella* (Hill, Phillips, and Walker-Smith 1991; Levine and Edelman 1984b; Nguyen *et al.* 2006). The reason behind this distinction remains unclear, although immunosuppressive activity of EPEC may impair pathogen clearance by the host. These data suggest that infection with EPEC may have higher mortality due to persistent symptoms.

In cases of severe dehydration caused by EPEC-induced diarrhoea, antibiotic treatment is considered a last resort. Although antibiotics have historically been a fast and effective treatment for EPEC infections, there are several drawbacks. Firstly, antibiotic treatment has been shown to stimulate shiga-toxin release during STEC infection, which can promote life-threatening HUS (Kakoullis *et al.* 2019). As STEC and EPEC diarrhoea affects individuals similarly, accurate classification is required before treatment with antibiotics. Furthermore, antimicrobial resistance amongst EPEC is increasing at an alarming rate. Clinical isolates of EPEC resistant to a wide array of antimicrobials (fluoroquinolones, cephalosporins, penicillin, aminoglycosides) have been identified worldwide (Canizalez-Roman *et al.* 2016; Canizalez-Roman *et al.* 2013; Huang *et al.* 2015; Karami *et al.* 2017; Mitra *et al.* 2011; Ochoa *et al.* 2009). Currently, systematic reviews of this literature have yet to be published, making it difficult to draw conclusions on the prevalence of antimicrobial resistance worldwide. Nevertheless, studies highlight high rates of multi-drug resistance (MDR) amongst EPEC, as almost half of clinical EPEC isolates identified are resistant to antibiotics from 3 or more classes (Canizalez-Roman *et al.* 2016; Canizalez-Roman *et al.* 2013; Huang *et al.* 2015; Karami *et al.* 2017; Mitra *et al.* 2011; Ochoa *et al.* 2009). In addition to high rates of resistance, other issues make antibiotics an unfavourable treatment option: the cost of antibiotics limits their availability, particularly for low-income families in LMICs who need them most; issues with infrastructure in LMICs makes transport and administration of antimicrobials challenging; and patient compliance is poor due to a lack of education surrounding antimicrobials (Ayukekbong, Ntemgwa, and Atabe 2017). Moreover, the broad-spectrum activity of many antimicrobials can cause a significant loss of microbiota abundance and diversity leading to dysbiosis. Microbial dysbiosis and loss of symbiotic gut bacteria are associated with a range of diseases including infections by gut pathogens, which can now colonise niches that were previously occupied by an established microbial population (Flint *et al.* 2012). As such, using antimicrobials to treat EPEC infection may result in recurrent infections leading to malnutrition, particularly if the individual is exposed to the same contaminated material.

The fact that alterations in the gut microbiota result in disease suggests that the majority of these organisms help to maintain intestinal homeostasis. This revelation has led to an explosion in research and commercialisation of ingestible “good” bacterial strains (commonly known as probiotics),

particularly as a potential alternative to antimicrobials. With regards to EPEC infection, an optimal treatment would be effective at resolving recurrent and persistent infections, affordable for the low-income demographic that is most susceptible to EPEC and easy to administer. Probiotics have the potential to fulfill these criteria.

1.2. Colonisation resistance

1.2.1 Role of the Microbiota in Gut Health

The gastrointestinal tract (GIT) is a complex system composed of multiple interconnecting organs with the primary function of digesting and extracting energy from food. It is also home to up to 10^{14} bacteria spanning an estimated 400 distinct species (Berg 1996; Vaughan *et al.* 2000). The majority of the bacterial species in this diverse ecosystem can be assigned to two distinct phyla, the Bacteroidetes and Firmicutes (Guarner and Malagelada 2003). Within each species are several strains, which may interact with the host in a different way, adding to the abundance of dynamic interactions between the host and resident microbiota. The human gut microbiota consists largely of anaerobic organisms, reflecting the low levels (<1% of atmospheric pressure) of oxygen in the ileal and colonic lumen (Friedman *et al.* 2018; Albenberg *et al.* 2014; Eckburg *et al.* 2005). Despite the emergence of anaerobic culturing techniques, 16S ribosomal RNA (rRNA) gene sequencing remains the technique of choice to determine the microbial composition of the intestine (Mizrahi-Man, Davenport, and Gilad 2013; Poretsky *et al.* 2014). While analysis of ileostomy effluent revealed a dominance of facultative anaerobes in the jejunum and ileum, metagenomic analysis of faecal samples highlighted an abundance of obligate anaerobic species including Bacteroides, *Ruminoccocus* and *Clostrium* spp. (Booijink *et al.* 2010; Zoetendal *et al.* 2012; Qin *et al.* 2010).

A healthy GIT is a hub for mutualism en masse. The host provides nutrients from exogenous (e.g. dietary fiber) and endogenous (e.g. mucus) sources, facilitates colonisation of the mucosa and exhibits immune tolerance to the microbiota. In return, the microbiota provides the host with essential nutrients, aids development and maintenance of the GIT immune system, and maintains barrier function of the intestinal epithelium (Flint *et al.* 2012; Round and Mazmanian 2009). One of the most established roles of the gut microbiota is defending the host from invading bacteria. This concept is termed “colonisation resistance” and was first hypothesised in the 1950-60s after observations that mice with an impaired intestinal microbiota (through antibiotic treatment) were susceptible to pathogens at abnormally low doses (Bohnhoff, Drake, and Miller 1954; Bohnhoff and Miller 1962; Osawa *et al.* 1964). A further study demonstrated that replacing the lost microbiota in antibiotic-

treated mice by faecal transplants from healthy controls restored their resistance to infection (van der Waaij, Berghuis-de Vries, and Lekkerkerk 1971). Almost 50 years after its discovery, the importance of colonisation resistance to human health is becoming clearer. There is now growing clinical data revealing the efficacy of probiotics in the treatment of infectious disease (Francavilla *et al.* 2012; Guandalini *et al.* 2000; McFarland 2006; Savino *et al.* 2015). In recurrent infections with *Clostridiodes difficile*, the European Society of Clinical Microbiology and Infectious Diseases strongly recommends the use of faecal microbiota transplantation (FMT) which has demonstrated an efficacy of 92% in treating recurrent and refractory *C. difficile* infections (Debast *et al.* 2014; Quraishi *et al.* 2017) Despite the success of FMT, detailed studies elucidating the mechanisms of colonisation resistance are scarce. A deeper understanding of how the microbiota protects from infection at a molecular level is required to improve confidence in their efficacy at the clinical level.

1.2.2 The mucosal barrier: mediator of host-bacterial interactions

1.2.2.1 Structure and function of intestinal mucus

As the intestinal mucus provides the main battleground for this interbacterial warfare, it is essential to understand the structure and function of the mucus layer before delving into mechanisms of colonisation resistance. Mucus is a gel-like substance that covers the epithelial surface of many organs, including the GIT (Johansson, Larsson, and Hansson 2011). At the epithelium, specialised goblet cells secrete mucins, which are heavily glycosylated proteins undergoing extracellular polymerisation to form mucus. Intestinal mucus is primarily composed of mucin-2 (MUC2) (Johansson, Thomsson, and Hansson 2009). MUC2 has been estimated as 20% protein and 80% oligosaccharide content by weight, indicating the considerable glycosylation that nascent MUC2 proteins undergo prior to secretion (Johansson, Larsson, and Hansson 2011). The range and breadth of glycosylation patterns in MUC2 glycoproteins (built from 47 potential *O*-linked and 30 *N*-linked glycans) yields a diverse set of colonisation sites and nutrients for the intestinal microbiota (Arike, Holmen-Larsson, and Hansson 2017; Juge 2012). Characterisation of MUC2 *O*-linked glycans from intestinal biopsy tissue of mice indicated distinct glycosylation patterns corresponding to different regions of the GIT (Holmén Larsson *et al.* 2013). Mucus on biopsy tissue from the ileum, jejunum and duodenum was predominantly composed of sialylated and sulphated glycans, whereas colonic MUC2 was dominated by fucosylated glycans. These regiospecific glycosylation patterns may explain why certain bacteria exclusively inhabit particular regions along the intestine.

(A) **(B)**

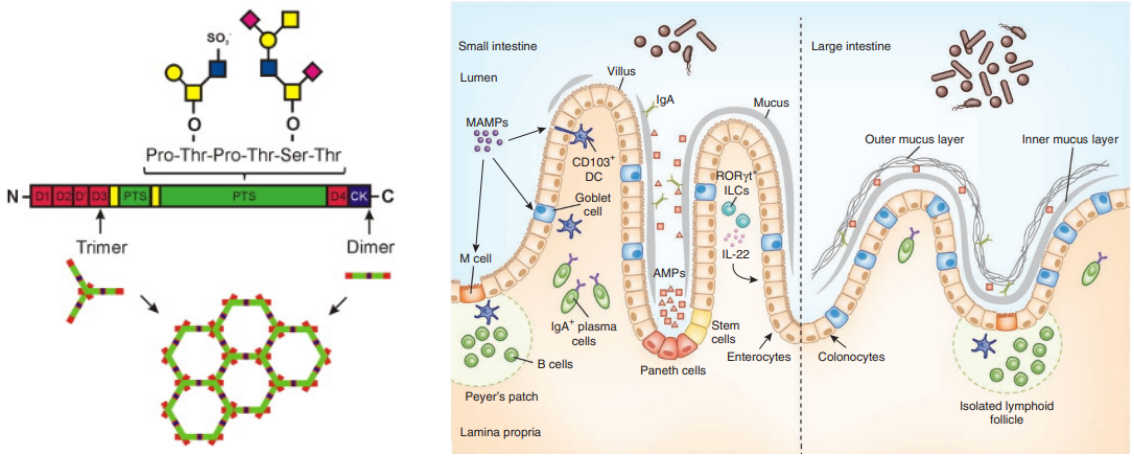


Figure 1.7 Structure and composition of the intestinal epithelium and mucus layer. (A) Overview of post-translational processing of nascent MUC2 polypeptides. Dimerisation occurs through disulphide bond formation between cysteine knot ("CK") domains, *O*-glycans are then added to Proline, Threonine, Serine ("PTS") domains, followed by trimer assembly via von Willebrand domain 3 ("D3"). Interactions between trimers is thought to result in the formation of dehydrated net-like structures, which form a gel-like substance upon extracellular hydration. Figure sourced from Arike & Hansson, 2016 (Arike and Hansson 2016). **(B)** Diagram showing the composition and immune response at the small and large intestinal mucosa. Although the main cell types are conserved between the two tissues, there are differences in epithelial morphology, mucus thickness, and presence/contribution of immune cells. Figure sourced from Brown, Sadarangani, and Finlay 2014.

Prior to *O*-linked glycosylation, nascent MUC2 undergoes *N*-linked glycosylation and dimerisation in the rough endoplasmic reticulum (see **Fig. 1.7A** for an overview of post-translational processing of MUC2) by forming disulphide bonds between the C-terminal cysteine-knot (CK) domains (Asker *et al.* 1998). Interestingly, *N*-glycosylation is essential for efficient dimerisation. (Bell *et al.* 2003; van Klinken *et al.* 1998). MUC2 dimers are subsequently transported to the Golgi apparatus where they undergo significant *O*-glycosylation at the PTS domain and are shuttled into secretory vesicles via the trans-Golgi network (Axelsson, Asker, and Hansson 1998). The low pH and high calcium levels in these vesicles facilitates the formation of N-terminal trimers supported by disulphide bonds (Godl *et al.* 2002). Although the structure of MUC2 polymers is yet to be directly visualised, this bonding implies a net-like pattern (**Fig. 1.7A**). Mucin polymers are stored in granulae of goblet cells and upon secretion, MUC2 binds water via the PTS domain to produce the gel-like consistency associated with secreted

mucus (Lang, Hansson, and Samuelsson 2007). Despite consisting predominantly of MUC2, there are many structural differences between the mucus layers of the small and large intestine. In addition to regiospecific *O*-glycosylation, differences in mucus structure are apparent in different parts of the intestine, and generally correlate with the number of bacterial residents. The concentration of bacteria is highest in the colon (~10^{11} CFU/mL), which is supported by a range of ecological niches offered by a thick (200-400 µm), loosely associated mucus layer (Johansson *et al.* 2008; Gustafsson *et al.* 2012; Pullan *et al.* 1994; Atuma *et al.* 2001; Sender, Fuchs, and Milo 2016). Beneath this is a thinner (100 µm) adherent layer devoid of microbes which protects the epithelium. Travelling back from the colon through the small intestine, bacterial numbers progressively decrease from 10^8 CFU/mL (ileum) to 10^3-10^4 CFU/mL (jejunum and duodenum) (Sender, Fuchs, and Milo 2016). Reflecting this, the mucus of the small intestine is characterised as thinner (200 µm) and more permeable to allow nutrient absorption (Gustafsson *et al.* 2012). Although little information is available on the thickness of the mucus within the different sections of the small intestine, the proportion of goblet cells from the ileum to the jejunum decreases from 12 to 4%, which is likely to reduce mucus thickness and may even disrupt the continuity of the mucus layer (Gustafsson *et al.* 2012). The mucus layer has been implicated in essential processes for gut homeostasis, such as facilitating colonisation by health-promoting microbes and regulating mucosal immunity. These functions will be discussed below in greater detail.

1.2.2.2 Host immunity at the intestinal mucosa

The intestinal mucosa has been described as “hyporesponsive” to the vast array of molecules derived from the microbiota and consumed food (Shao, Serrano, and Mayer 2001). Intestinal mucus plays an integral part in regulating this tolerance as mice carrying a deletion in the MUC2 gene spontaneously develop colitis (Wenzel *et al.* 2014). Interestingly, removal of the gut microbiota attenuated pro-inflammatory cytokine production, indicating that a lack of mucus in combination with luminal bacteria drives inflammation (Wang, Moniruzzaman, *et al.* 2021). This suggests that secreted mucus serves as a physical barrier restricting exposure of the intestinal epithelium to gut bacteria in order to maintain mucosal immunity. Indeed, the importance of secreted mucus in reducing pathogenesis has been demonstrated in infection of MUC2-deficient mice with *Salmonella enterica* serovar Typhimurium which resulted in higher pathogen loads, epithelial colonisation and reduced survival relative to wild-type mice (Zarepour *et al.* 2013).

Despite the protective effect of colonic mucus, studies in mice have revealed increased bacterial permeability of small intestinal mucus which likely reflects a compromise to facilitate nutrient uptake

(Ermund *et al.* 2013). The greater risk of bacterial exposure at the small intestinal epithelium is mitigated by antimicrobial proteins and peptides secreted by Paneth cells including REG3 derivatives and defensins (**Fig 1.7B**) (Vaishnava *et al.* 2011; Ghosh *et al.* 2002). In addition, MAMPs from the intestinal lumen are recognised by DCs and M cells which interact with underlying lymphoid structures known as Peyer's patches (PPs) (see **Fig 1.7B**). Activation of PP lymphocytes results in IgA release into the small intestine mucus layer, which agglutinates pathogens and thereby impairs mucosal colonisation and activity of secretion systems (Mantis, Rol, and Corthesy 2011).

Another defence mechanism of the small intestine is the glycocalyx, a thin continuous layer of transmembrane (TM) mucins produced by absorptive epithelial cells (Sun *et al.* 2020; Egberts *et al.* 1984). The small intestinal glycocalyx is predominantly composed of the TM mucins MUC13 and MUC17 (Schneider *et al.* 2018). Many of these have demonstrated protective activities against pathogens *in vitro*. Knock-down of *MUC13* in porcine small intestine epithelial cells resulted in increased ETEC adhesion (Zhou *et al.* 2013). In addition, overexpression of MUC17 and MUC3 genes reduced EPEC adherence to human IECs (Schneider *et al.* 2019; Larson *et al.* 2003). As TM mucins form a "bottle-brush" structure at the epithelial surface, inhibition of pathogen binding might be mediated by blocking access to epithelial receptors. In addition, extracellular TM mucin domains can be released and thereby act as decoy receptors for pathogen adhesins (Pelaseyed and Hansson 2020; Bork and Patthy 1995; Cone 2009; Linden *et al.* 2008; Pelaseyed *et al.* 2013; van Putten and Strijbis 2017).

In addition to these host-derived factors, colonisation resistance provided through the various activities of commensals at the intestinal mucosa (see **Fig. 1.8** for an overview) is also crucial in the defence against enteric pathogens.

1.2.3 Mechanisms of Colonisation Resistance: Antagonism

The number and diversity in the mechanisms of colonisation resistance are vast, consisting of a wealth of published literature investigating different bacterial species and strains in a variety of model systems. The following sections on the Mechanisms of Colonisation Resistance aim to give a concise overview of the proposed mechanisms, for which examples from more relevant commensal bacteria (Lactobacilli and *Ruminococcus gnavus*) will be provided in later sections.

1.2.3.1 Competition for Nutrients

The gut is home to an array of niches, many of which are formed due to the unique set of nutrients available for bacterial utilisation. Breakdown of consumed food by the host is incomplete without the

aid of bacteria-derived catabolic pathways, which release nutrients and metabolic by-products (e.g. liberated saccharides and short-chain fatty acids) for host utilisation but also into the surrounding environment (Myhrstad *et al.* 2020). Passing microbes with the capability to consume these products can proliferate at these sites and release their own by-products for use by other microbes, creating niches with complex and dynamic metabolic networks (Sorbara and Pamer 2019). This concept, known as cross-feeding, has been shown by computational models to be a stable method of efficient nutrient utilisation in an environment with diverse nutrient availability (Crombach and Hogeweg 2009; van Hoek and Merks 2017). The significance of cross-feeding is that it generates diverse bacterial sub-populations which are highly adapted to a given nutritional niche. These established bacteria are constantly faced with challenges by passing bacteria, driving adaption and increasing competitiveness.

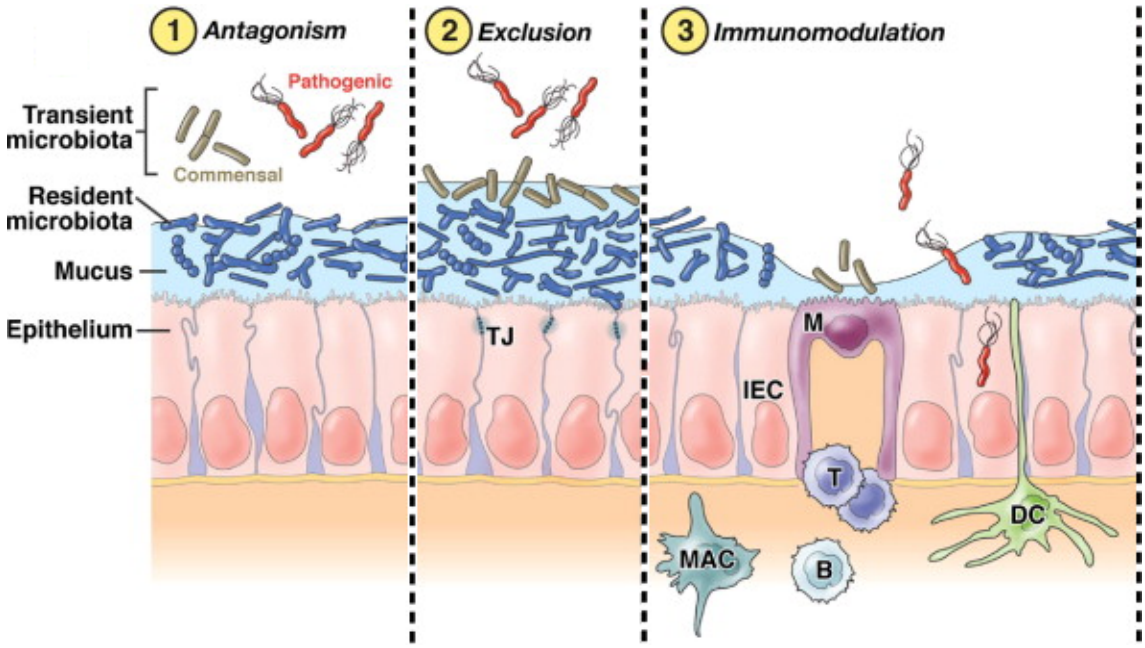


Figure 1.8 Mechanisms of colonisation resistance at the intestinal epithelium. Illustration showing three overarching mechanisms of colonisation resistance: direct antagonism (targeting pathogen growth and viability); exclusion (targeting epithelial colonisation) and immunomodulation (modifying the host immune response). TJ = tight junction, IEC = intestinal epithelium cell, M = M cell, T = T cell, B = B cell, MAC = macrophage, DC = DC. Adapted from Preidis *et al.* 2011.

Competition for nutrients proves to be a substantial barrier to overcome for many pathogens. Several studies have demonstrated that nutrient competition between pathogenic and commensal strains can hamper pathogen colonisation. Compounds such as saccharides, amino acids and free iron are often

critical for survival and proliferation of invading pathogens, including DEC (Maltby *et al.* 2013; Momose, Hirayama, and Itoh 2008; Schinner *et al.* 2015; Yilmaz and Li 2018). Germ-free (GF) mice lacking a microbiota are frequently used to perform these studies, as they offer the complex physiological conditions expected in the human gut. Nevertheless, their relevance to natural infection by DEC is questionable. Infection of GF mice with *C. rodentium* revealed minimal LEE expression in the absence of a complex microbiota, impairing colonisation (Kamada *et al.* 2012). Consequently, studies into colonisation resistance should seek to use models that allow a more accurate representation of infection by a given pathogen.

1.2.3.2 Antimicrobial compounds

Many bacteria produce anti-microbial (AM) secondary metabolites to better compete with challengers for nearby nutrients. Bacteriocins are a broad and heterogeneous group of AM compounds that can be produced by members of the gut microbiota and are strongly associated with spoilage prevention in fermented food products (Silva, Silva, and Ribeiro 2018). Bacteriocins can elicit their AM activity via an array of mechanisms, including membrane disruption or targeting crucial cellular functions such as DNA and protein metabolism (Cotter, Ross, and Hill 2013). Class I and II bacteriocins predominantly target Gram-positive bacteria, inhibiting growth of common gut pathogens such as *C. difficile* and *Listeria monocytogenes* (Bartoloni *et al.* 2004; Eijsink *et al.* 1998; Shelburne *et al.* 2007). With regards to bacteriocins targeting Gram-negative organisms, microcins are generally the most potent. Microcins are small peptides that insert into target membranes and form pores, thereby compromising membrane integrity and resulting in bacterial death (Moll, Konings, and Driessen 1999). Microcins S, 7 and J25 have all demonstrated colonisation resistance activity against E. coli and Salmonella spp., reducing pathogen growth in liquid culture and colonisation of Caco-2 IECs (Garcia-Bustos, Pezzi, and Mendez 1985; Soudy, Wang, and Kaur 2012; Lopez *et al.* 2007; Zschuttig *et al.* 2012). Consequently, these could be an important tool utilised by the microbiota to protect from EPEC infection.

In addition to short peptides, larger proteins with AM activity have also been identified from gut commensals, although these are poorly characterised. Several *Bacteroides* spp. produce AM proteins that target closely related strains, yielding an advantage over rivals for ecological niches (Chatzidaki-Livanis, Coyne, and Comstock 2014; Roelofs *et al.* 2016). Colicins are a large group of narrow-spectrum AM proteins that have been characterised from a range of bacterial species (Cascales *et al.* 2007). These proteins generally exhibit activity against closely-related strains vying for similar ecological niches (Cascales *et al.* 2007).

The narrow spectrum of bacteriocin activity drives high levels of competition between bacteria adapted to similar niches in the gut. Whilst competition is fundamental for effective colonisation resistance, this restricts the capacity of bacteriocins to target invading pathogens. Furthermore, the role of bacteriocins in colonisation resistance is largely presumed, rather than proven, as investigations of bacteriocin activity in the gut, using host cells or *in vivo* models, are lacking.

1.2.4 Mechanisms of Colonisation Resistance: Exclusion

Exclusion refers to the capability of the microbiota to restrict access of pathogens to the intestinal mucosa and augment host defence. The mechanisms of exclusion are diverse, encompassing commensal-derived changes to the mucosal barrier and inter-bacterial competition for epithelial colonisation sites. Host-mediated mechanisms of exclusion discovered so far include enhancement of epithelial barrier function, stimulation of mucus production and augmenting AM activity of the mucus (Preidis *et al.* 2011).

1.2.4.1 Competitive Exclusion

Mucosal adhesion, and subsequent nutrient utilisation, facilitates successful colonisation of gut bacteria to a given niche. Without effective adhesion, gut bacteria are removed by the flow of the intestinal chyme. This is particularly important in the small intestine, where strong peristaltic waves result in considerable shear stress, necessitating strong adhesion of bacteria to the intestinal mucosa. Consequently, attenuating pathogen adherence provides a potent mechanism by which commensal organisms enforce colonisation resistance. Studies have highlighted the challenge that pathogens face in adhering to IECs that have already been colonised. Pre-incubation of epithelia with commensals prior to infection is routinely used and considerably attenuates pathogen colonisation sites (Bernet *et al.* 1994; Ren *et al.* 2012). Interestingly, commensal mucosal adhesins can reduce pathogen colonisation on their own, indicating direct competition for binding sites (Chen *et al.* 2007). Notably, most studies investigating pathogen exclusion have utilised IECs that secrete little or no mucus. Nevertheless, mucus adhesion is presumed to be essential *in vivo*, as the intestinal epithelium is covered by mucus layer(s). Using more appropriate models to study competitive exclusion may provide novel insights into the underlying mechanisms of this phenomenon.

1.2.4.2 Strengthening the mucosal barrier

The mucosal barrier is composed of the secreted mucus layer, TM mucins and secreted factors that act to restrict epithelial access to luminal bacteria. The importance of this barrier has been demonstrated in MUC2-deficient *Winnie* mice, where expression of pro-inflammatory cytokine genes (*Ifn-γ*, *Il-1β*, *Il-17, Mip*-2) was reduced in germ-free counterparts (Wang, Moniruzzaman, *et al.* 2021). No difference was observed in WT mice, suggesting that an inflammatory response to the gut microbiota is only mounted in the absence of secreted mucus. *C. rodentium* infection of MUC2-deficient mice resulted in considerably more effective colonisation compared to WT mice, which appeared to up-regulate MUC2 production during infection (Bergstrom *et al.* 2010). This could translate to human AE pathogens, such as EPEC, highlighting the importance of an uncompromised mucosal barrier. As several commensals have demonstrated the ability to stimulate TM and secreted mucin production from IECs, this could be a powerful mechanism of colonisation resistance.

In addition to promoting mucus production, commensals can alter the AM function of the mucosal barrier. IECs secrete a range of AM compounds, such as the REG3 and defensin protein families, targeting bacteria that come too close to the epithelium (Dupont *et al.* 2014). Multiple studies have revealed upregulation and increased secretion of human β-defensin 2 in Caco-2 cells following incubation with a range of commensal bacteria, including *E. coli* Nissle 1917 and various lactobacilli (Möndel *et al.* 2009; Schlee *et al.* 2008). In addition, *Bifidobacterium breve* stimulated production of REG3γ in the intestine of GF mice (Natividad *et al.* 2013). Interestingly, *in vivo* studies have revealed a potential role for microbial propionate in REG3 production as induction of *Reg3β* and *Reg3γ* expression was observed in human intestinal organoids treated with propionate and removal of propionate-producing bacteria resulted in decreased faecal REG3β and REG3γ levels in mice (Bajic *et al.* 2020).

IgA secretion is an interface between mucosal immunity and pathogen exclusion in the gut. IgA is secreted into the intestinal mucus by B cells residing in the lamina propria following activation by immune cells such as DCs and follicular T helper (T_h) cells (Tezuka and Ohteki 2019). In the small intestinal this occurs at Peyer's patches (PPs), which contain M cells and DC protrusions with the function of translocating bacterial antigens into the underlying lamina propria (Tezuka and Ohteki 2019). Secretory IgA (SIgA) are antibodies with broad-specificity and function as a molecular barrier between the intestinal microbiota and host epithelium (Corthesy 2013; Bos, Jiang, and Cebra 2001; Kawashima *et al.* 2018; Wijburg *et al.* 2006). Several *Lactobacillus* and *Bifidobacterium* species have been shown to stimulate SIgA production in mice (Galdeano and Perdigón 2006; Martins *et al.* 2009; Qiao *et al.* 2002; Shu and Gill 2001). In animal studies using enteric pathogens such as STEC, enhanced

SIgA production by commensal organisms was associated with reduced pathogen numbers and mortality (Ogawa, Shimizu, Nomoto, Takahashi, *et al.* 2001; Qiao *et al.* 2002; Shu and Gill 2001). These studies demonstrate how the microbiota can reinforce the mucosal barrier by influencing host defence mechanisms.

In addition to restricting access to the epithelial surface, the host also regulates access to the underlying basal tissue through the activity of tight-junction complexes between IECs. Enhancement of intestinal epithelial barrier function could ameliorate disease caused by enteric pathogens disrupting tight junctions, such as EPEC. It has been suggested that increased intestinal permeability can contribute to diarrhoeal disease through down-regulated water transport and enhanced inflammation due to paracellular transport of pathogen MAMPs (Eichner *et al.* 2017; Farré *et al.* 2020). Several probiotic strains have demonstrated up-regulation of TJ proteins and barrier function in cell lines and mice, in some cases reversing disruption caused by pathogens (Ukena *et al.* 2007; Zyrek *et al.* 2007b; Resta–Lenert and Barrett 2006). Nevertheless a greater understanding of how TJ disruption impacts intestinal function during infection is required to validate this as a mechanism of colonisation resistance.

1.2.5 Mechanisms of Colonisation Resistance: Immunomodulation

The microbiota is involved in maturation of the immune system as well as modulating the immune response by affecting cytokine production and release, through a process known as immunomodulation (Belkaid and Hand 2014). Although not originally regarded as a mechanism of colonisation resistance, impaired immunomodulation has since been linked to increased susceptibility to pathogen colonisation. Enterobacterial "blooms" are common in inflammatory bowel disease patients and in mouse models of colitis (Stecher, Maier, and Hardt 2013). Other enteric pathogens including *Campylobacter jejuni*, *S.* Typhimurium, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Vibrio cholerae*, *Clostridiodes difficile*, and *Enterococcus* species have also been shown to benefit from the inflamed gut environment (Gevers *et al.* 2014; Lawley *et al.* 2009; Lupp *et al.* 2007; Ma and Mekalanos 2010; Stecher *et al.* 2007). There is no definitive explanation for this phenomenon, although evidence is available for multiple hypotheses. Immune cells of the inflamed gut produce terminal electron acceptors that *Enterobacteriaceae*, such as S. Typhimurium, can use to gain an advantage over the microbiota (Winter *et al.* 2010). Alternative nutrients are also available in the inflamed gut due to shedding of dead cells and release of free sugars from excessive mucus degradation (Stecher 2015). In this respect, anti-inflammatory mechanisms elicited by the microbiota would confer colonisation

resistance. Generally, the immunomodulatory activity of gut bacteria is mediated by interaction with PRRs or secretion of microbial compounds such as short chain fatty acids (SCFAs).

As discussed in **section 1.1.3.3.1**, exposure of IECs to MAMPs from luminal bacteria promotes pro-inflammatory cytokine production via activation of PRRs such as TLRs (see **Fig. 1.9** for an overview of TLRs). Nevertheless, several MAMPs (LPS, flagellin, PG) are shared amongst pathogenic and commensal bacteria, posing the question as to whether they trigger the same immune responses. Evidence of anti-inflammatory responses mediated by TLR activation was observed in mice lacking the adapter protein MyD88 (linking TLR activation to downstream intracellular pathways) as they were more susceptible to dextran sulphate sodium (DSS)-induced colitis compared to WT mice (Rakoff-Nahoum *et al.* 2004). Interestingly, removal of the gut bacteria by antibiotic treatment exacerbated mortality in DSS-treated WT mice, suggesting that activation of TLRs by the microbiota underpinned protection from DSS-colitis. Although the mechanisms that drive this differential inflammatory response of TLRs have not been elucidated, recognition of subtle differences in MAMP structure between pathogenic and commensal bacteria has been proposed (Chu and Mazmanian 2013).

In addition to differentiating between different forms of common TLR agonists, the suite of MAMPs recognised by TLRs may extend to commensal-specific molecules. One example is polysaccharide A (PSA) from Bacteroides fragilis, which has been implicated in maturation of naïve CD4+ T cells and production of anti-inflammatory cytokine IL-10 in mice (Round and Mazmanian 2010). Consequently, these mice exhibited less tissue damage in response experimental colitis.

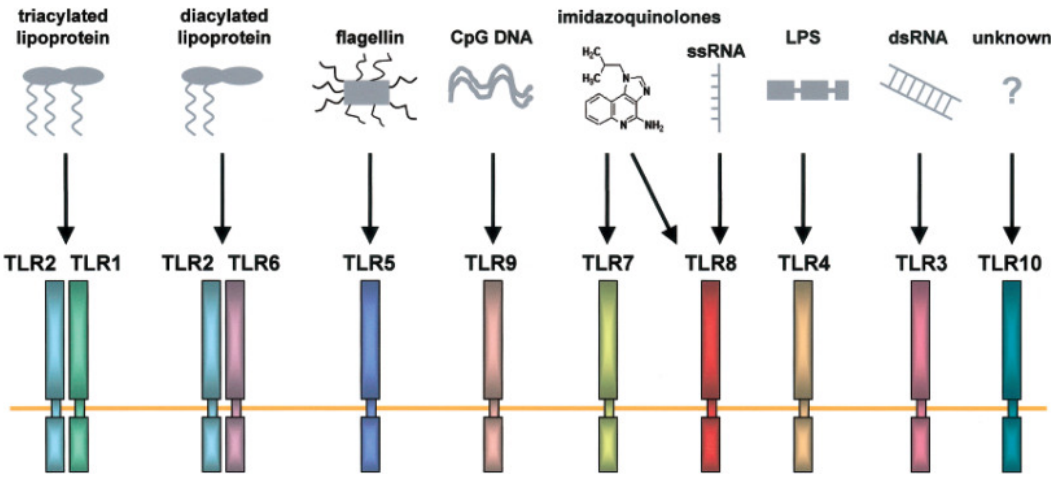


Figure 1.9 Human toll-like receptors (TLRs) and recognised MAMPs. The diagram indicates the full suite of TLRs currently identified in humans and the specific MAMP each is known to interact with. Figure sourced from McInturff, Modlin, and Kim 2005.

Immunomodulation by gut commensals can also be mediated through the interaction of secreted bacterial compounds and IECs. Several studies have demonstrated immunomodulatory activity of organic acids, particularly SCFAs produced during bacterial fermentation of dietary fibre. Whilst these molecules are thought to diffuse freely across the host cell membrane, specific transporters (e.g. monocarboxylate transporter and SLC5A8) have been characterised that greatly increase uptake, suggesting beneficial functions to the host (Miyauchi *et al.* 2004; Ritzhaupt *et al.* 1998). Furthermore, SCFAs can activate G-protein coupled receptors (GPCRs) on the host cell surface and interfere with immune signalling cascades. In particular, the SCFA acetate has demonstrated anti-inflammatory activity in DSS-colitis mice, reducing secretion of pro-inflammatory TNF-α, IL-6 and IL-1β and epithelial damage, although little is known about the mechanism (Laffin *et al.* 2019). Although the immunomodulatory activity of the SCFA butyrate has been well documented, reports of its effect on inflammation are conflicting. Several studies have revealed inhibition of NF-KB activation in response to butyrate treatment in murine macrophages, IECs and small-scale clinical trials, resulting in decreased pro-inflammatory cytokine secretion and inflammation markers (Lührs *et al.* 2002; Park *et al.* 2007; Singh *et al.* 2014; Thangaraju *et al.* 2009). Notably, work by Singh *et al.* suggested a role for the butyrate-binding GPCR GPR109A in this anti-inflammatory effect, as NF-κB activity of IECs lacking this receptor was unaffected by butyrate (Singh *et al.* 2014). Further anti-inflammatory properties of butyrate are evidenced by reduced IL-8 secretion and neutrophil migration in butyrate-treated Caco-2 cells and reduced inflammation in patients with ulcerative colitis (Bocker *et al.* 2003; Lührs *et al.* 2002). In contrast, enhanced NF-κB production and IL-8 secretion has been reported in butyrate-treated Caco-2 cells stimulated with peptidoglycan or LPS (Fusunyan *et al.* 1999; Leung *et al.* 2009). The authors hypothesised that butyrate exhibits pro-inflammatory activity following activation of TLRs while mediators of chronic inflammation (e.g. IL-1β) drive an anti-inflammatory response. Whilst the mechanism underpinning differential activity of butyrate has yet to be identified, it is clear that it plays an important role in immunomodulation.

In addition to freely diffusible metabolites such as SCFA, bacteria may also interact with IECs through extracellular vesicles (EVs). EVs are lipid bilayer nanoparticles capable of transporting biological compounds and may play a crucial role in inter-kingdom communication (Brown *et al.* 2015). Indeed, uptake of bacterial-derived EVs by IECs has been reported in numerous studies (Cañas *et al.* 2018; Li *et al.* 2017; Rodovalho *et al.* 2020). The effect of EVs on the host appear to vary, as pro- and anti-inflammatory responses have been noted in studies using EVs derived from different bacteria (Cañas *et al.* 2018; Rodovalho *et al.* 2020). Consequently, the involvement of EVs in immunomodulation are likely to be species-specific.

Through activation of PRRs, secretion of diffusible metabolites and delivery of cargo via EVs, commensal bacteria exhibit a range of mechanisms by which they can influence host immunity at the intestinal epithelium.

1.2.6 Lactobacilli and their Benefits to Human Health

1.2.6.1 Lactobacilli: A heterogenous group of probiotic bacteria

From its conception in 1901 up until 2020, the genus *Lactobacillus* comprised 261 distinct species grouped based on the following shared characteristics: Gram-positive, fermentative (producing lactic acid as a by-product of glucose metabolism), facultatively anaerobic and non-spore forming (Zheng *et al.* 2020). As more data was collected on the species, the vast heterogeneity of this genus was revealed, highlighting diversity at the genotypic, phenotypic and ecological level. To improve classification, these species have been re-organised into 26 genera (23 novel genera and amended *Lactobacillus*, *Paralactobacillus* and *Pediococcus genera*) based on genomic analysis, which agreed well with ecological and phenotypic classification (Zheng *et al.* 2020). The term "lactobacilli" refers to all members of the *Lactobacillus* genus prior to re-classification.

Many *Lactobacillus* species naturally reside the human intestinal tract, although their abundance in the adult faecal microbiota is low (0.01-0.6% of total bacteria) (Kimura *et al.* 1997; Sghir *et al.* 2000; Tannock *et al.* 2000). This agrees with studies quantifying lactobacilli from small intestinal biopsies which indicate an abundance of 0.3-0.6% (Walter 2008). Interestingly, lactobacilli represent 45% of the faecal microbiota in infants > 6 months, suggesting an important role in intestinal homeostasis prior to maturation of the microbiota (Ahrné *et al.* 2005; Grönlund *et al.* 2000). Despite uncertainty over their contribution to gut health in adults, lactobacilli are commonplace in the market on commercial probiotics, reflecting the large number of studies demonstrating health-promoting activities. The WHO define probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2001). Randomised controlled trials (RCTs) assessing the probiotic activity of lactobacilli are common, as their consumption is "generally regarded as safe" by the American Food and Drug Administration, limiting the ethical requirements for study. A meta-analysis of existing RCTs revealed consistent positive outcomes in patients suffering with acute diarrhoea (reduced length of diarrhoea and/or frequency of stools) following oral administration of lactobacilli (Sazawal *et al.* 2006). Further RCTs have yielded promising results of lactobacilli on diarrhoea caused by enteric infection, necrotising enterocolitis in neonates and IBD (Deshpande, Rao, and Patole 2007; Dinleyici *et al.* 2015; Jia *et al.* 2018; Szymański *et al.* 2006). Whilst Health Canada

approves a multi-strain probiotic (including several Lactobacillus strains) to alleviate inflammatory bowel disease (IBD) symptoms, widescale adoption of probiotics to improve gut health is scarce (Plaza-Diaz *et al.* 2019).

1.2.6.2 Colonisation resistance by lactobacilli

1.2.6.2.1 Clinical efficacy of *Lactobacillus* treatment against diarrhoea

Colonisation resistance activity of lactobacilli has been extensively studied, and a wealth of data is available from both *in vitro* and *in vivo* studies. Of the *in vivo* studies, RCTs are the most relevant to investigating therapeutic potential. Two groups of RCT are commonly observed in literature: antibiotic-associated diarrhoea (AAD) and infectious diarrhoea (ID). AAD is seen in patients following broad-spectrum antibiotic treatment, which targets important members of the intestinal microbiota leading to dysbiosis. In contrast, trials on ID focus on disease caused by an external pathogen transmitted between individuals. Colonisation resistance is crucial in both groups, protecting the host from overgrowth of indigenous bacteria that could destabilise intestinal homeostasis as well as external threats. A comprehensive meta-analysis of studies investigating incidence of AAD in patients given probiotics revealed a statistically significant decrease following oral administration of lactobacilli (Goodman *et al.* 2021). Sub-group analysis by species indicated significant reduction in AAD incidence by *L. acidophilus*, *Lactobacillus delbrueckii subsp. bulgaricus*, *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei* and *Lacticaseibacillus rhamnosus* compared to non-treated controls. With regards to ID, Lactobacilli reduced duration and frequency of diarrhoea caused by rotavirus or bacteria (Van Niel *et al.* 2002). Recent meta-analyses focusing on particular strains indicated decreased duration of diarrhoea in ID by *L. rhamnosus* GG (Li *et al.* 2019).

Whilst RCTs allow to study beneficial effects of lactobacilli in the human GIT, they are small scale and difficult to control due the uniqueness of an individual's gut environment, often leading to considerable variation between studies. Hence, model systems of the human intestinal epithelium are routinely used to investigate colonisation resistance of lactobacilli in greater detail. These studies have revealed various potential mechanisms underpinning the antagonistic activity of lactobacilli against enteric pathogens.

1.2.6.2.2 Antagonism by lactobacilli

Growth inhibition of common enteric pathogens, such as *S.* Typhimurium, *Shigella sonnei* and EPEC, has been demonstrated by several *Lactobacillus* spp. *in vitro* (Drago *et al.* 1997; Piatek *et al.* 2020). Secretion of AM compounds appears to be a main mechanism as CFS of Lactobacillus cultures exhibit a similar degree of antagonism as whole culture (Chen *et al.* 2019; George-Okafor *et al.* 2020; Koohestani *et al.* 2018). Although bacteriocin production by lactobacilli is associated with growth inhibition, this is largely restricted to studies on uropathogens (Mokoena 2017). Lactobacilli produce lactic and, to a lesser degree, acetic acid during fermentation. Lactic acid production by *L. casei* and *L. plantarum* inhibited the growth of *L. monocytogenes*, interrupting proton pump function by decreasing the intracellular pH (Asahara *et al.* 2011; Nielsen *et al.* 2010). Although AM activity has been demonstrated in *E. coli* and *S. enteritidis* treated with organic acids, studies have been unable to directly show growth inhibition by organic acids produced during lactobacilli culture (ADAMS and HALL 1988; Marianelli, Cifani, and Pasquali 2010). This disparity might be due to different concentrations of lactic acid in experimental set-ups. While growth inhibition by lactic acid was evident at concentrations of 2-63 mM, bacterial lactic acid production in culture did not exceed 1.25 mM (ADAMS and HALL 1988; Marianelli, Cifani, and Pasquali 2010). Organic acids may therefore target more pH-sensitive enteric pathogens, such as *L. monocytogenes*. Hydrogen peroxide, a potent AM substance, is also produced by lactobacilli. Growth inhibition of *S.* Typhimurium by H_2O_2 produced by intestinal strains of *Lactobacillus johnsonii* has been demonstrated *in vitro* (Pridmore *et al.* 2008). Nevertheless, investigations into its activity have focused primarily on pathogens of the vaginal tract where it is associated with a decreased risk of bacterial vaginosis (Martín and Suárez 2010; Mitchell *et al.* 2015). Even in this domain, there is debate over the importance of H_2O_2 production to these benefits *in vivo* (Tachedjian, O'Hanlon, and Ravel 2018). Further studies are required to elucidate the impact of H_2O_2 on intestinal colonisation resistance by lactobacilli.

The species *L. reuteri* is distinct from other Lactobacilli in its ability to produce the AM compound reuterin from the fermentation of glycerol. In contrast to bacteriocins, which target closely related strains, reuterin exhibits activity against a range of organisms, particularly Gram-negative pathogens (Cleusix *et al.* 2007; Spinler *et al.* 2008). Interestingly, reuterin production is increased during co-culture with *E. coli*, suggesting that *L. reuteri* can recognise bacteria and upregulate production of the AM (Chung *et al.* 1989; Schaefer *et al.* 2010). Production of reuterin from PTA 6475, the strain used in this study, has been demonstrated *in vitro*. PTA 6475-derived reuterin can inhibit growth of a range of diarrhoeagenic organisms (including STEC and ETEC) in liquid culture (Spinler *et al.* 2008). Notably, PTA 6475 exhibited more potent growth inhibition of these pathogens compared to other *L. reuteri* strains.

1.2.6.2.3 Competitive Exclusion by lactobacilli

A large number of protein adhesins facilitating binding to the epithelial surface and/or secreted mucus have been identified in lactobacilli (Vélez, De Keersmaecker, and Vanderleyden 2007). Mucus-binding proteins (MUBs) and S-layer proteins (Slps) are families of adhesins targeting mucus and epithelial surfaces, respectively, and are widespread amongst lactobacilli (Hynönen *et al.* 2002; Johnson-Henry *et al.* 2007; Vélez, De Keersmaecker, and Vanderleyden 2007). In addition to these shared adhesin families, other adhesins have been characterised from individual species which often exhibit heterogenous expression between strains (Gross *et al.* 2010; MacKenzie *et al.* 2010; Matsuo *et al.* 2012; Miyoshi *et al.* 2006). Interestingly, several adhesins are associated with adhesion to purified mucins and non-mucus producing cell lines (e.g. Caco-2), suggesting a dual-role in mucosal adherence (Bergonzelli *et al.* 2006; Granato *et al.* 2004). The contribution of Lactobacillus adhesins to colonisation resistance has been investigated by assessing pathogen exclusion by recombinant adhesins. Pre-treatment of IECs with purified lactobacilli adhesins such as CbsA, MUBs5s6 and Slps, considerably attenuated adherence of ETEC, STEC and *S.* Typhimurium (Chen *et al.* 2007; Horie *et al.* 2002; Johnson-Henry *et al.* 2007; Singh *et al.* 2018). One study has also highlighted a role for steric hindrance, as inhibition of host cell adherence by common uropathogens was retained by cell wall fragments from *Lactobacillus* spp. even after protein denaturation by SDS treatment (Chan *et al.* 1985). The probiotic Lactobacillus species *L. reuteri* reduced adherence of *S.* Typhimurium and *Enterococcus faecalis* to Caco-2 cells without affecting pathogen viability (Todoriki *et al.* 2001). Likewise, human faecal isolates of *L. reuteri* inhibited adherence of *S. enterica* serovar Typhi, *L. monocytogenes* and *E. faecalis* to Caco-2 cells regardless of whether the commensal was added before or with the pathogen (Singh *et al.* 2017). This suggests direct competition of *L. reuteri* strains native to the human gut with enteric pathogens. Pathogen exclusion activity extends to the strain PTA 6475, which significantly reduced EPEC epithelial adherence to human small intestinal biopsies (Walsham *et al.* 2016). Mouse models infected with the closely-related pathovar STEC showed significantly reduced pathogen colonisation in the presence of PTA 6475 after 3 weeks (Eaton *et al.* 2011).

There is also evidence that *L. reuteri* can enhance the mucosal barrier. In DSS-colitis mice, oral supplementation of *L. reuteri* partially restored microbial composition and mucus layer integrity (Dicksved *et al.* 2012). In addition, ingestion of *L. reuteri* D8 increased gene expression of β-defensin 1 and MUC2 in the jejunum of neonatal pigs, corresponding to elevated numbers of goblet cells in the crypts (Wang *et al.* 2020). Furthermore, increased mucus thickness following exposure to *L. reuteri* ATCC PTA 4659 and R2LC has been observed in DSS-colitis mice (Ahl *et al.* 2016).

1.2.6.2.4 Lactobacilli: augmenting the mucosal barrier

Lactobacilli can stimulate production of TM and secretory mucins from IECs (Mack *et al.* 2003; Mack *et al.* 1999; Wang *et al.* 2014). In the case of TM MUC3, increased expression after incubation with lactobacilli was associated with attenuated EPEC adherence (Mack *et al.* 1999). Stabilisation of intestinal barrier function has been demonstrated for numerous lactobacilli. Both *L. rhamnosus* ATCC 53103 and *L. plantarum* ATCC 10241 ameliorated disruption of barrier function in Caco-2 cells treated with LPS (Blackwood *et al.* 2017). Similarly, pre-treatment with *L. plantarum* ZLP001 prevented loss of barrier function in IPEC-J2 porcine enterocytes infected with ETEC (Wang *et al.* 2018). In addition, *L. salivarius* and *L. rhamnosus* GG restored epithelial resistance in Caco-2 cells exposed to H_2O_2 (Miyauchi *et al.* 2012) (Seth *et al.* 2008). In DSS-colitis mice, oral supplementation of *L. reuteri* partially restored microbial composition and mucus layer integrity (Dicksved *et al.* 2012). Ingestion of *L. reuteri* D8 increased gene expression of β-defensin 1 and MUC2 in the jejunum of neonatal pigs, corresponding to elevated numbers of goblet cells in the crypts (Wang *et al.* 2020). Furthermore, increased mucus thickness following exposure to *L. reuteri* ATCC PTA 4659 and R2LC has been observed in DSS-colitis mice (Ahl *et al.* 2016). These data highlight that Lactobacilli can aid in maintaining the thickness of the mucosal barrier, which is crucial in restricting epithelial access to pathogens.

Colonisation of commensal bacteria, including lactobacilli, have been associated with secretion of SIgA in GF mice (Shroff, Meslin, and Cebra 1995). Studies have linked long-term consumption with lactobacilli with enhanced SIgA-dependent immune response in the gut (de Moreno de LeBlanc *et al.* 2008; Perdigón *et al.* 1999). *In vitro* analysis has confirmed that many lactobacilli induce SIgA secretion in PP cells harvested from the SIs of mice, frequently observed alongside increased activation of SIgA-secretin B cells (Arai *et al.* 2018; Kawashima *et al.* 2018; Kikuchi *et al.* 2014; Sakai *et al.* 2014). Activation of B cells by lactobacilli typically require the presence of DCs and TLR activation. In mice studies, innate SIgA protected against rotavirus and *S.* Typhimurium, as SIgA-secretion mutants exhibited increased pathogen colonisation and mortality (Franco and Greenberg 1997; Wijburg *et al.* 2006). The importance of SIgA in ameliorating infectious enteric disease combined with the induction of SIgA in B cells by lactobacilli suggests that this could be a crucial mechanism of colonisation resistance.

In addition to improving thickness and antagonistic activity of the mucosal barrier, Lactobacilli can also target intestinal permeability to augment the mucosal barrier. Numerous studies have associated *L. reuteri* consumption with improved intestinal barrier function. Enhanced expression of genes

encoding the TJ proteins occludin, ZO-1 and claudins was observed in IECs of lupus-prone mice following orally administration of *L. reuteri* CF48-3A (Mu *et al.* 2017). Furthermore, culture with *L. reuteri* I5007 attenuated LPS translocation and protected cells from loss of barrier function during ETEC infection in IPEC-J2 porcine IECs (Wang *et al.* 2016; Yang *et al.* 2015). Notably, restoration of epithelial barrier function was also reported in neonatal piglets following consumption of *L. reuteri* I5007, as well as children with atopic dermatitis treated with *L. reuteri* DSM 12246 (Rosenfeldt *et al.* 2004; Yang *et al.* 2015). Improved intestinal barrier function is thought to restrict access of bacterial toxins and MAMPs to the basal tissue, resulting in greater resilience to infection.

1.2.6.2.5 Immunomodulation by lactobacilli

Further to promoting mucosal IgA production, lactobacilli can also influence inflammation and T cell responses by direct interaction with PRRs (TLRs, NOD-like receptors (NLRs) and C-type lectin receptors (CLRs) or secreted immunomodulatory metabolites. The interactions between Lactobacilli and host immune system are complex, driven by a wide array of mediators that can differ between species and strains.

Cell wall components of lactobacilli have been frequently shown to modulate the host adaptive immune response (see **Fig. 1.10** for more detail on cell wall components of lactobacilli). Peptidoglycan (PG), teichoic acid (TA), proteinaceous pili, Slps and MUB can all influence crucial aspects of this response (Bene *et al.* 2017; Konieczna *et al.* 2015; Martínez *et al.* 2012; Smits *et al.* 2005; Fernandez *et al.* 2011; Grangette *et al.* 2005; Lebeer, Claes, and Vanderleyden 2012; Mohamadzadeh *et al.* 2011). Influencing T cell proliferation and maturation into T_h cells appears to be a conserved function of these immunomodulatory compounds. Due to these immunomodulatory activities, several studies have been successful at treating experimental colitis in mice with lactobacilli (Fernandez *et al.* 2011; Mohamadzadeh *et al.* 2011; Park *et al.* 2018).

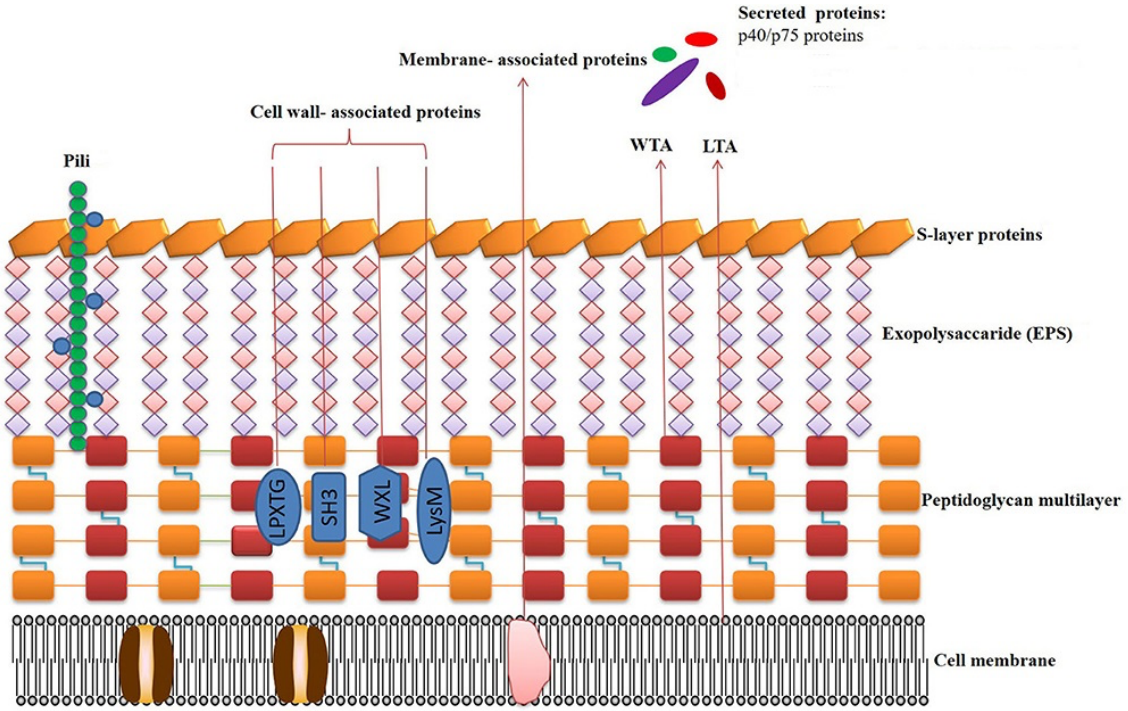


Figure 1.10 Cell-wall architecture of lactobacilli. Lactobacilli have a multi-layer cell wall composed of peptidoglycan, exopolysaccharide and s-layer proteins. Whilst this general structure is conserved, species- and strain-specific variations are present that can affect host interactions. WTA = wall teichoic acid, LTA = lipoteichoic acid. Figure adapted from Teame *et al.* 2020.

Lactobacilli DNA could play an important role in mediating inflammation. Bacterial DNA is recognised by TLR9, which distinguishes itself from vertebrate DNA by enhanced frequency of methylated cytosine-guanidine dinucleotides (CpG motif) (Stacey *et al.* 2003). Unlike immune cells, where TLR9 is only found within the cell, polarised IECs exhibit basal and apical surface expression of the receptor, suggesting that early detection of bacterial DNA is crucial to mucosal immunity (Hemmi *et al.* 2000; Wells 2011). Interestingly, different response to bacterial DNA have been observed from basal and apical TLR9; basal responses result in a pro-inflammatory response via NF-κb activation, whereas apical exposure does not, suggesting that IECs may be tolerant to bacterial DNA (Lee, Mo, Katakura, Alkalay, Rucker, Liu, Lee, Shen, Cojocaru, and Shenouda 2006). This extends to lactobacilli, as apical exposure of HT-29 monolayers to DNA from a probiotic mixture (VSL3) containing four *Lactobacillus* spp. failed to initiate IL-8 secretion (Jijon *et al.* 2004). Interestingly, DNA from enteric pathogens *Salmonella dublin* and *S.* Typhimurium elicited a strong IL-8 response (two-fold increase) from the monolayers, demonstrating a different response than to VSL3 DNA. This highlights a potential mechanism of differentiation between the DNA of pathogenic and commensal bacteria, although this such a mechanism has yet to be identified. Not only is lactobacilli DNA tolerated by IECs, but it also

appears to have anti-inflammatory activity. Further experiments by Jijon *et al.* revealed that treatment of HT-29 monolayers with VSL3 DNA decreased IL-8 secretion in response to *S.* Typhimurium, regardless of whether VSL3 DNA was incubated prior to or with pathogen DNA. The anti-inflammatory activity of lactobacilli DNA also extends to *L. rhamnosus* GG, as pre-treatment of polarised HT-29 and T84 monolayers reduced IL-8 secretion following TNF-α activation via stabilisation of IκB (Ghadimi *et al.* 2010).

One major question facing the involvement of these MAMPs is how frequently they are exposed to the intestinal epithelium given the mucosal barrier. Instead, secreted factors could be crucial in modifying the immune response. Indeed, immunomodulatory activity of lactobacilli CFS has been frequently reported (Bermudez-Brito *et al.* 2014; De Marco *et al.* 2018; Engevik *et al.* 2021; Gao *et al.* 2015a; Rieu *et al.* 2014; Vincenti 2010; Yan *et al.* 2007). These effects are often anti-inflammatory; suppression of pro-inflammatory cytokine secretion has been observed in human IECs and monocytes. in particular, CFS from *L. acidophilus*, *L. casei* and *L. delbrueckii subsp. lactis* attenuated IL-8 secretion from LPS-stimulated HT-29 cells, suggesting modulation of neutrophil chemotaxis following bacterial infection (De Marco *et al.* 2018). Other studies investigating immunomodulation by *L. rhamnosus* GG and *L. casei* ATCC 334 highlighted roles for the secreted proteins p40 and p75. The study using *L. rhamnosus* GG revealed anti-apoptotic activity in TNF-α-stimulated HT-29 cells and mouse colonic explants, whereas p40 and p75 from *L. casei* ATCC 334 was associated with suppression of TNF-α in LPS-stimulated THP-1 cells (Rieu *et al.* 2014; Yan *et al.* 2007).

SCFA production has been strongly linked to immunomodulation by the gut microbiota (see **section 1.2.5**). Although lactobacilli produce the SCFAs acetate, butyrate and propionate as well as lactic acid during anaerobic fermentation, there is very little evidence of their contribution to immunomodulation (Parada Venegas *et al.* 2019; Tan *et al.* 2014).

In contrast, EVs from lactobacilli have immunomodulatory effects, and EVs from *L. rhamnosus* GG attenuated IFN-γ secretion by peripheral blood mononuclear cells (PBMCs) stimulated with *S. aureus* CFS (Mata Forsberg *et al.* 2019). In addition, uptake of EVs from *L. plantarum* WCFS1 into Caco-2 cells IECs resulted in enhanced cathepsin and *Reg3γ* gene expression (Li *et al.* 2017). These data indicate that lactobacilli EVs may represent an emerging immunomodulatory feature of lactobacilli.

Taken together, these studies demonstrate a highly complex crosstalk between lactobacilli and the intestinal mucosa. Whilst some of the interactions are shared, some appear to be species- and strain-specific. Strain-specificity is a crucial consideration when discussing species with demonstrated immunomodulatory activity. *L. reuteri* is an excellent example of this, as certain strains (e.g. BM36301, CRL1098, PTA 6475, PTA 4659 and PTA 5289) have been associated with anti-inflammatory cytokine

profiles in cell lines and mouse models, whereas others (e.g. BM36304 and DSM 17938) have been linked to increases in pro-inflammatory cytokine release (Gao *et al.* 2015b; Griet *et al.* 2014; Lee *et al.* 2016; Liu *et al.* 2010).

With regards to immunomodulatory effects, PTA 6475 falls into the anti-inflammatory group of *L. reuteri* strains, and LPS-induced IL-8 secretion was significantly reduced in the presence of PTA 6475 in porcine IPEC-J2 cells (Liu *et al.* 2010). Similarly, PTA 6475 inhibited LPS-induced secretion of pro-inflammatory TNF-α from monocytic cell lines and macrophages from patients with Crohn's disease (Lin *et al.* 2008). This was associated with decreased phosphorylation of c-Jun, impacting its ability to interact with c-Fos to form the pro-inflammatory transcription regulator AP-1. Furthermore, recent data has demonstrated that this effect is dependent on the *L. reuteri* folate synthase FolC2. Interestingly FolC2 also has an anti-inflammatory role and mediates histamine production which decreases pro-inflammatory cytokine secretion in LPS-stimulated monocytes via the H2 histamine receptor (Dohlsten *et al.* 1988; Elenkov *et al.* 1998; Vannier, Miller, and Dinarello 1991). The anti-inflammatory effects of PTA 6475 on IECs exposed to LPS may also protect the epithelium from damage caused by inflammation during infection. In addition to production of AMs, effective epithelial colonisation and enhancement of the mucosal barrier, the anti-inflammatory activity of PTA 6475 is thought to contribute to the gut health benefits seen in mouse studies and RCTs (see **Fig 1.11**). Hence, it is an excellent choice as a potential probiotic treatment for EPEC infection.

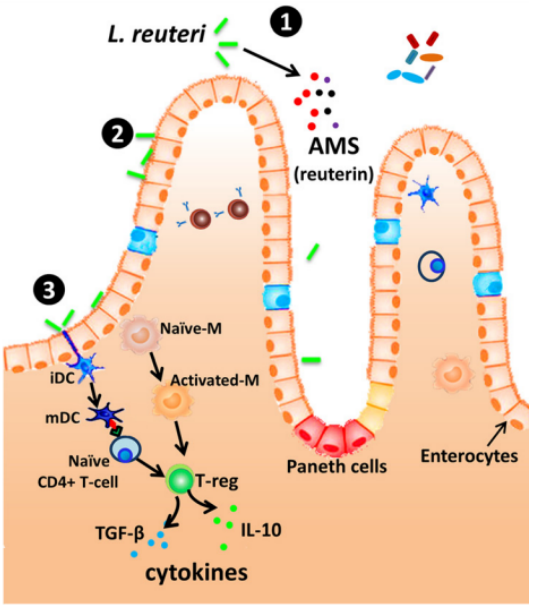


Figure 1.11 General mechanisms of colonisation resistance by *L. reuteri.* *L. reuteri* can protect from infection by (1) producing anti-microbial substances (AMS), (2) competing for epithelial binding sites and (3) modulating the host immune response. Figure adapted from Hou *et al.* 2015.

1.2.7 *Ruminococcus gnavus*

Ruminococcus gnavus is a Gram-positive, oxygen-sensitive microbe and a prominent member of the human gut microbiota. Metagenomic analysis of faecal matter from 124 Europeans identified *R. gnavus* in the top 57 organisms present in more than 90% of individuals (Qin *et al.* 2010). Its prominence is likely due to its capability to degrade a wide variety of mucins and dietary oligosaccharides (Cervera-Tison *et al.* 2012; Crost *et al.* 2013). Consequently, *R. gnavus* may be a crucial organism in shaping the microbiota by freeing easily digestible sugars from oligosaccharides that are inaccessible to many of the neighbouring organisms. Much of the research on *R. gnavus* has focused on its role in metabolism rather than colonisation resistance. Indeed, there is no commercially available probiotic containing *R. gnavus.* Nevertheless, there is some data to suggest that *R. gnavus* has the potential to protect the gut from bacterial infection. A class of bacteriocins, called ruminococcins, have been purified from *R. gnavus* both in culture and in rat models (Crost *et al.* 2011; Dabard *et al.* 2001; Marcille *et al.* 2002; Ramare *et al.* 1993). Ruminococcin A (RumA), is produced by *R. gnavus* in liquid culture and has been shown to inhibit growth of pathogenic strains of *Clostridium*, including *C. difficile* and *Clostridium botulinum* (Dabard *et al.* 2001). Nevertheless, minimal activity against Gram-negative bacteria including *E. coli* and *Salmonella spp*. suggests that the spectrum of activity is restricted to Gram-positive bacteria (Dabard *et al.* 2001). More recent data, however, has shown that expression of *rumA* in rat models is low and antimicrobial effects observed *in vitro* may not be relevant (Dabard *et al.* 2001). Instead, this study identified a distinct ruminococcin, RumC, which is expressed sufficiently to result in inhibition of *C. difficile* growth in plate cultures. Nevertheless, RumC extracted from GF rats colonised by *R. gnavus* failed to inhibit growth of *Salmonella enteritidis in vitro*, suggesting a similar spectrum of activity as RumA (Chiumento *et al.* 2019; Crost *et al.* 2011; Dabard *et al.* 2001). Therefore, ruminococcins are unlikely to play a role in colonisation resistance against EPEC infection.

Nevertheless, a recent publication has highlighted a potential role for *R. gnavus* in out-competing pathogens as culture of *C. difficile* with a biofilm of nine common gut commensals including *R. gnavus,* significantly reduced pathogen numbers (Hassall *et al.* 2021). Although the use of multiple species makes it difficult to conclude the contribution of *R. gnavus,* elevated numbers of *R. gnavus* were observed and may indicate a specific response to the pathogen. In addition, *R. gnavus* decreased

proliferation of commensal *E. coli* in mixed-culture biofilms, suggesting competition between the two species.

RG has been strongly associated with intestinal inflammation due to its expansion in the gut microbiota of IBD patients (Hall *et al.* 2017b). Further phylogenetic analysis categorised *R. gnavus* into two clades: Clade I, which was associated with a healthy gut and Clade II which was predominant in IBD. Although the mechanisms behind the different inflammatory phenotypes of the two clades have not been unravelled, recent publications have provided some insight into the involvement of *R. gnavus* in intestinal immune responses. Interestingly, expression of the antimicrobial protein Reg3γ was upregulated by *R. gnavus* ATCC 21949 in humanised mice confirming beneficial impact of this Clade I strain. Unexpectedly, a glucorhamnan secreted by *R. gnavus* ATCC 21949 elicited TNF-α secretion from DCs in a TLR4-dependent manner (Henke *et al.* 2019). Nevertheless, this glucorhamnan appears to be conserved amongst all sequenced strains regardless of their Clade, raising doubts over its involvement in IBD. A follow-up study demonstrated that the pro-inflammatory activity of *R. gnavus* depends on the absence of a capsular polysaccharide (Cps) surrounding the bacteria as strains lacking Cps stimulated TNF-α secretion in murine DCs, whereas Cps^{+} strains did not (Henke *et al.* 2021). Interestingly the cps contains several components that are often involved in immunomodulation, such as TAs and PG, which are likely to be crucial to the activity shown in this study.

Taken together, these studies highlight a crucial role of *R. gnavus* in the healthy microbiota by liberation of mucus-derived sugars and interactions with the immune system. Nevertheless, very little information is available about the involvement of *R. gnavus* in protection from enteric pathogens, which may be due to differing oxygen requirements of the obligate anaerobe and the intestinal epithelium. Development of novel model systems that permit side-by-side culture may elucidate protective effects of *R. gnavus*.

1.3 Model Systems of the Human Gut

The physiology of the human gut is highly dynamic due to an array of exogenous and endogenous factors. Oxygen concentration, pH, mucus thickness, transit rate (peristalsis), epithelial morphology (microvilli, villi, crypts of Lieberkühn) and barrier function can change along the gut and also at specific sites due to infection, inflammation and shifts in microbiota populations (see **Fig. 1.12A**). The gut epithelium is composed of six main cell types: absorptive enterocytes, mucus-producing goblet cells; enteroendocrine cells which release hormones; AM-secreting Paneth cells; M-cells, which act as the interface between the lumen and the immune cells of the lamina propria; Tuft cells and Cup cells, both of which remain relatively unexplored (**Fig 1.12B**) (Gerbe, Legraverend, and Jay 2012; van der Flier and Clevers 2009). This level of complexity is a considerable hurdle that model systems of the gut have to

overcome. Although mouse models share many of these features that are difficult to replicate *in vitro*, the specificity of adaption of the microbiota to the host makes it difficult to translate the results to the human gut. In addition, the use of non-native models is a particular problem for many human pathogens such as EPEC, which do not colonise the mouse epithelium or stimulate diarrhoea (Dupont *et al.* 2016). Consequently, human-derived model systems are required to conduct meaningful experiments which can eventually have an impact at the clinical level.

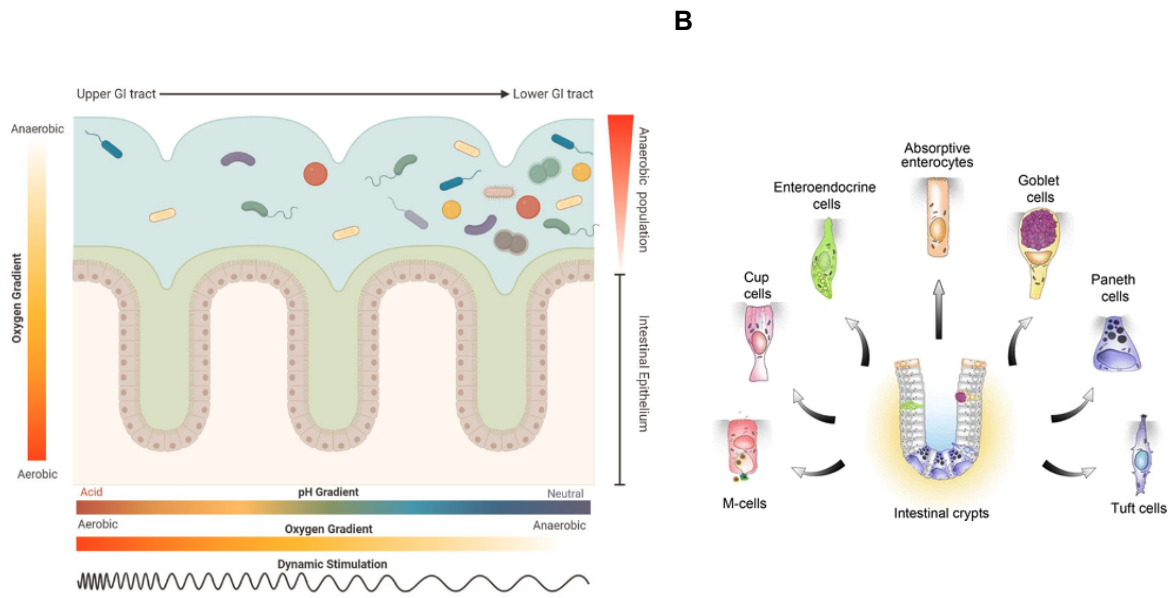


Figure 1.12. Features of the GIT relevant for the development of human experimental models. (**A**) Illustration outlining the key physiological conditions of the GIT and how they vary at each site. (**B**) Major cell types of the intestinal epithelium. Figures sourced from Gerbe, Legraverend, and Jay 2012, and Sardelli *et al.* 2021.

1.3.1 Cell line-based Model Systems

1.3.1.2 Traditional cell culture

In terms of simplicity, cell lines are an attractive option as a gut model system. Caco-2, HT-29 and T84 are commonly used cell lines derived from human colon carcinomas. These cell lines are able to form differentiated monolayers when cultured on permeable membrane supports, exhibiting tight junctions between cells and apical brush border microvilli (Devriese *et al.* 2017; Martínez-Maqueda, Miralles, and Recio 2015). As TJs restrict paracellular transport, expression of water and ion transporters is increased at the apical surface of polarised IECs to facilitate transcellular uptake (Engevik and Goldenring 2018; Kipp *et al.* 2003; Noel, Roux, and Pouysségur 1996). As some of these,

such as NHE3 and SGLT1, are targeted by EPEC and other diarrhoeagenic pathogens, correct localisation is necessary to study virulence more accurately. In addition, polarisation is important for biologically relevant immune responses of the epithelium as TLRs can be distributed asymmetrically on apical and basal cell membranes (Cario *et al.* 2002; Gewirtz *et al.* 2001; Stanifer *et al.* 2020). (Schuller *et al.* 2009). A common application of polarised IEC culture is the Transwell system where cells are cultured on a permeable filter membrane in a plastic support which is suspended in a well plate. This generates separate apical and basal compartments which can be modified accordingly (Ulluwishewa *et al.* 2015; von Martels *et al.* 2017). Whilst T84, Caco-2 and HT-29 cells readily form polarised monolayers, they only represent a single cell type (i.e. absorptive enterocyte/colonocyte) of the intestinal epithelium.

1.3.1.2 Mucus-producing models

The intestinal epithelium is covered by mucus, formed predominantly of the secreted MUC2 glycoprotein. Secretion of MUC2 is the primary function of specialised goblet cells, which constantly synthesise and export stores of intracellular MUC2 to maintain the intestinal mucus layer(s) (Allen, Hutton, and Pearson 1998; Asker *et al.* 1998). Mucus forms a barrier between luminal bacteria and the host epithelium, which is crucial for retaining intestinal homeostasis (Apella *et al.* 1992; Johansson *et al.* 2008; Van der Sluis *et al.* 2006). Additionally, mucus acts as a nutrient source and colonisation site for the microbiota (Arike, Holmen-Larsson, and Hansson 2017; Juge 2012). Consequently, intestinal epithelial models that incorporate a mucus layer allow researchers to gain more insight into host-microbe interactions. Cell lines derived from goblet cells have been routinely used to study host-bacteria interactions in the presence of a mucus layer. Although not a mucus-secreting cell line, treatment of HT-29 with 10 µM methotrexate produces a homogenous subpopulation exhibiting goblet cell-like properties such as intracellular storage and secretion of mucins (Lesuffleur *et al.* 1990). Mucin gene expression in the HT29-MTX subpopulation was enhanced relative to HT-29, particularly for MUC3 (TM) and MUC5AC (major component of secreted gastric mucus), suggesting differentiation into goblet cells of the gastric epithelium (Lesuffleur *et al.* 1993). HT29-MTX cells have been used to assess host-microbe interactions with gut bacteria, from mucus-degradation by pathogens to adherence efficiency relative to non-mucus secreting cell lines (Gagnon *et al.* 2013; Gopal *et al.* 2001; Lee *et al.* 2015). Despite their mucus-secreting properties, the resemblance of HT29-MTX to goblet cells of the gastric epithelium raises doubts over their application to study intestinal mucus, which is composed primarily of MUC2. In contrast, the LS174T cell line, derived from colonic adenocarcinoma tissue, expresses MUC2 at similar levels as human jejunal biopsy tissue (van Klinken *et al.* 1996).

Furthermore, MUC2 protein is present in cell lysates and culture medium of LS174T cells, demonstrating mucin secretion (McGuckin, Devine, and Ward 1996; van Klinken *et al.* 1996). A handful of studies have utilised LS174T to investigate adherence of gut bacteria and virulence properties of DEC, though their use to study host-microbe interactions is uncommon (Hews *et al.* 2017; Walsham *et al.* 2016).

Despite their practicality, cancer-derived cell lines exhibit considerable genetic and phenotypic changes compared with native intestinal epithelium which likely affects their response to infection and interaction with commensal organisms (Vogelstein and Kinzler 2004).

1.3.2 *Ex vivo* systems

1.3.2.1 *In vitro* organ culture

In vitro organ culture (IVOC) of human intestinal mucosal biopsy tissue has been used extensively to investigate A/E lesion formation in EPEC and STEC (Knutton, Lloyd, and McNeish 1987). Explant culture offers several advantages over cell lines including the presence of all epithelial cell types and a continuous mucus layer (Fang, Schüller, and Phillips 2013; Walsham *et al.* 2016). Polarised IVOC (pIVOC) is a modification which allows restrictive bacterial access to the mucosal biopsy surface and is thus suitable to assess the inflammatory response to EPEC infection. In addition, reduced EPEC adherence in the presence of *L. reuteri* PTA 6475 was observed in pIVOC of duodenal biopsy samples.

Disadvantages of the IVOC system include frequent access to patient samples, limited tissue viability (8-24 h), and the requirement of hyper-oxygenated conditions (to keep the tissue alive) which is unsuitable for the study of oxygen-sensitive gut microbes.

1.3.2.2 Human intestinal organoids

Human intestinal organoids are a newly developed model system derived from native tissue that contain many of the different cell types of the intestinal epithelium. In addition, organoids can be propagated and stored in liquid nitrogen (Sato *et al.* 2011). Intestinal organoids can be established from small intestinal (enteroids) or colonic tissue; (colonoids) (Zachos *et al.* 2016). Traditionally, enteroids/colonoids are 3-dimensional, self-assembling intestinal epithelial spheroids propagated from intestinal biopsy samples (see **Fig. 1.13**). This is possible due to the presence of pluripotent stem cells located at the base of intestinal crypts. Culturing these stem cells with the signalling factors

Wnt3a, R-spondin and Noggin results in the formation of a 3D spheroid with the luminal surface facing inwards (Sato *et al.* 2011). These structures contain all major IEC types and form villus-like structures which are organised spatially as seen in biopsy tissue (Zachos *et al.* 2016). Initial application of hIOs for studying host-bacteria interactions was technically challenging due to the "inverse" nature of the 3D spheroids, making luminal access only possible through microinjection (Karve *et al.* 2017). Since then, protocols to reverse polarity of spheroids and culture them as 2D monolayers on Transwell supports have been developed (Co *et al.* 2019; Foulke-Abel *et al.* 2014; VanDussen *et al.* 2015).

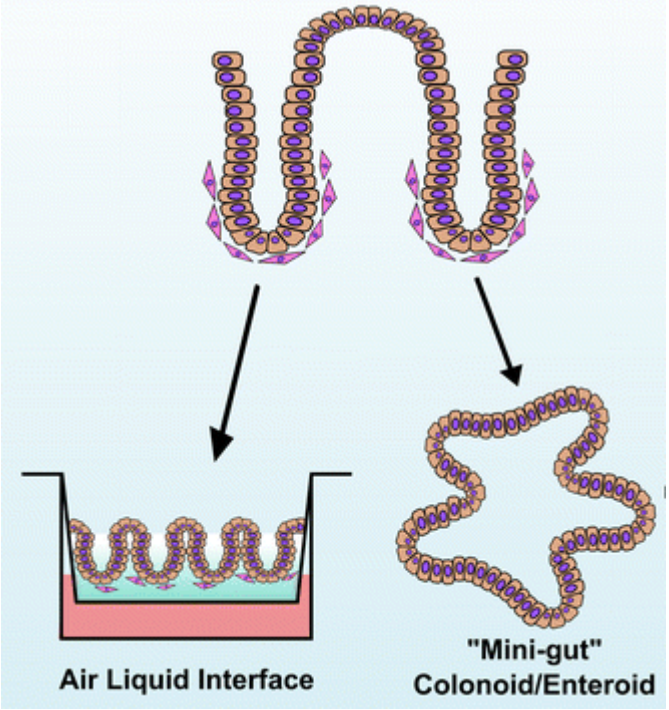


Figure 1.13 Diagram showing the different applications of the colonoid/enteroid model. "Mini-guts" are the 3D, spheroid models and the Air Liquid Interface refers to monolayers grown on Transwells. Figure sourced from Short, Costacurta, and Williams 2017.

Enteroid/colonoid monolayers have proven particularly useful for investigating host-pathogen interactions. Their capability to produce a continuous mucus layer has facilitated the study of mucus-degradation by EAEC and STEC (In *et al.* 2016; Liu *et al.* 2020). Interestingly, brush border microvilli damage, A/E lesion formation and TJ breakdown was observed following STEC infection of colonoids, indicating that these virulence features are retained (In *et al.* 2016). An interesting adaptation to 2D enteroid culture, where macrophages are cultured beneath the monolayer, has been developed to study the immune response to ETEC infection (Noel *et al.* 2018). In addition to studying host-pathogen interactions, these organoid models have the potential to provide insights into how the microbiota influences these relationships. The presence of cells involved in the immune response, as well as

hormone and mucus secretion, could elucidate novel mechanisms of colonisation resistance. Nevertheless, conventional culture of organoids in an air/5% CO2 incubator restricts their application to oxygen-tolerant bacteria.

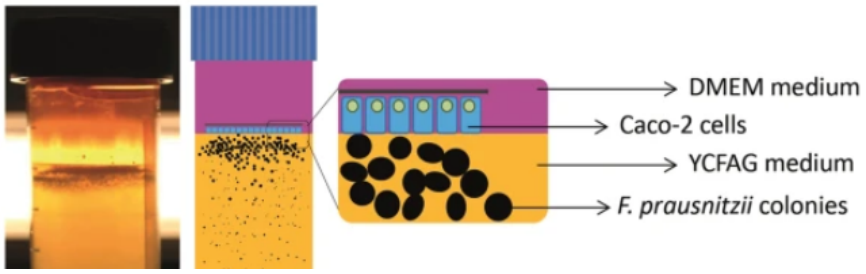
1.3.3 Microaerobic models

The intestinal lumen is extremely low in oxygen and mainly colonised by anaerobic microbes which(Albenberg *et al.* 2014; Crompton, Shrimpton, and Silver 1965; Lind Due *et al.* 2003; Macfarlane and Macfarlane 2012). The luminal oxygen pressure (pO_2) in the GIT decreases along a longitudinal axis, and measurements in mice estimate pO_2 at 7.6%, 4.2%, 1.3% and 0.4% in the stomach, duodenum, mid-small intestine and distal colon, respectively (He *et al.* 1999). These values agree with the bacterial populations of each location, as strict anaerobes dominate in the colon whereas facultative anaerobes are common in the small intestine (Booijink *et al.* 2010; Qin *et al.* 2010; Zoetendal, Akkermans, and De Vos 1998; Zoetendal *et al.* 2012). Low luminal oxygen concentrations allow anaerobic fermentation of dietary fibre and resistant starches consumed by the host. Products of fermentation, such as SCFAs and reuterin, may have considerable implications on colonisation resistance mediated by the intestinal microbiota. In addition to decreasing luminal O_2 concentrations along the GIT, a steep O_2 gradient exists between the lumen and submucosa. Oxygen levels at the vascularised submucosa of mice are an estimated 6%, compared to <1% of atmospheric pressure measured in the lumen (Albenberg *et al.* 2014; Schwerdtfeger *et al.* 2019). It is probable that bacteria, in particular adherent pathogens, utilise this gradient to sense their proximity to the epithelial surface. Indeed, some preliminary studies demonstrated that expression of the STEC T3SS increases with oxygen concentration (Carlson-Banning and Sperandio 2016). This agreed with an *in vitro* study indicating enhanced adherence and effector translocation into host cells by STEC under microaerobic conditions (Schuller and Phillips 2010). The potential to gain new insight into host-microbe interactions under low oxygen concentrations has driven the development of novel microaerobic model systems of the GIT.

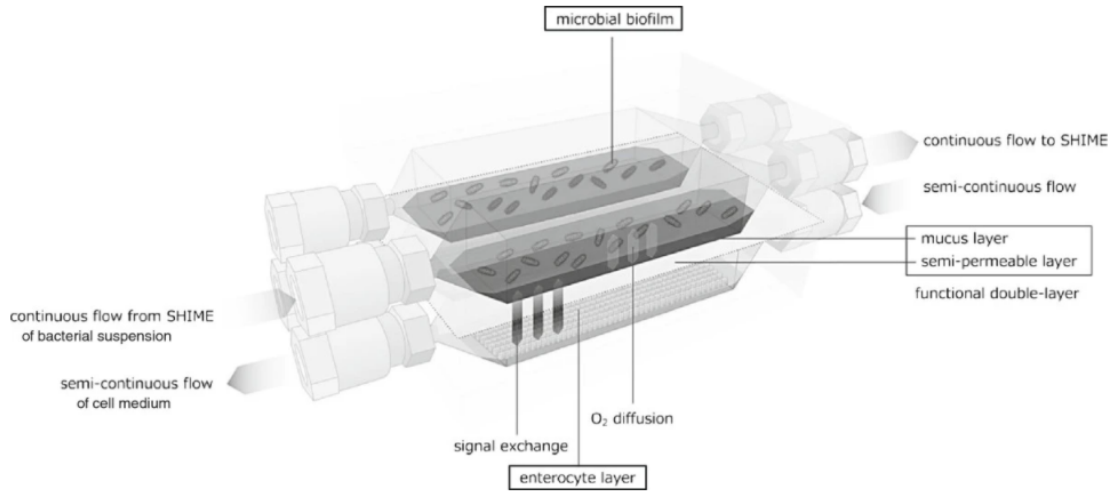
To overcome the challenge of culturing IECs in an apical anaerobic environment, several next-generation model systems have been developed (see **Fig. 1.14**). Many of these involve some form of compartmentalisation of the apical and basal areas surrounding the IECs, allowing to maintain a low-oxygen apical environment whilst supporting the host cells with oxygenation at the basal side. The way in which this is achieved is different between each model, as is the complexity. Models with high

levels of complexity often offer additional features which may be of interest to researchers depending on the aim of their study.

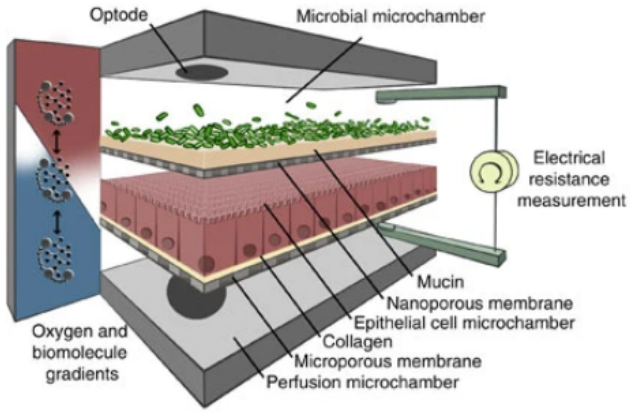
A - HoxBan



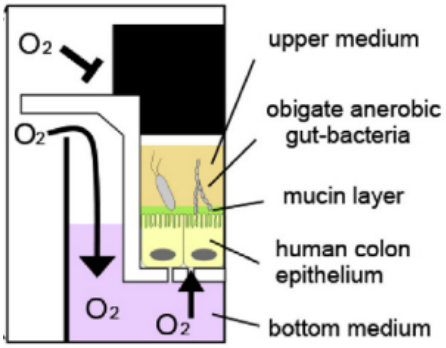
B – HMI™ module



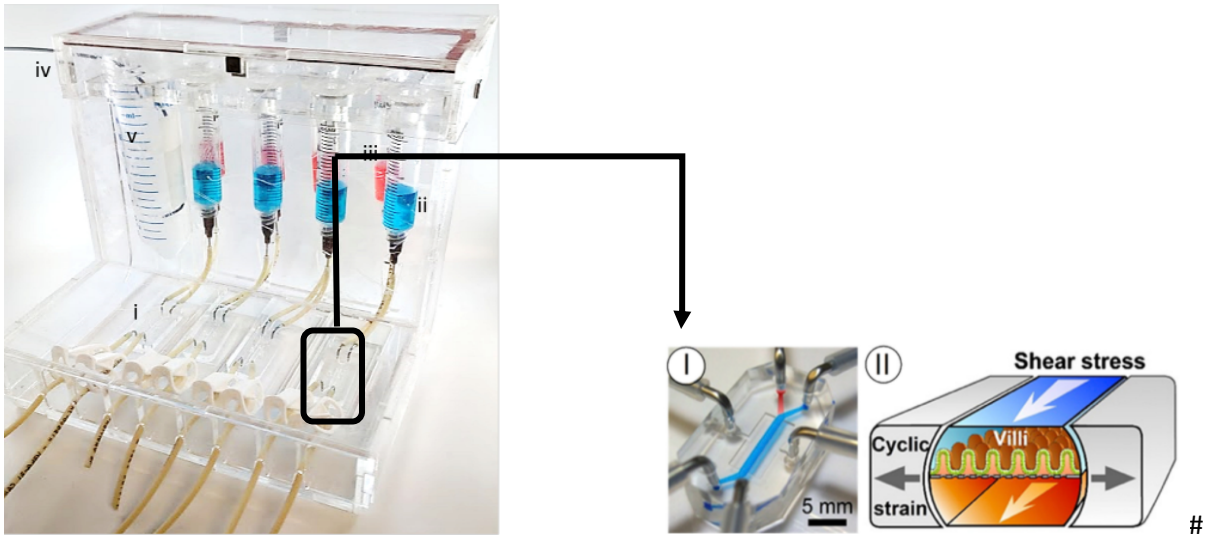
C – HuMiX



D – Anaerobic Transwell



E – Anaerobic Gut-on-a-Chip



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F – GuMi

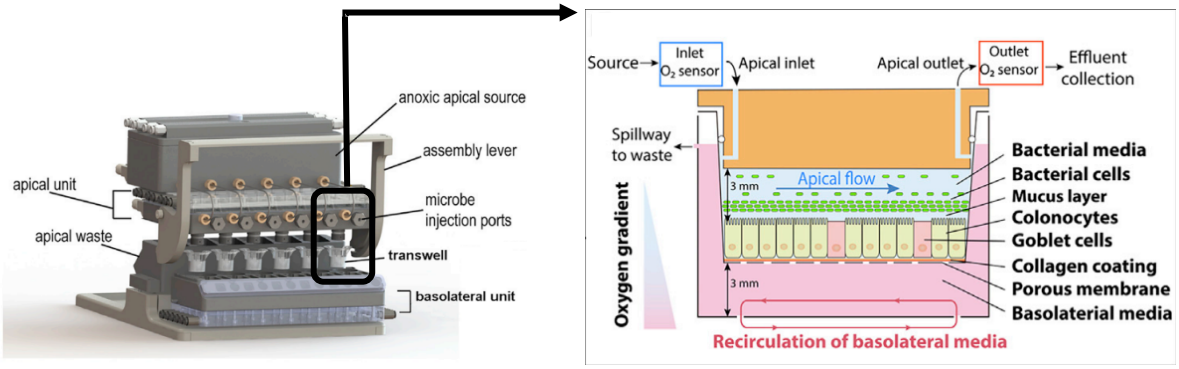

Figure 1.14 Microaerobic model systems of the intestinal epithelium. (**A**) Human-oxygen Bacteria-anaerobic (HoxBan) (Sadabad *et al.* 2015), (B) Host-Microbiota module (HMI™) (Marzorati *et al.* 2014), (C) Human-Microbe Crosstalk (HuMiX) (Shah *et al.* 2016), (D) Anaerobic Transwell (Sasaki *et al.* 2020), (E) Anaerobic Gut-on-a-Chip (Jalili-Firoozinezhad *et al.* 2019), (F) Gut Microbiome (GuMi) physiomimetic platform (Zhang *et al.* 2020).

1.3.3.1 Human-oxygen Bacteria-anaerobic system

The **H**uman **ox**ygen-**B**acteria **an**aerobic (HoxBan) system is the least complex of the next-gen models, requiring standard laboratory reagents (Sadabad *et al.* 2015). Host cells, grown on glass coverslips, are placed on semi-solid agar containing anaerobic bacteria, allowing direct microbe-host cell contact. The cells are then submerged in aerobic cell culture medium. This system enabled culture of the anaerobic gut commensal *Faecalibacterium prausnitzii* in the presence of Caco-2 cells. Whilst this system is relatively simple and inexpensive to set up, it is also lacking features (e.g. polarised epithelia, diffusion of secreted metabolites, compatibility with downstream assays) that would have considerable impact on studying host-microbe interactions.

1.3.3.2 Host-Microbiota™ module

The **H**ost-**MI**crobiota interaction (HMI™) module is an advancement of the **S**imulator of the **H**uman **I**ntestinal **M**icrobial **E**cosystem (SHIME), which is a series of bioreactors that simulate the pH and digestive environment of the GIT (Marzorati *et al.* 2014). The module comprises a microbial compartment with inlets and outlet to permit the flow of the SHIME effluent, and a host cell compartment containing cells grown on a membrane with a supply of oxygenated cell culture medium beneath. The two compartments are separated by a semi-permeable membrane, which is necessary as exposure of host cells to the SHIME effluent reduces viability up to 80% within 8 hours. Nevertheless, this model allows the study of contact-independent interactions between a complex microbiota and host epithelium.

1.3.3.3 Human-Microbe Crosstalk system

The **Hu**man-**Mi**crobe crosstalk (HuMiX) is another dynamic fluid system with artificial physical separation between the host cells and bacteria, which can then be supplied with oxygenation and anaerobic medium (Shah *et al.* 2016). Whilst it lacks the complex microbiota and physiological conditions of the HMI (offered by the SHIME), it allows more focused studies on specific bacteria,

which can be introduced at precise amounts by the user. Nevertheless, the lack of direct interaction with host may be a hurdle to studying contact-dependent host-microbe interactions.

1.3.3.4 Anaerobic Transwells

The anaerobic Transwell system is generated by adding anaerobic culture medium to the apical side of cells grown on Transwell membranes and sealing apical compartments with a cap to restrict gas transfer (Ulluwishewa *et al.* 2015). Until recently, its use was restricted to cell lines, but a recent study cultured enteroids on Transwells under low oxygen conditions (Sasaki *et al.* 2020).

1.3.3.5 Anaerobic Gut-on-a-Chip

The anaerobic gut-on-a-chip model is a recent adaption of the existing gut-on-a-chip system (Jalili-Firoozinezhad *et al.* 2019; Kim *et al.* 2012). Chips are placed in chamber composed of gas-permeable plastic, which is then perfused with anaerobic gas (95% N_2 + 5% CO_2), producing O_2 levels of ~1% after 2 days. Viable Caco-2 cells were maintained in this system for up to 6 days, utilising oxygen from the continuous supply of medium to the basal side of the epithelia. An additional feature of the gut-on-a-chip is the simulation of peristalsis through stretching and relaxing of the membrane beneath the IECs (Kim *et al.* 2012). The gut-on-a-chip has been successfully used to culture Caco-2 cells and enteroids with single commensal species (e.g. *B. fragilis)* as well as a complex human gut microbiota (Kim *et al.* 2012; Kim *et al.* 2016).

1.3.3.6 Gut Microbiome physiomimetic platform

Another dynamic fluidic system, the **Gu**t **Mi**crobiome physiomimetic platform (GuMi), has been recently developed utilising colonoids grown on Transwells (Zhang *et al.* 2020). The innovative system utilises an artificially generated vacuum to drive the flow of anaerobic medium over the apical surface of the cells, mimicking the shear stress created by the flow of intestinal chyme *in vivo*. Replenishment of anaerobic medium from an external reservoir maintains the low oxygen apical conditions. Application of this system to investigate interactions between *F. prausnitzii* and colonoids over several days demonstrated enhanced butyrate concentration in apical effluent (0.1 to 19 µM). Commensal-dependent butyrate production was associated with reduced *NF-κB*, *TLR2* and *TLR3* expression.

1.3.3.7 Vertical Diffusion Chamber

The Vertical Diffusion Chamber (VDC) is a dual compartment system suitable for culturing polarised IECs on membrane filters (Fig. 1.15). Apical and basolateral compartments can be filled with defined culture media and perfused with different gas mixtures. Under anaerobic gas perfusion, oxygen levels in the VDC have been measured at 1-1.7% of atmospheric pressure, indicating a microaerobic environment similar to that at the gut mucosal surface (Schuller and Phillips 2010). So far, the VDC model has been used to determine the influence of oxygen on intestinal pathogenesis of STEC, *H. pylori*, *C. jejuni* and *C. difficile* (Anonye *et al.* 2019; Cottet *et al.* 2002; Mills *et al.* 2012; Schuller and Phillips 2010). In addition, co-culture of intestinal epithelia with the anaerobic commensal *Bacteroides dorei* has been demonstrated (Anonye *et al.* 2019).

The VDC has many features which make it a suitable model system to investigate the influence of gut commensals on EPEC infection. Firstly, this system allows direct interaction between the bacteria and host. Epithelial attachment is fundamental to pathogenesis of adherent pathogens such as EPEC, and competition for adherence sites could be a potent mechanism of reducing EPEC virulence by gut commensals. Notably, virulence traits, such as A/E lesion formation and adherence, are retained during culture of STEC in the VDC (Schuller and Phillips 2010). Furthermore, the use of polarised IECs is crucial to study EPEC pathogenesis as polarisation influences the expression of apical receptors (e.g. TLRs, ion channels) and tight junctions, which are targets for EPEC effector proteins and commensal colonisation resistance activities. The VDC is also compatible with a range of downstream assays. Assessment of host cytokine secretion, barrier function, epithelial structure, and pathogen adherence and invasion have all been demonstrated in studies utilising the VDC (Lewis *et al.* 2015; Mills *et al.* 2012; Naz *et al.* 2013). The VDC affords the user significant experimental control. Bacterial inoculums, apical and basal media can all be regulated to suit the user's needs. Moreover, the apical medium can be sampled for assessments (e.g. growth or cytokine secretion) without significantly disrupting the microaerobic conditions due to constant perfusion of anaerobic gas and affixable bungs. The reasons make the VDC a potentially powerful tool to assess host-microbe interactions, particularly the influence of commensal organisms on pathogen virulence.

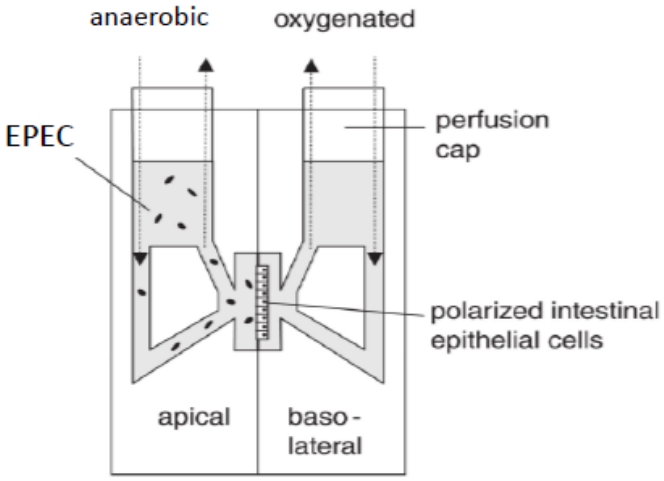

Figure 1.15 Schematic diagram of the Vertical Diffusion Chamber. This diagram shows infection with EPEC, although other pathogens have been used (e.g. STEC, *C. jejuni*, *C. difficile*). Diagram modified from Schuller and Phillips 2010.

1.4 Aims and Objectives

Understanding the interactions between members of the gut microbiota and EPEC may provide crucial insight for the development of novel therapeutics. The overarching aim of this study was to investigate colonisation resistance activities of the gut commensals *L. reuteri* PTA 6475 and *R. gnavus* ATCC 35913 in intestinal EPEC infection. This was achieved by 1) establishing a microaerobic Vertical Diffusion Chamber system allowing the culture of EPEC, *L. reuteri* and *R. gnavus* with mucus-producing human intestinal epithelia, 2) determining the influence of mucus-secreting LS174T cells on bacterial growth, adherence and pro-inflammatory host response and 3) investigating the influence of *L. reuteri* and *R. gnavus* on EPEC proliferation, host cell adhesion, T3S and host interleukin-8 response.

Chapter 2 – Methods & Materials

2.1 Bacterial Strains

2.1.1 Strains and culture conditions

EPEC strain E2348/69, serotype O127:H6, was provided by Jim Kaper (University of Maryland, Baltimore, US) and the Δ*escN* mutant from Gad Frankel (Imperial College London, UK), *L. reuteri* strain MM4-1A (ATCC PTA 6475) and *R. gnavus* (ATCC 35913) were obtained from Nathalie Juge. EPEC was grown either statically in Brain Heart Infusion broth (BHI, Oxoid) for liquid cultures, or on Lysogeny broth agar (1.5% w/v) (LB-Lennox, Merck) for plate cultures, at 37°C overnight. EPEC liquid cultures were prepared by inoculating bacteria directly from a streak plate into BHI. Streak plates were stored for up to one month at 4°C, after which fresh streak plates were prepared. For *L. reuteri* liquid cultures, frozen glycerol stocks were inoculated directly into de Man, Rogosa and Sharpe (MRS) broth (Oxoid) and cultured statically at 37°C overnight. To limit oxygen exposure, the lid of the culture vessel was tightened to reduce gas exchange. For *L. reuteri* plate culture, bacteria were grown overnight on MRS agar in an anaerobic cabinet (5% CO_2, 10% H_2 and 85% N_2, Don Whitley Scientific) at 37°C. *R. gnavus* liquid cultures were prepared by inoculating defrosted glycerol stock directly into oxygen-free BHI supplemented with yeast extract and haemin (BHI-YH) and incubating in an anaerobic cabinet at 37°C overnight. Removal of oxygen from the medium was achieved using the Hungate technique (Hungate, 1950). Briefly, medium was boiled, then anoxic gas bubbled through to remove oxygen trapped within the medium. For plate culture, *R. gnavus* was grown on BHI-YH agar in an anaerobic cabinet for 48-72 hours. BHI-YH was made by supplementing BHI broth with 5 g/L of BD Bacto™ Yeast Extract (Becton Dickinson) and 5 mg/L Haemin (Merck).

2.1.2 Cryopreservation and thawing of strains

For EPEC and *L. reuteri*, stock cultures were preserved in 15% (v/v) glycerol. A 50% glycerol solution was prepared in distilled H2O (dH_2O) and sterilized by autoclaving. 300 µL 50% glycerol solution was mixed with 700 µL fresh overnight culture in a sterile ampoule by pipetting and snap frozen on dry ice before long term storage at -70 °C. For *R. gnavus*, stock cultures were preserved in 40% (v/v) glycerol. An 80% glycerol solution was prepared in dH_2O, sterilized by autoclaving and placed in an anaerobic cabinet at least 48 hours prior to use to remove oxygen. 250 µL 80% glycerol solution was mixed with 250 µL fresh overnight culture in a sterile ampoule by pipetting and transferred directly to -70°C for long term storage.

2.1.3 Growth curve analysis

Growth curves were performed in 96-well plates by diluting bacterial overnight cultures 1:100 in culture medium containing reagent for assessment. For polymyxin B (Merck) MICs, a two-fold serial dilution was performed to assess bacterial growth across a range of concentrations (1-32 μg/mL). For porcine gastric mucin (PGM, Merck), concentrations of 0.125-1% (w/v) were tested. Growth was assessed by optical density at 600 nm (OD_{600}). For EPEC, plates were cultured at 37°C in aerobic conditions, and OD_{600} measurements taken using a FLUOstar Optima microplate reader (BMG) and analysed with the MARS data analysis software. For *L. reuteri* and *R. gnavus*, experiments were performed in an anaerobic cabinet at 37°C and readings taken with an Infinite F50 microplate reader (Tecan) and data collected with Magellan software.

2.2 Cell lines and culture conditions

2.2.1 General cell culture

Human colon carcinoma T84 cells (ATCC CCL-248) were cultured in Dulbecco's Modified Eagle's Medium/Ham's F-12 Nutrient (DMEM/F-12) medium (Merck) supplemented with 10% foetal bovine serum (FBS) (Merck) and 2.5 mM L-glutamine (Merck). Human colon carcinoma LS174T cells (ATCC CL-188) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 4 mM L-glutamine and 1x Non-Essential Amino Acid solution (Merck). Cells were used between passages 46-65 and split at a ratio of 1:5-1:8 (T84), or between passages 7-27 and split at a ratio of 1:8-1:24 (LS174T). Cells were grown in 25 cm^2 culture flasks (Sarstedt) and passaged at full confluency. To passage cells, spent medium was removed from culture flasks and cells rinsed with 4 mL of sterile phosphate-buffered saline (PBS) to remove any residual FBS. Cells were then washed in 0.5 mL 0.25% (w/v) trypsin + 0.02% (w/v) EDTA (Trypsin-EDTA, Merck) followed by incubation in 0.5 mL fresh Trypsin-EDTA at 37°C until cell detachment from the base of the flask. Detached cells were resuspended in 5 mL of fresh medium to neutralize trypsin activity and seeded into new flasks at the ratios described above.

Cells were maintained at 37°C in a 5% CO_2 atmosphere.

2.2.2 Seeding of cells for plate culture or polarization

Seeding of cells was performed during the passaging process. Following resuspension in fresh medium, 50 µL of trypsinized cells were mixed with 50 µL of 0.4% (w/v) trypan blue and loaded into a Neubauer counting chamber with 0.1 mm depth (Hawksley). Viable cells (not stained by trypan blue) were counted from all four counting areas, including all cells that touched internal boundaries and excluding those that touched external boundaries (see **Fig. 2.1**). The number of cells per mL was calculated according to the following equation:

$$cells\ per\ mL = (average\ cells\ per\ counting\ area \times 2) \times 10^4$$

The volume of cell suspension required for seeding was then calculated by the following equation:

$$x = (y \times w) \div z$$

where x is the volume of cell suspension required, y is the desired number of cells per well, w is the number of wells for seeding and z is the cell concentration.

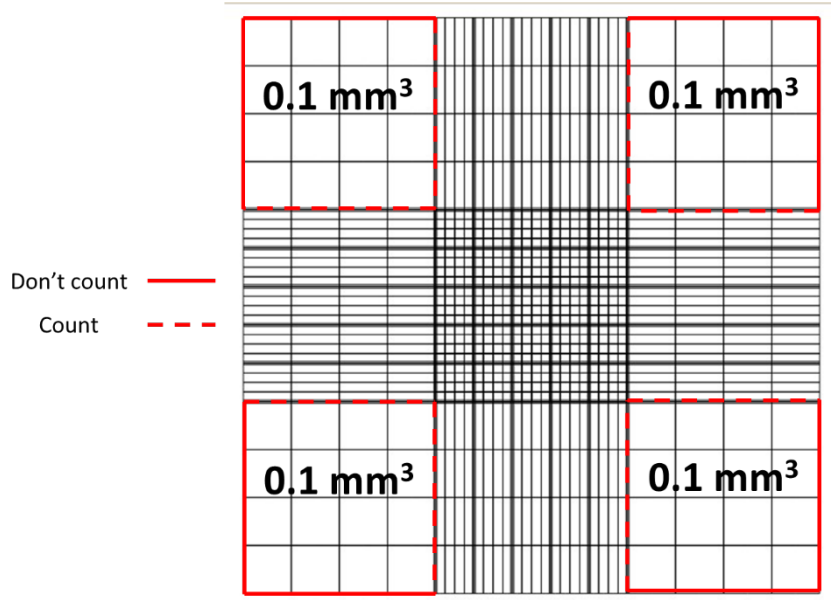

Figure 2.1 Neubauer counting chamber. Red boxes indicate counting areas with 0.1 mm^3, i.e. 10^{-4} mL, capacity. Internal and external boundaries are denoted by dashed and solid lines respectively.

For plate cultures, 1.5 x 10^5 cells were seeded out into 24-well plates (Greiner) in 1 ml of medium per well and grown until ~70% confluency. Cultures used for immunofluorescence staining were grown in wells containing sterile round coverslips (13 mm diameter, 0.13-0.16 mm thickness, Academy).

For experiments requiring polarized cells, 5 x 10^5 T84 cells were seeded out on collagen coated polyester Snapwell filter inserts (Corning, 1.2 cm diameter, 0.4 um pore). For T84/LS cultures, 5 x 10^4 LS174T cells were added. For collagen coating of filters, a stock solution was prepared by dissolving rat tail collagen type I (Merck) at a concentration of 1.25 mg/mL in 0.1 N acetic acid at 4°C on a tube rotator for 4 h. The stock solution was diluted to 50 µg/mL in 60% (v/v) ethanol, and 200 µL (i.e. 10 µg) were added to each membrane. Plates were incubated at room temperature in a microbiology safety cabinet until the ethanol had evaporated. Coated plates were stored at room temperature until use. Cells seeded on Snapwell inserts were maintained in medium containing penicillin (100 units/mL) and streptomycin (100 µg/mL) (P/S) (Merck), with medium exchange after four days and every two days thereafter. Medium was changed to P/S-free medium on the day before the experiment. Cell polarisation was monitored by TEER determined using an EVOM resistance meter and EndOhm electrode (World Precision Instruments), and cells were used after TEER reached >1,000 Ω x cm^2 (T84 monolayers) or 30-80 Ω x cm^2 (T84/LS).

Cells were maintained at 37°C in a 5% CO_2 atmosphere.

2.2.3 Cryopreservation and thawing of cells

Cells were trypsinised and resuspended in 5 mL supplemented DMEM/F-12 (T84) or DMEM (LS174T), and the cell count was determined using a haemocytometer. The cell concentration was adjusted to 2-4 x 10^6 cells/ mL, and 950 µL cell suspension was added to a freezing vial. Dimethyl sulfoxide (DMSO) (50 ul, Merck) was added and vials were placed in a freezing container (Mr Frosty, Nalgene) containing isopropanol (Merck), and cooled to -80 °C overnight at a cooling rate of 1 °C/ min to reduce ice crystal formation. Frozen vials were transferred to liquid nitrogen storage (vapour phase; -190 °C) for long term preservation.

To resurrect cryostocks, vials were thawed rapidly at 37°C and cells transferred to 5 mL of pre-warmed supplemented cell culture medium (DMEM/F-12 for T84, DMEM for LS174T). Cells were then spun at 112 x g for 6 minutes to pellet cells, and supernatants containing DMSO were removed. Cell pellets were resuspended in 7 mL of fresh medium, transferred to a 25 cm^2 culture flask and cultured until desired confluence.

2.3 Vertical diffusion chamber

Snapwell filter inserts with polarised epithelia were mounted between apical and basal half chambers of a VDC system as shown in **Fig. 2.2**. Apical compartments were filled with 4 mL of pre-reduced DMEM/F-12, BHI-YH or a 1:1 mixture of both (F-12/BHI-YH) while basal compartments contained 4 mL of non-supplemented DMEM/F-12 medium. Apical media were pre-reduced by storing in an anaerobic cabinet for >48 hours to remove oxygen (DMEM/F-12) or using the Hungate technique (BHI-YH). Apical compartments were perfused with an anaerobic gas mixture (90% N_2, 5% H_2, 5% CO_2) whereas basal compartments were gassed with 95% air, 5% CO_2. Media were allowed to equilibrate to these gas conditions for 30 mins prior to bacterial inoculation.

For VDC experiments without host cells, empty Snapwell inserts were mounted between the two compartments and the VDC was filled with 8 mL pre-reduced F-12/BHI-YH and gassed with anaerobic gas mixture from both apical and basal outlets.

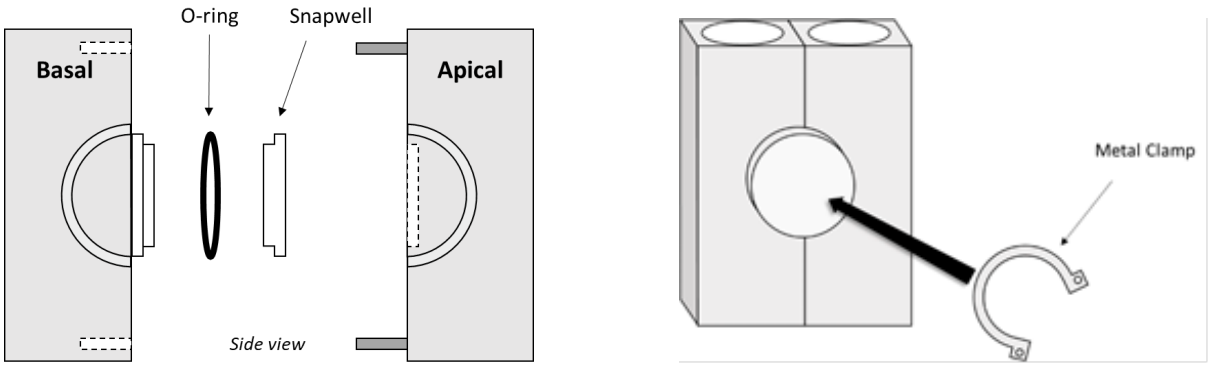

Figure 2.2 Schematic diagram of VDC assembly.

For bacterial inoculation, overnight cultures were centrifuged for 5 minutes at 5,000 x g, supernatants were removed, and bacterial pellets resuspended in apical medium. Unless otherwise stated, EPEC, *L. reuteri* and *R. gnavus* were inoculated into the apical compartment at 6.25 x 10^4 colony forming units/mL (CFU/mL), 7 x 10^7 CFU/mL or 1 x 10^7 CFU/mL, respectively. These bacterial inoculums yielded multiplicity of infection (MOI) values of 0.2, 225, 32 for EPEC, *L. reuteri* and *R. gnavus*, respectively. Following inoculation, chambers were incubated for 4 hours at 37°C. Bacterial growth was determined by optical density at 600 nm (OD_{600}) using a spectrophotometer (Uvikon xs). Details on alternative downstream analyses are outlined below.

Once experiments were complete, chambers were disassembled and disinfected in 2.5 L of dH_2O containing 5g Presept™ (Johnson & Johnson) for 16-24 hours. The following day, the cleaning solution was removed, and chambers were washed three times with dH_2O to remove any residual cleaning reagents. Cleaned chambers were stored in a clip top box until use.

2.4 Immunofluorescence staining

2.4.1 Primary antibodies and fluorescent stains

Antigen	Host species	Working conc.	Source	Product code
CmbA	Rabbit	1:250	N. Juge	N/A
E. coli LPS	Mouse	1:200	Abcam	Ab35654
MUC2	Mouse	1:250	Santa Cruz	Sc-7314
MUC2	Rabbit	1:250	Santa Cruz	Sc-15334
Occludin	Rabbit	1:20	Invitrogen	71-1500
Sialidase	Rabbit	1:200	N. Juge	N/A
Lipoteichoic acid (LTA)	Mouse	1:30	Invitrogen	MA1-40134

Table 2.1 List of primary antibodies and dilutions used for immunofluorescence in this study.

All primary antibodies were used in combination with Alexa Fluor 488, 568 or 647- conjugated donkey anti- rabbit or -mouse IgG secondary antibodies (1:400, Invitrogen).

In addition, cells were stained with fluorescein isothiocyanate-labelled phalloidin (FITC-phalloidin) (1:200, Merck) and 4',6-diamidino-2-phenylindole (DAPI) (1:5000, Merck) to label filamentous actin and nuclei, respectively.

2.4.2 Bacterial staining

After removal of non-adherent bacteria by two washes in PBS, cells on Snapwells or coverslips were fixed in 3.7% (v/v) formaldehyde (Acros Organics) in PBS for 10 mins at room temperature (RT), washed with PBS and stored at 4°C until use. Samples were permeabilised and blocked by incubation in 0.1% (v/v) Triton X-100 (Merck), 0.5% (w/v) bovine serum albumin (BSA) in PBS for 20 mins at RT, and then washed in PBS. Samples were incubated with one of the following primary antibodies diluted

in 0.5% BSA in PBS for 60 mins at RT: rabbit anti-CmbA (*L. reuteri*), rabbit anti-sialidase (*R. gnavus)*; or mouse anti-*E. coli* LPS (EPEC). Cells were washed in PBS and incubated with respective secondary antibodies, FITC-phalloidin and DAPI for 30 mins at RT in the dark. Samples were then washed in PBS for 30 mins in the dark at RT, mounted onto slides using Vectashield mounting medium (Vector Laboratories) and imaged using an Axioimager fluorescent light or LSM800 confocal laser-scanning microscope (Zeiss).

2.4.3 Occludin staining

Cells were washed twice in PBS, pre-extracted in 0.2% (v/v) Triton X-100 in PBS for 2 mins on ice, washed in PBS, fixed in 3.7% (v/v) formaldehyde for 10 mins at RT, washed again and stored at 4°C until use. After permeabilization and blocking in 0.05% (v/v) Triton X-100 and 0.5% (w/v) BSA in PBS on ice, cells were incubated in diluted anti-occludin for 60 min on ice and subsequent staining was performed as described in **section 2.4.2.**

2.4.4 MUC2 Staining

After washing in PBS, cells were fixed and permeabilised in ice cold methanol/acetone (1:1) for 4 mins on ice to preserve mucus. The fixative was removed, cells washed in PBS and stored at 4°C until use. Immunofluorescence staining was performed as described in **section 2.4.2** without permeabilization in Tx-100.

2.4.4.3 Assessing activity of mucolytic reagents

To assess the activity of mucolytic reagents dithiothreitol (DTT, Merck) and N-acetyl cysteine (NAC, Merck), epithelia were incubated with the reagents and MUC2 staining was performed. NAC was used at 5 or 10 mM for 48 hours at 37°C, whereas DTT was used at 10-100 mM for 15 minutes at room temperature. Following this, MUC2 staining was performed and quantitative comparison of MUC2 staining between conditions was performed by integrated density (i.e. signal intensity per μm^2) measurements.

2.5 Bacterial Growth and Adherence

To quantify planktonic bacteria, apical media from VDC incubations were serially diluted in sterile PBS and either 100 µL (whole plates) or 20 µL (spot plates) plated. Spot plates were performed by drying agar plates in a MSC for 20 minutes, spotting 20 µL drops onto the agar and allowing the drops to soak into the agar. For EPEC, bacterial suspensions were plated on LB agar and incubated overnight at 37°C. For *L. reuteri* and *R. gnavus*, bacterial suspensions were plated on MRS or BHI-YH agar, respectively, and incubated at 37°C in an anaerobic cabinet until colonies were formed. CFUs were quantified by counting colonies on plate..

To quantify adherent bacteria, Snapwell inserts were washed twice with 1 mL PBS for 10 minutes on a rocker at RT. Cells were lysed with 300 µL of 1% Triton X-100 (v/v) in PBS for 20 mins at RT. For *L. reuteri* and *R. gnavus*, cell lysis was performed in an anaerobic cabinet to prevent oxygen exposure. For *R. gnavus*, bacteria were dissociated in PBS only due to bacterial sensitivity to Triton X-100 . After incubation, cells were carefully scraped from the membrane using a sterile P1000 tip and homogenised by vigorous pipetting. Cell lysates were transferred to sterile 1.5 mL microcentrifuge tubes and volumes were adjusted to 1 mL with sterile PBS. Cell lysates were then serially diluted and plated onto appropriate agar according to **section 2.5.1**.

2.6 Interleukin-8 Release

VDC incubations were performed for 4 hours as described in **section 2.3**. Apical media were removed, replaced with pre-warmed DMEM/BHI-YH containing 50 µg/mL gentamicin or 16 µg/mL polymyxin B, and incubations were continued for 18 hours. Samples of 1 mL were taken from apical and/or basal chambers and bacteria were removed by centrifugation at 4000 x g, 4°C for 10 minutes. Supernatants were kept at 4°C if analysed within 24 hours, or at -20°C for long-term storage. IL-8 was subsequently quantified using a human IL-8 ELISA kit (PeproTech) according to the manufacturer's instructions.

2.7 SDS-PAGE and Western Blotting

To determine EspB translocation into host cells, cell monolayers were incubated in ice-cold lysis buffer (20 mM HEPES pH 7.4, 140 mM NaCl, 1% Triton X-100) containing protease inhibitor cocktail (1:100, Merck) for 20 minutes on ice. Cell lysates were transferred to a microcentrifuge tube and centrifuged at 14,000 x g, 4°C for 15 minutes. Triton-soluble (Tx-soluble) fractions were transferred to Amicon Ultra-0.5 centrifugal filter units (Merck Millipore) with a 10 kDa molecular weight cut-off (10K MWCO)

and concentrated 6x by centrifugation at 14,000 x g, 4°C for 25 minutes. Triton-insoluble (Tx-insoluble) pellets were suspended in 1x reducing sample buffer (RSB, Table 2.3).

EspB secretion into the medium was determined as described in Cameron *et al.* (2018). Briefly, apical media were transferred to 15 mL centrifuge tubes on ice and centrifuged at 4000 x g, 4°C for 10 minutes. Bacterial pellets were suspended in 1x RSB. Supernatants were filtered through a polyethersulfone membrane filter with 0.22 µm pore size, and protease inhibitor cocktail (1:100) was added. Supernatants were concentrated 8x in an Amicon 10K MWCO filter unit. All samples were stored at -20°C until use.

To prepare samples for SDS-PAGE, 5x RSB was added to Tx-soluble fractions and apical supernatants for a 1x final concentration. Samples were heat denatured at 95 °C for 5 minutes and electrophoresed in 12% SDS-polyacrylamide gels submerged in Running Buffer using a Mini-PROTEAN Tetra Cell device (Bio-Rad) for 60 minutes at 150 V, 100 mA, 10 W. Proteins were transferred to polyvinylidene difluoride membranes (Cytiva) by wet blotting at 100 V constant for 60 minutes in Transfer Buffer. All following incubations were performed on a rocking platform unless stated otherwise. Membranes were blocked in 3% BSA in tris-buffered saline (TBS) + 0.05% Tween-20 (TBST), at RT for 60 min. After a 10-minute wash in TBST, membranes were incubated with primary antibody (Table 2.2) diluted in TBST overnight at 4 °C. After a TBST wash, blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:200,000, Merck) for 45 min. Membranes were washed for 30 minutes with TBST and developed using enhanced chemiluminescence (Pierce™, Thermo Scientific). Blots were imaged with a G:Box Chemi XRG Imager (Syngene), and densitometric analysis was performed with ImageJ Fiji software.

Table 2.2 Primary antibodies used for Western blotting.

Antigen	Host species	Dilution	Source
EspB	Rabbit	1:1000	G. Frankel
Tir	Rabbit	1:500	G. Frankel
GroEL	Rabbit	1:50,000	Merck

Table 2.3 Reagents for SDS-PAGE and Western Blotting. All reagents were prepared using dH_2O.

5x Reducing Sample Buffer:

Reagent	Final Concentration
Glycerol	50% (v/v)
Sodium dodecyl sulphate	100 mg/mL
(SDS)	250 mM
Tris pH 6.8	78 mg/mL
Dithiothreitol (DTT)	1 mg/mL
Bromophenol blue	

4x Stacking Gel Buffer (SGB) pH 6.8:

Reagent	Concentration
Tris base	60.5 g/L
SDS	400 mg/L

4x Running Gel Buffer (RGB) pH 8.8:

Reagent	Concentration
Tris base	181.6 g/L
SDS	4 g/L

12% SDS-polyacrylamide Gel:

Reagent	Running Gel	Stacking Gel
4x RGB/SGB	1x	1x
30% acrylamide/bis-aa	12%	3%
10% Ammonium persulphate (APS)	5 µL/mL	5 µL/mL
TEMED	1 µL/mL	0.5 µL/mL

Table 2.3 Reagents for SDS-PAGE and Western Blotting. All reagents were prepared using dH_2O.

Running Buffer pH 8.3:

Reagent	Concentration
Tris base	3 g/L
Glycine	14.4 g/L
SDS	1 g/L

Transfer Buffer pH 8.8:

Reagent	Concentration
Tris base	3 g/L
Glycine	14.4 g/L

2.8 Graphs & Statistical Analysis

Data was analysed using GraphPad Prism Version 8 software. Normality of data was determined using Shapiro-Wilk test and equal variances confirmed using F-test to compare two variances. For the comparison of two groups, parametric student's t-test was used. Bar graphs show individual data points plus a bar at the mean value, unless otherwise stated. Three or more groups were compared using parametric one-way analysis of variance (One-way ANOVA) with Tukey's multiple comparison test. Analysis of data with more than one independent variable was carried out using parametric two-way ANOVA, with Tukey's post-hoc test. For regression analysis, data was plotted as a scatter plot with a trendline and boundaries indicating a 95% confidence interval. Correlation was determined using Pearson's correlation co-efficient (R) and the fit of data to the trendline was assessed by R^2. For all data analysis, a P value of < 0.05 was considered statistically significant, and degrees of statistical significance are presented as follows: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$.

Chapter Three – Developing a Model System of the Intestinal Epithelium to Study Colonisation Resistance by the Microbiota

3.1 Introduction

The intestinal environment is fiendishly complex, composed of a wide array of interplaying factors. Oxygen concentration, pH, mucus thickness and composition, transit rate (peristalsis), epithelial morphology (microvilli, villi, crypts of Lieberkühn), and barrier function are all factors which change along the various regions of the gut (Albenberg *et al.* 2014; Kararli 1995; Gustafsson *et al.* 2012; van der Flier and Clevers 2009). Rigorous competition between members of the microbiota for colonisation sites has resulted in highly specific adaptation to these physiological conditions, as shown by the defined spatial organisation of microbial inhabitants (Albenberg *et al.* 2014; Lu *et al.* 2014; Kudelka *et al.* 2016). As a result of this exquisite adaption, recreating *in vivo* conditions is essential to study interactions between the gut microbiota and host. Despite this, much of the work investigating these interactions has been performed using traditional cell culture models that do not take these factors into account. The Vertical Diffusion Chamber used in this study allows culture of human intestinal epithelial cell lines in an apical environment low in oxygen. This is crucial as the human gut microbiota is thought to consist largely of anaerobic organisms. Although there is currently no method to identify intestinal bacteria *in situ*, the low oxygen levels of the ileal and colonic lumen (<1% of atmospheric pressure), which harbour the majority of the microbiota, imply dominance of facultative and strict anaerobes (Friedman *et al.* 2018; Albenberg *et al.* 2014; Eckburg *et al.* 2005). The application of 16S ribosomal RNA (rRNA) sequencing, which utilises the species specificity of 16S rRNA for classification, has provided insight into the composition of the gut microbiota that was previously impossible due to the lack of anaerobic culture techniques (Mizrahi-Man, Davenport, and Gilad 2013; Poretsky *et al.* 2014). Studies employing rRNA sequencing to analyse ileal effluent from individuals with ileostomies have concluded that the jejunum and ileum are dominated by facultative anaerobes (Booijink *et al.* 2010; Zoetendal *et al.* 2012). Metagenomic analyses of human faecal samples confirmed previous studies indicating the dominance of anaerobic bacteria belonging to Bacteroidetes and Firmicutes phyla in the colonic microbiota, particularly *Bacteroides* and *Ruminococcus ssp*. (Qin *et al.* 2010; Zoetendal, Akkermans, and De Vos 1998). Therefore, the benefit of the VDC is two-fold. Firstly, the system has the capacity to support culture of anaerobic organisms alongside intestinal epithelial cells. Not only does this facilitate investigation into colonisation resistance activities of a plethora of untested strictly anaerobic organisms (such as *Ruminococcus gnavus*), but it could also enhance competitiveness of well-studied oxygen-tolerant probiotics. For example, *Limosilactobacillus reuteri* (*L. reuteri*) produces anti-bacterial secondary metabolites during anaerobic growth (e.g. lactic acid and reuterin) that may mediate colonisation resistance (Talarico *et al.* 1988). Secondly, culturing oxygen-tolerant gut pathogens in a low-oxygen environment similar to that of the intestinal lumen has been shown to influence virulence. For A/E lesion-forming *E. coli*, the low oxygen conditions

offered by the VDC have been shown to stimulate expression of the T3SS, resulting in enhanced epithelial adherence (Schuller and Phillips 2010). Studies on other pathogens in the VDC have also investigated virulence characteristics under low oxygen conditions (Jafari *et al.* 2016; Mills *et al.* 2012; Naz *et al.* 2013). Infection of T84 and Caco-2 cells with *Campylobacter jejuni* in the VDC demonstrated increased epithelial association and invasion of host cells under microaerobic conditions, compared to atmospheric oxygen conditions (Mills *et al.* 2012; Naz *et al.* 2013). In a different study investigating virulence of *Clostridiodes difficile*, infection of T84 monolayers under a microaerobic apical environment resulted in enhanced expression of genes encoding pro-inflammatory mediators (such as IL-8, human β-defensin 2 and TNF-α) compared to aerobic conditions (Jafari *et al.* 2016). In addition, barrier function assessments of T84 epithelia infected with *C. difficile* indicated a steeper reduction in epithelial resistance under microaerobic conditions (Anonye *et al.* 2019).

In addition to low oxygen concentrations, mucus is a crucial factor in the physiology of the intestinal epithelium. The human colonic mucus layer consists of a thick (200-400 μm) loosely attached outer layer that facilitates bacterial colonisation and penetration, and a thin (100 μm) adherent inner layer that protects the epithelium from contact with gut microbes and luminal contents (Johansson *et al.* 2008; Gustafsson *et al.* 2012; Pullan *et al.* 1994). In contrast, the secreted mucus in the small intestine forms a single, easily dissociated layer that is permeable to allow nutrient absorption but charged with antimicrobial defensins to minimise bacterial access to the epithelium (Meyer-Hoffert *et al.* 2008). In porcine enteric biopsies, the thickness of the mucus layers is dependent on the region, measured at 25.6, 35.3 and 53.8 μm for the duodenum, jejunum and ileum respectively (Gustafsson *et al.* 2012). The observation of a progressive increase in mucus thickness in the small intestine agrees with a study demonstrating increasing ratios of goblet cells along the small intestine tract (4% and 12% for duodenum and ileum, respectively) (Cheng 1974). Despite differences in thickness and function, the small intestinal and colonic mucus layers are both predominantly comprised of secreted glycoprotein MUC2 (Allen, Hutton, and Pearson 1998). Nascent MUC2 undergoes significant glycosylation and oligomerisation and is stored in the granulae of goblet cells until extracellular export, where it becomes hydrated to form the observed mucus layers (Asker *et al.* 1998; Godl *et al.* 2002; Lidell *et al.* 2003; Johansson *et al.* 2008). Glycosylation is indispensable for mucus structure and function; N-glycosylation has been shown to be crucial for MUC2 dimerisation, and O-glycosylation provides the monosaccharides (or glycans) utilised by the microbiota for nutrients and colonisation sites (Arike and Hansson 2016; Juge 2012; Asker *et al.* 1998; Bell *et al.* 2003).

As the primary component of intestinal mucus, inclusion of MUC2 is essential when developing a model system of the intestinal mucosa. This is particularly relevant when studying host-microbe interactions and colonisation resistance, as the microbiota are highly adapted to the ecological niches

offered by intestinal mucus. Despite this, the most commonly utilised cell lines (T84, Caco-2, HT-29) are only capable of producing small amounts of mucus. Mucin can be introduced to intestinal epithelial models by several methods: the addition of crude mucin (isolated from pigs or cattle) to non-secreting IECs; using *ex vivo* intestinal biopsies with the native mucus layer preserved and utilising mucus secreting IECs (via cell lines or organoids). The latter two of these methods have the benefit of a mucus layer which is produced and secreted by endogenous goblet cells. On the other hand, the use of mucus-secreting colon carcinoma cell lines allows for more practicality than biopsies or organoids, albeit at the cost of a highly mutagenic background. Furthermore, the high oxygen levels required by *in vitro* organ culture of biopsy tissue precludes studying host-microbe interactions under low oxygen conditions. In order to simulate the proportion of goblet cells in the small intestinal epithelium (4-12%), mixed cultures of mucus-producing HT29-MTX cells alongside absorptive enterocyte-like Caco-2 cells has be performed to generate a mucus-producing epithelial model (Chen *et al.* 2015; Navabi, McGuckin, and Lindén 2013; Walter *et al.* 1996).

In this study, colonocyte-like T84 cells were selected as they polarise during culture on Transwell membranes and exhibit microvilli and TJs which are crucial for epithelial barrier function (Dharmsathaphorn *et al.* 1984). Enhanced permeability is common in individuals with inflammatory bowel diseases, and TJ proteins are frequently targeted by diarrhoeagenic pathogens such as EPEC (Turner 2009; Edelblum and Turner 2009; Hollander 1988). Infection of T84 cells with EPEC has implicated several effectors (EspF, Map, Intimin) in the disruption of TJs (Dean and Kenny 2004b; McNamara *et al.* 2001; Simonovic *et al.* 2000). Furthermore, T84 cells have been utilised frequently to unravel the underlying mechanisms of EPEC pathogenesis including A/E lesion formation and release of pro-inflammatory IL-8 (Cepeda-Molero *et al.* 2017; Zhou *et al.* 2003; Czerucka *et al.* 2001; Ruchaud-Sparagano, Maresca, and Kenny 2007). Several studies implementing the VDC model system have used it alongside T84 cells, which retain polarity and epithelial structures under microaerobic apical conditions (Ellis *et al.* 2019; Naz *et al.* 2013; Tran *et al.* 2014). To introduce mucus into the system, the goblet cell-like cell line LS174T has been chosen as it exhibits higher levels of MUC2 production than other commonly used cell lines (Bu *et al.* 2011; van Klinken *et al.* 1996). Consequently, LS174T cells have been utilised in numerous publications investigating the influence of secreted mucus on host-microbe interactions, often highlighting differences in microbial adherence and host cell mucin expression (Walsham *et al.* 2016; Wang *et al.* 2014). In particular, previous studies have demonstrated *L. reuteri* adherence and EPEC A/E lesion formation to LS174T cells indicating translocation of effector proteins into this cell line through the type III secretion system (T3SS) (Walsham *et al.* 2016).

In addition to establishing a microaerobic co-culture of T84 and LS174T cells, we aimed to identify an apical medium that enables the growth of EPEC, *Limosilactobacillus reuteri* and *Ruminococcus gnavus*

in the VDC. EPEC strain E2348/69 was chosen for this study as it is the prototype strain for typical EPEC and its virulence traits, such as adherence and A/E lesion formation, have been well-characterised (Iguchi *et al.* 2009). *L. reuteri* probiotic strain PTA 6475 was selected as it has demonstrated protective effects during enteric infection both *in vitro* and *in vivo* (Mu, Tavella, and Luo 2018). The *R. gnavus* strain ATCC 35913 was chosen as it was isolated from the faeces of a healthy human, and its interaction with glycans of the mucus layer has been characterised (Crost *et al.* 2016). The apical medium should also support EPEC pathogenesis and expression of the type III secretion system. In addition to enabling bacterial growth, the chosen medium should maintain the structure and function of the host epithelium.

3.2 Results

3.2.1 The Vertical Diffusion Chamber allows culture of EPEC, *R. gnavus* and *L. reuteri*

Initial experiments focused on developing a growth medium that supported growth of EPEC E2348/69, *L. reuteri* ATCC PTA 6475 and *R. gnavus* ATCC 35913 in the VDC. Previous studies demonstrated microaerobic growth of EPEC, *L. reuteri* and *R. gnavus* in BHI-YH medium, but further experiments indicated loss of T84 epithelial barrier function after 4h of incubation in apical BHI-YH medium (Laveckis 2017). Therefore, a 1:1 mixture of BHI-YH and DMEM/F-12 cell culture medium (DMEM/F-12 + BHI-YH) was tested. Bacteria were inoculated at 1 x 10^7 CFU (EPEC), 2 x 10^8 CFU (*L. reuteri*) and 4.8 x 10^8 CFU (*R. gnavus*) in VDCs without host cells, and chambers were gassed with an anaerobic gas mixture (90% N_2, 5% H_2, 5% CO_2). Bacterial growth was monitored over 6 hours (EPEC and *L. reuteri*) or 23 hours (*R. gnavus*) by taking regular optical density measurements at 600 nm (OD_{600}). Graphs shown in **Fig. 3.1** indicate increased OD_{600} values for all species following incubation in BHI-YH and DMEM/F-12 + BHI-YH medium, suggesting effective bacterial growth and suitability for future co-culture experiments. As expected, EPEC exhibited the fastest growth out of all organisms, with OD_{600} values increasing from ~0.1 to 1.0 after only 2 hours post-inoculation. For *L. reuteri*, an increase in OD_{600} from initial inoculation was noticeable after 4 hours whereas for *R. gnavus* 6 hours were required, indicating that longer incubation times were required for growth. Importantly, the microaerobic environment generated by the VDC supported culture of the oxygen-sensitive gut microbe *R. gnavus*. In contrast, only a small increase in OD_{600} values was observed for all organisms during culture in DMEM/F-12 medium.

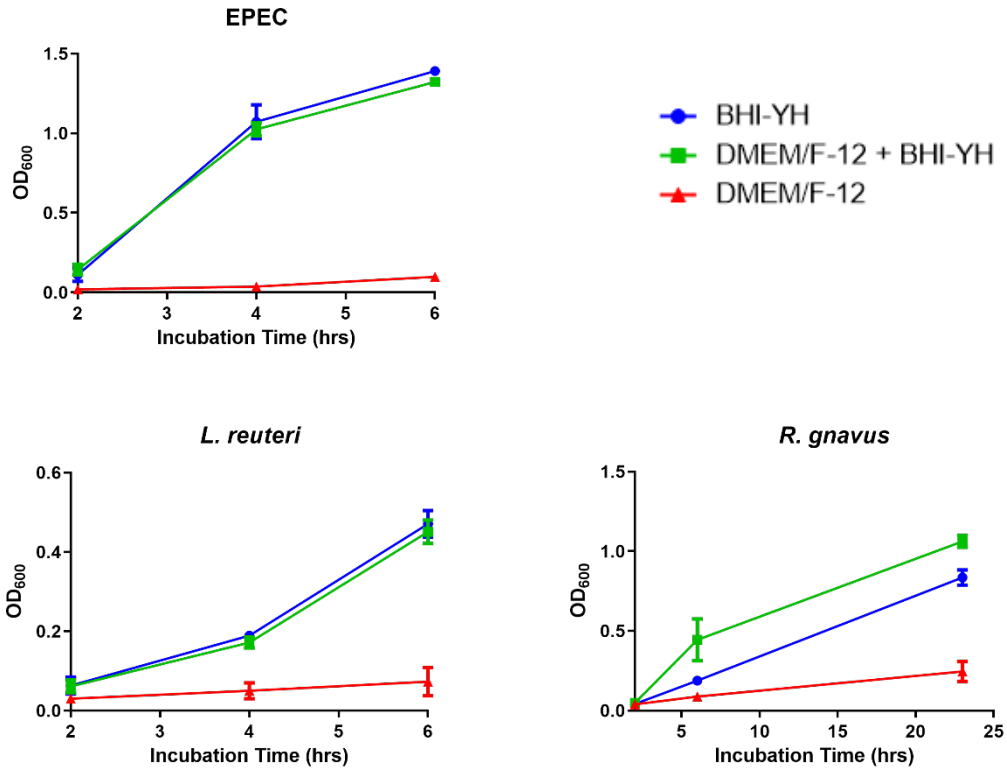

Figure 3.1 Bacterial growth in the microaerobic VDC system using different media Bacterial strains were inoculated at 1 x 10^7 CFU (EPEC), 2 x 10^8 CFU (*L. reuteri*) and 4.8 x 10^8 CFU (*R. gnavus*) in VDC chambers without cells. Chambers were filled with either DMEM/F-12 (red), BHI-YH (blue) or a 1:1 mixture of both media (DMEM/F-12 + BHI-YH, green) and gassed with anaerobic gas mixture. Growth was assessed by measuring optical density (OD_{600}) over indicated periods of incubation. Data shown is from two independent experiments performed in duplicate (n = 4).

3.2.2 Epithelial integrity and barrier function is retained in DMEM/F-12 + BHI-YH Medium

To determine the influence of BHI-YH and DMEM/F-12 + BHI-YH medium on epithelial barrier function, T84 epithelia were cultured for 4 or 22 hours in the VDC with BHI-YH and DMEM/F-12 + BHI-YH apical medium, as well as DMEM/F-12 cell culture medium as a control. DMEM/F-12 was also added to the basal compartment for all chambers. Epithelial structure was evaluated by immunofluorescence staining (IFS) for the microvillous brush border (filamentous actin) and tight junction protein occludin. In addition to IFS, transepithelial electrical resistance (TEER) was determined to quantitatively assess barrier function. This was done by measuring TEER before and after the incubation, calculating the

change in TEER and then normalising the data to the DMEM/F-12 control. Normalisation is crucial as TEER values decrease when transferring epithelia from DMEM/F-12 supplemented with FBS and L-glutamine into the VDC where non-supplemented DMEM/F-12 is used, hence the DMEM/F-12 control can be utilised as a reference point to account for this.

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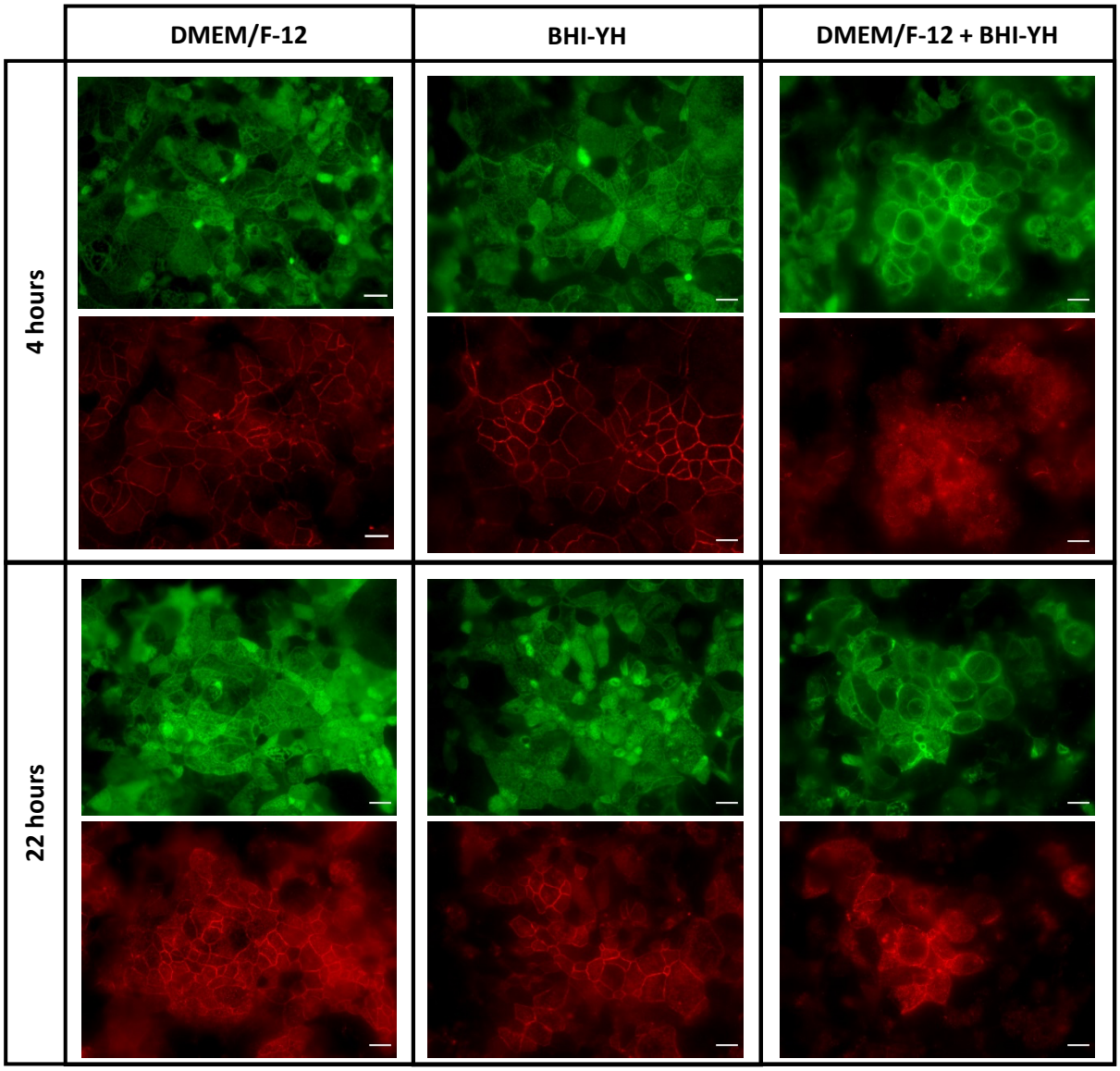

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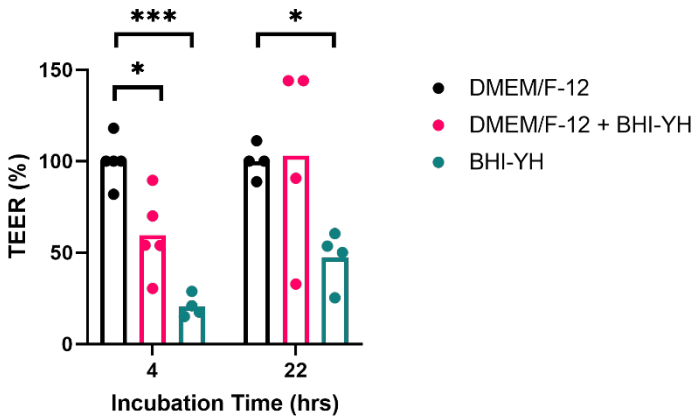

Figure 3.2 Influence of media on epithelial structure and barrier function. (**A**) T84 monolayers were incubated with F-12, BHI-YH or DMEM/F-12 + BHI-YH on the apical side for 4 or 22 hours. Epithelia were then stained with antibodies targeting F-actin (green) or occludin (red). Scale bar = 10 µm. Representative images of three independent experiments performed in duplicate (n = 6). (**B**) Graph indicating TEER values of epithelia incubated with different media. TEER values have been normalised to the DMEM/F-12 control. Data collected for DMEM/F-12 + BHI-YH are from five replicates collected from two independent experiments (n = 5) whereas four replicates are shown for all other conditions (n = 4). *$P < 0.05$, ***$P < 0.001$.

As expected, epithelial structure was best preserved in DMEM/F-12 medium. IFS for F-actin indicated distinct cell edges and well established microvillous brush borders while occludin staining confirmed the preservation of tight junctions between cells. Preservation of tight junctions in DMEM/F-12 was also confirmed quantitatively; barrier function assessments indicated greater retention of TEER in DMEM/F-12 compared to DMEM/F-12 + BHI-YH and BHI-YH. In contrast, incubation of T84 monolayers with BHI-YH medium had a considerable effect on epithelial structure, demonstrated by rounding of cells, loss of microvillous brush border and disruption of tight junctions (occludin staining no longer localised to cell edges). The latter is reflected by a significant decrease in TEER reaching 17% and 57% of the control values at 4 and 22 hours, respectively. Interestingly, the epithelial structure of cells in DMEM/F-12 + BHI-YH medium resembled that of epithelia incubated in the control medium, indicating that addition of DMEM/F-12 to BHI-YH was sufficient to preserve crucial epithelial features. Despite this, barrier function appeared weakened after a 4h incubation in DMEM/F-12 + BHI-YH medium (46% relative to control), indicating some reduction in tight junction integrity. Surprisingly, barrier function in DMEM/F-12 + BHI-YH returned to that seen in the control condition after 22 hours (90% of control TEER), suggesting that the conditions become less detrimental to barrier function over time, or the

epithelia adapt to them. Nevertheless, it is challenging to draw conclusions from this condition as the data collected were highly variable. Although barrier function was somewhat reduced in DMEM/F-12 + BHI-YH medium, raw TEER values were still higher than 500 Ω x cm^2, which is similar to that of other differentiated intestinal cell lines such as Caco-2 and HT-29 (Alemka *et al.* 2010; Srinivasan *et al.* 2015). This coupled with preservation of epithelial structures, demonstrates that DMEM/F-12 + BHI-YH medium is a suitable apical medium for retention of key features of epithelial polarisation.

3.2.3 EPEC type III secretion system activity and actin pedestal formation are supported in DMEM/F-12 + BHI-YH medium

In addition to supporting bacterial growth and epithelial polarity, it was imperative to confirm that EPEC pathogenesis was retained during culture in DMEM/F-12 + BHI-YH. Of particular interest was expression of the T3SS as this facilitates translocation of effectors essential for EPEC virulence but responds to nutrient composition and availability via a range of transcriptional regulators (Platenkamp and Mellies 2018).

To assess T3SS activity, translocation of the translocon protein EspB was assessed by western blotting (Ide *et al.* 2001). EspB was selected as it is secreted into the medium during planktonic growth or directly translocated into the host cell membrane during adhesion (Knutton *et al.* 1998b; Wolff *et al.* 1998). For infected cell lysates, Triton-soluble (host cell derived) and Triton-insoluble (bacteria-derived) fractions were assessed.

Western blotting for EspB from culture supernatants yielded bands for EPEC grown in both DMEM/F-12 and DMEM/F-12 + BHI-YH, whereas no band was observed for the T3SS-deficient mutant Δ*escN* (**Fig. 3.3A**). These data indicate that the T3SS from EPEC is active after 4 hours of culture in DMEM/F-12 and DMEM/F-12 + BHI-YH. EspB was also detected from T84 cells infected with WT EPEC in the VDC (**Fig. 3.3B**). Infections with the Δ*escN* mutant performed in parallel showed the presence of bacterial-derived EspB but no band for the host lysate fraction, indicating that the lack of EspB translocation is due to a non-functioning T3SS. Optical density measurements of apical media demonstrated similar bacterial loads for both WT and Δ*escN*.

In addition to EspB secretion, actin pedestal formation by EPEC was examined by fluorescent actin staining (FAS) (Knutton *et al.* 1989). As shown in **Fig. 3.4**, pedestal formation by EPEC was evident on T84 epithelia after a 4-hour incubation in DMEM/F-12 + BHI-YH medium as indicated by the bacterial shaped F-actin staining co-localising with the EPEC-specific LPS stain. No difference in the size or number of pedestals was noted between infections in DMEM/F-12 or DMEM/F-12 + BHI-YH medium.

Taken together, these results indicate that DMEM/F-12 + BHI-YH medium supports EPEC T3S and actin pedestal formation in T84 cells.

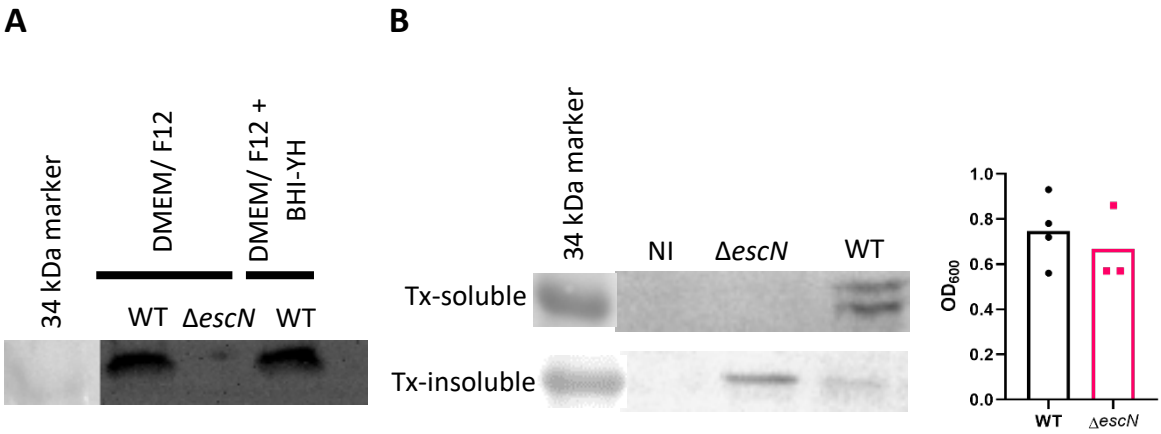

Figure 3.3 EPEC translocates EspB in a T3SS-dependent manner in DMEM/F-12 and DMEM/F-12 + BHI-YH media. (**A**) EPEC (1 x 10^7 CFU/mL) was cultured in DMEM/F-12 or DMEM/F-12 + BHI-YH for 4 hours. EspB secretion into culture supernatants was determined by Western blot analysis. Culture supernatants of an isogenic T3SS-inactive mutant (Δ*escN*) were included as a negative control. (**B**) T84 epithelia were infected with EPEC in the VDC for 4 hours. Translocation of EspB into host cells was determined by western blot analysis of the Triton-soluble protein fraction. The Triton-insoluble fraction was also included to indicate production of EspB by the bacteria. Non-infected (NI) cells were included as a negative control for EspB detection and Δ*escN*-infected cells were included as a negative control for T3SS-dependent translocation. Bacterial growth in apical media was measured by optical density measurements at 600 nm (OD_{600}). Blots are representative of a single experiment performed in duplicate (n = 1). Bars indicate mean values of four (WT, n = 4) or three (Δ*escN*, n = 3) replicates from two independent experiments (n = 3).

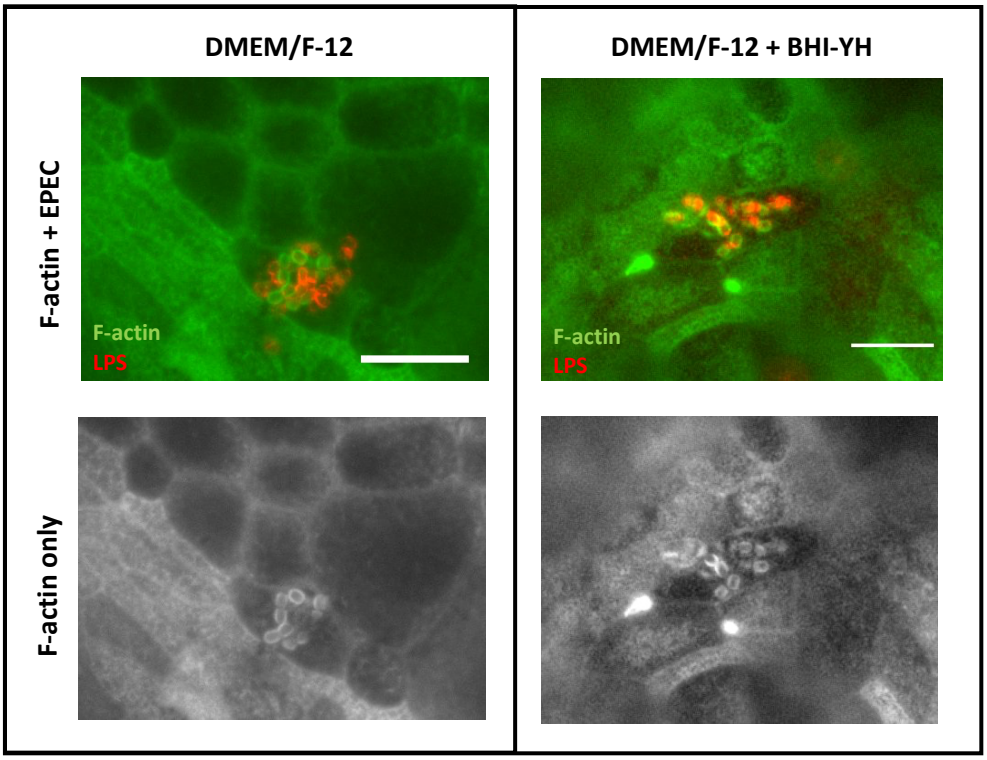

Figure 3.4 EPEC forms actin pedestals on T84 monolayers in DMEM/F-12 + BHI-YH medium. T84 monolayers were infected with EPEC in DMEM/F-12 or DMEM/F-12 + BHI-YH apical medium for 4h. Samples were stained for F-actin (green) and EPEC LPS (red) (top row). Images in the bottom row show the F-actin channel only in monochrome. Images are representative of three independent experiments performed in duplicate (n = 6). Bar = 10 µm.

3.2.4 Optimisation of *L. reuteri* and *R. gnavus* inoculums

Selection of commensal inoculums is a crucial factor to consider when designing colonisation resistance experiments, as several publications have demonstrated enhanced antagonistic activities with increasing numbers of commensal microbes (Bernet *et al.* 1994; Walsham *et al.* 2016). This is likely due to competition for binding sites and nutrients, highlighting the need to strike a balance between pathogen and commensal to enable effective pathogenesis but also antagonistic activity. Consequently, a 1:1 ratio of EPEC to commensal after a 4-hour culture period in the VDC was targeted. For these experiments, EPEC was cultured with a range of commensal inoculums for 4 hours in the VDC without cells and the amount of *L. reuteri* PTA 6475 and *R. gnavus* ATCC 35913 relative to EPEC was determined. The EPEC inoculum was fixed at 6.25 x 10^4 CFU/mL as this has been shown to produce A/E lesions on T84 cells after a 4-hour infection in DMEM/F-12 + BHI-YH. *L. reuteri* was co-cultured with EPEC at 2.4 x 10^6, 2.4 x10^7 and 2.4 x10^8 CFU and EPEC and *L. reuteri* were quantified by plate

counting on LB under aerobic conditions and MRS under anaerobic conditions, respectively. CFU data indicated the absence of colonies on MRS agar and LB agar for EPEC and *L. reuteri* monocultures respectively, confirming selective growth. This allowed quantification of EPEC and *L. reuteri* from co-cultures by counting colonies that grew after plating on LB and MRS agar, respectively. As expected, increased amounts of *L. reuteri CFUs* were observed from co-cultures with more concentrated inoculums. To identify a *L. reuteri* inoculum that corresponded to ~1:1 ratio with EPEC, the point at which the lines crossed for EPEC and *L. reuteri* CFU data was calculated. From this, the optimal *L. reuteri* inoculum for use in future experiments was calculated as 3.5 x 10^7 CFU/mL.

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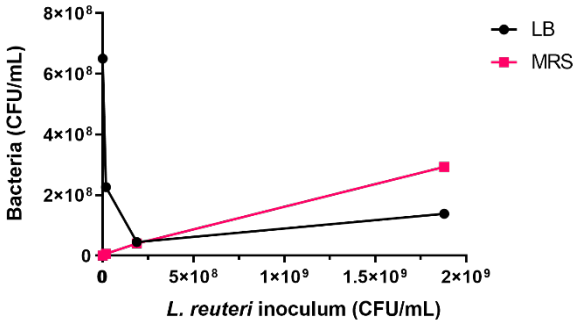

Figure 3.5 Identifying optimal inoculum of *L. reuteri*. EPEC and *L. reuteri* were co-cultured in the VDC for 4 hours, plated onto LB and MRS agar, and CFUs were determined by counting colonies. Data was collected from a single experiment performed in duplicate (n = 2).

For *R. gnavus*, bacteria were cultured at 9.8 x 10^5, 4.9 x 10^6, 2 x 10^7 and 6.1 x 10^7 CFU/mL with EPEC and the relative quantity of *R. gnavus versus* EPEC was assessed by IFS using an antibody targeting *R. gnavus* NanH. As shown in **Fig. 4.6**, the number of stained bacteria increased with *R. gnavus* inoculum indicating that this method was suitable for determining relative amounts of each organism. EPEC was present in greater numbers with an *R. gnavus* inoculum of the 4.9 x 10^6, while *R. gnavus* was more prevalent following inoculation with 2 x 10^7 CFU/mL. Hence, the mid-point of these inoculums (9.8 x 10^6 CFU/mL) was chosen for future experiments.

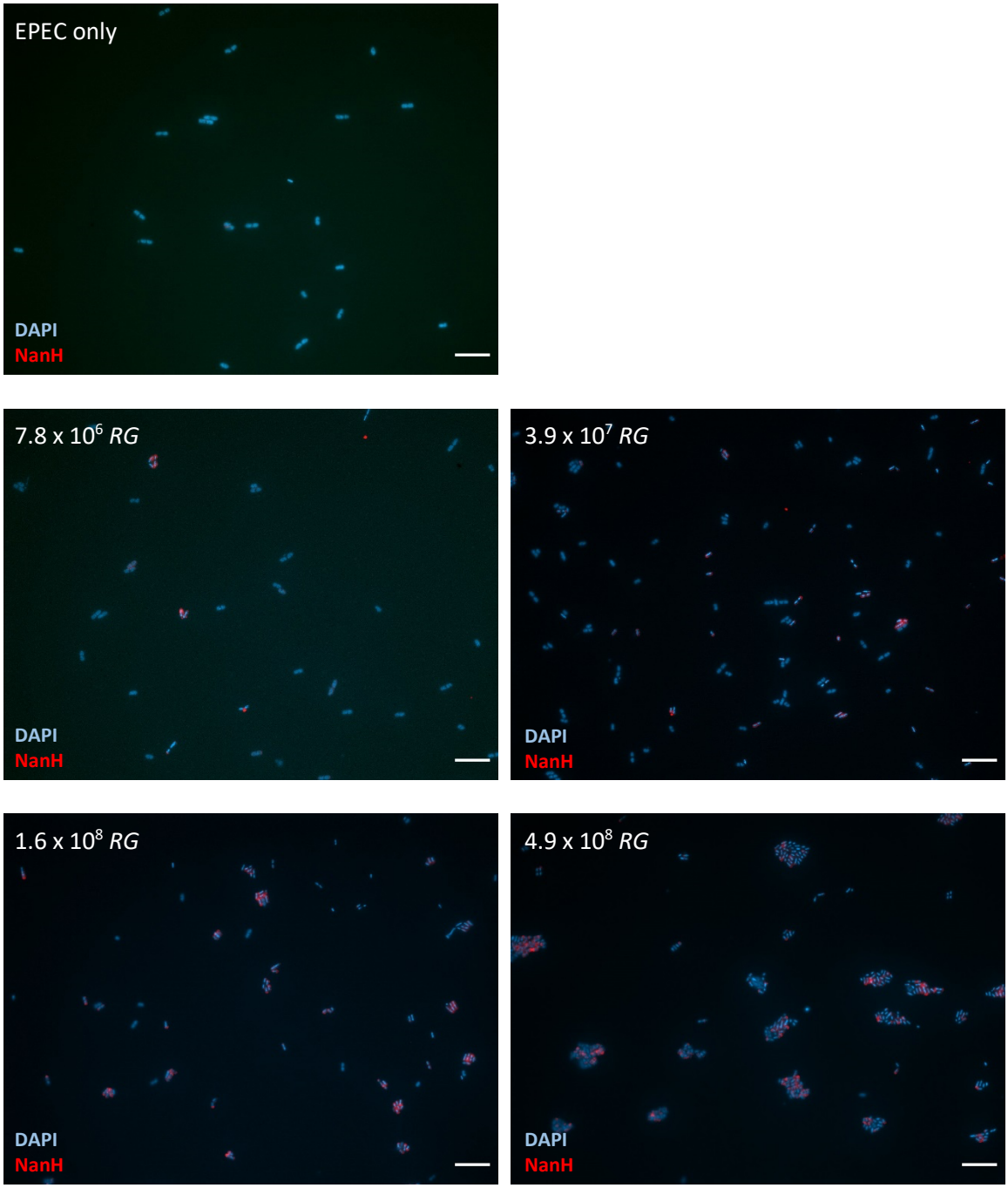

Figure 3.6 Identifying optimal inoculum of *R. gnavus*. EPEC and *R. gnavus* (*RG*) were co-cultured in the VDC for 4 hours and then stained for NanH (red) with DAPI counterstain (blue). Images are representative of a single experiment performed in duplicate (n = 2). Scale bar = 10 µm.

3.2.5 T84/LS174T co-cultures form mucus-secreting epithelia

In order to generate a mucus-producing epithelium, the MUC2-secreting cell line LS174T was introduced into the VDC system. To mimic the ratio of enterocytes versus goblet cells in the small

intestine mucosa, a ratio of 9:1 (T84 versus LS174T cells) was chosen. Mixed cultures and monoculture controls were grown on Snapwell inserts for 9-13 days, incubated in the VDC for 4 hours, and MUC2 production was evaluated by IFS (**Fig. 3.7**).

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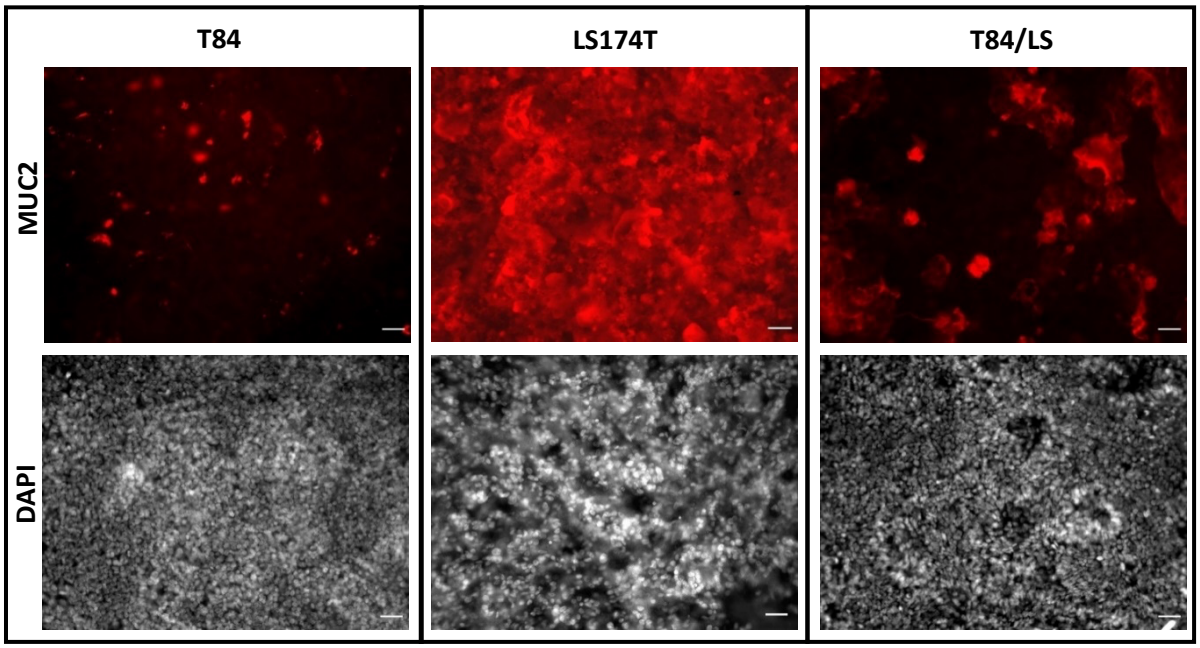

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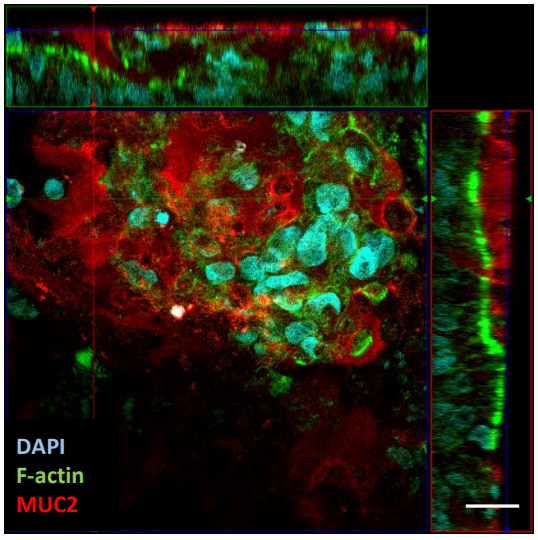


Figure 3.7 MUC2 secretion by LS174T, T84 and T84/LS epithelia. (**A**) Epifluorescence images of T84, LS174T and T84/LS cells (9:1) grown on Snapwell filters and incubated for 4 hours in the VDC. Cells were stained for MUC2 and nuclei were counterstained with DAPI. (**B**) Confocal laser scanning microscopy image of T84/LS epithelia (9:1) stained for F-actin (green) and MUC2 (red). Ortho-view image of a z-stack constructed by layering 1 µm xz slices. Images are representative of two independent experiments performed in duplicate. Scale bar = 25 µm.

LS174T monocultures demonstrated a continuous, wave-like MUC2 pattern which is reminiscent of a mucus layer. This phenotype was maintained in T84/LS co-cultures but broken up by the presence of T84 cells which demonstrated low mucus production. Further analysis by confocal microscopy confirmed that mucus was secreted by LS174T cells, as seen by the extracellular MUC2 staining. Therefore, co-cultures of T84 and LS174T cells at a ratio of 9:1 will be used for future experiments. These images also highlight clumping of LS174T cells, hence T84/LS epithelia do not form monolayers.

3.3 Discussion

In response to the growing recognition of the importance of low oxygen levels and the presence of mucus for microbiota establishment in the human gut, several model systems aiming to mimic these conditions are emerging. Much of the *in vitro* work investigating host-microbiota interactions have historically used models that do not consider these key features, ignoring the contribution of oxygen-sensitive anaerobes (which make up the majority of the intestinal flora) and activity at the mucosal interface.

In this study, we have developed a model system of the human intestinal mucosa by introducing a mucus-producing T84/LS cell epithelium into the microaerobic VDC system and optimising media compositions and bacterial inoculums to support host-commensal-pathogen co-culture. The integrity and barrier function of the intestinal epithelium was maintained in a microaerobic apical environment. In addition the VDC system supported growth of pathogenic EPEC E2348/69, probiotic *L. reuteri* PTA 6475 and the oxygen-sensitive gut symbiont *R. gnavus*. Furthermore, EPEC T3S and A/E lesion formation was confirmed in the VDC model.

3.3.1 Identifying a suitable apical medium for incubation of host epithelia with EPEC, *L. reuteri* and *R. gnavus* in the VDC

Growth curves of EPEC, *L. reuteri* and *R. gnavus* indicated growth of all organisms in BHI-YH and DMEM/F-12 + BHI-YH medium, but not in DMEM/F-12. These data indicate incomplete nutritional composition of DMEM/F-12 medium for growth of all 3 organisms, which is overcome following the addition of BHI-YH. Growth of the oxygen-sensitive anaerobe *R. gnavus* demonstrates that the gaseous environment produced by the VDC during perfusion of anaerobic gas to both apical and basal compartments is sufficiently low in oxygen to culture strictly anaerobic gut commensals. However,

during culture with host epithelia, the basal compartment is perfused with an aerobic gas mixture, which may diffuse into the apical compartment and thereby raise oxygen levels. Experimental data on this setting will be provided in Chapter 4 and confirms the suitability of the VDC for culture of obligate anaerobes. This has been confirmed by previous studies demonstrating the capability of the VDC to support pathogenesis of the strict anaerobe *Clostriodes difficile* and probiotic activity of *Bacteroides dorei* alongside IECs (Anonye *et al.* 2019).

The observation that each organism exhibited distinct growth kinetics in DMEM/F-12 + BHI-YH highlighted the need to balance bacterial loads. This was crucial to support EPEC pathogenesis whilst preventing EPEC overgrowth, and to yield sufficient commensal organisms to facilitate competition with the pathogen. The results shown here indicated that an EPEC inoculum of 6.25 x 10^4 CFU/mL supported adherence, T3SS effector translocation and actin pedestal formation in polarised T84 cells. This is orders of magnitude lower than the inoculums used in other studies utilising conventional cell culture methods and DMEM/F-12 medium (10^7 CFU/mL), however enhanced growth in DMEM/F-12 + BHI-YH and the microaerobic conditions of the VDC could facilitate similar levels of T3SS effector translocation and actin pedestal formation (Pal *et al.* 2019; Walsham *et al.* 2016; Knutton *et al.* 1997). Comparison of enterohaemorrhagic *E. coli* (STEC) virulence traits under standard cell culture gas conditions and the VDC microaerobic environment indicated elevated adherence and T3SS effector translocation under low oxygen (Schuller and Phillips 2010). It is difficult to compare the commensal loads used here with those in other studies on colonisation resistance due to rapid EPEC growth in DMEM/F-12 + BHI-YH. Hence, whilst the starting loads of *L. reuteri* and *R. gnavus* relative to EPEC are very high (560x and 160x excess), a 1:1 ratio is achieved after 4 hours of growth. Nevertheless, the bacterial load of *L. reuteri* PTA 6475 used here is in line with other studies demonstrating colonisation resistance activities of lactobacilli. Attenuation of EPEC adherence to Caco-2 cells by *Lactobacillus acidophilus* La1 was observed using a 1x or 10x excess of the commensal (Bernet *et al.* 1994; Coconnier *et al.* 1993). Adherence of STEC and EPEC to polarised T84 cells was reduced during co-culture with *L. acidophilus* and *Lactobacillus rhamnosus* at a 10x and 1000x excess, respectively (Sherman *et al.* 2005). Furthermore, Walsham *et al.* demonstrated attenuation of EPEC adherence to HT-29 cells by both *L. reuteri* PTA 6475 and ATCC 53608 when 100x excess was used, but not with an excess of 10x or 1x (Walsham *et al.* 2016). There is limited information on the colonisation resistance activity of *R. gnavus,* hence the load required facilitate effective competition with pathogens has not been established.

It is interesting that DMEM/F-12 + BHI-YH medium supports structural integrity, polarity and barrier function of T84 monolayers whereas BHI-YH does not. This suggests that BHI-YH medium is lacking certain nutrients required by host cells to maintain epithelial structure and function, and/or that BHI-YH contains cytotoxic components whose deleterious effects are reduced by dilution with F-12.

Interestingly, incubating Caco-2 cells in glucose-free DMEM under hypoxic conditions has been shown to significantly reduce barrier function after 8 hours, compared to controls under atmospheric oxygen conditions (Huang *et al.* 2013; Huang, Pai, and Yu 2017). Supplementation of glucose to the medium reversed the barrier function disruption observed under hypoxic stress. This phenomenon was associated with an increase in anaerobic glycolytic metabolism, as introduction of a glycolysis inhibitor abolished the protective effect of glucose. As glucose is present in F-12, but not BHI-YH, this may explain why epithelia cultured in DMEM/F-12 resist these deleterious effects on barrier function most effectively, followed by epithelia maintained in DMEM/F-12 + BHI-YH and BHI-YH (40%, 74% and 90% reduction in barrier function, respectively). These results could also be due to cytotoxic components in BHI-YH medium but studies investigating the influence of microbial culture media on eukaryotic cell viability are rare. and the nutritional compositions of DMEM/F-12 and BHI-YH are too complex for systematic evaluations.

The observation that EPEC forms actin pedestals on T84 monolayers after a 4-hour infection in DMEM/F-12 + BHI-YH medium is consistent with previous studies. Whilst publications investigating actin pedestal formation on T84 cells infected by EPEC are scarce, studies using other epithelial cell lines (e.g. Hep-2 or Caco-2) or using T84 cells with other A/E pathogens (such as STEC) show pedestal formation within 3-5 hours of infection (Batchelor *et al.* 2004; Johnson-Henry *et al.* 2001; Johnson-Henry *et al.* 2008; Yamamoto *et al.* 2009; Lewis *et al.* 2015). This observation is important as many factors can affect the kinetics of T3SS secretion and subsequently EPEC pathogenesis. In the closely related pathogen STEC, environmental cues such as oxygen, mucus-derived sugars and nutrient availability influence bacterial T3S to enable optimal expression in the colon (Ando *et al.* 2007; Carlson-Banning and Sperandio 2016; Schuller and Phillips 2010). Carbon metabolism and T3S are inextricably linked through the activities of the transcriptional regulators Cra and KdpE. Expression of *ler*, the master regulator of the T3SS, increased two-fold during growth in 0.1% glucose DMEM, compared to 0.4% glucose (Njoroge *et al.* 2012). Glucose-dependent expression of *ler* was abolished in *Δcra* and *ΔkdpE* strains, implicating these regulators in T3SS activation. This is likely to extend to EPEC, where culture in low glucose DMEM is frequently used to induce T3SS expression in a process called 'DMEM priming' (Abe *et al.* 2002; Rosenshine, Ruschkowski, and Finlay 1996; Khan *et al.* 2008; Ruchaud-Sparagano, Maresca, and Kenny 2007; Furniss and Clements 2018). Furthermore, dilution of sodium bicarbonate, which has been shown to increase production of T3SS proteins, in DMEM/F-12 + BHI-YH may have influenced actin pedestal formation. Results shown here demonstrate EPEC effector translocation and actin pedestal formation in DMEM/F-12 + BHI-YH medium in a microaerobic VDC which is a prerequisite for subsequent studies on colonisation resistance by commensal bacteria.

3.3.2 Establishment of a mucus-producing epithelium using intestinal epithelial cell lines

Here, a mucus-secreting epithelium has been established by co-culturing the columnar crypt-like T84 cell line with the mucus-producing goblet-like cell line LS174T. These epithelia were successfully cultured in the VDC, yielding a mucus-secreting epithelium with a low oxygen apical environment. Culturing the two cell lines at a 9:1 ratio (similar to the enterocyte to goblet cell ratio seen in the mouse SI) produced epithelia which secreted MUC2, the major component of intestinal mucus layers (Allen, Hutton, and Pearson 1998; Cheng 1974). Notably, T84/LS epithelia shared features with the commonly used Caco-2/HT29-MTX enterocyte-goblet cell model including patchy mucus secretion corresponding to the proportion of goblet-like cells and mucus localisation to the microvilli (Walter *et al.* 1996).

It is challenging to ascertain how representative the mucus secretion observed here is of native small intestine mucosa as very little is known about its architecture. Some studies on small intestinal biopsies suggest a patchy mucus layer, whereas others suggest an undisturbed layer, likely due to different tissue processing and fixation techniques (Atuma *et al.* 2001; Johansson, Sjovall, and Hansson 2013; Lim *et al.* 2013b; Szentkuti and Lorenz 1995). Furthermore, most of the information on the physiology of small intestine mucus production has been collected using rodent models. Despite sharing many fundamental features of the small intestine with humans, mice vary in numerous crucial aspects including differing microbiota composition due to host-adaption, epithelial physiology and species-specific MUC2 O-glycosylation patterns (Frese *et al.* 2011; Hugenholtz and de Vos 2018; Nagpal *et al.* 2018; Thomsson *et al.* 2012; Mundy *et al.* 2006). The implications of these differences are unknown, and therefore great care must be taken when applying results obtained on rodent small intestine mucus to humans. Importantly, the advent of organoid culture has allowed the regeneration of native goblet cell-containing human intestinal epithelia and subsequent studies on mucus secretion (Sato *et al.* 2011; In *et al.* 2019). However, much of this work has been performed on colonic organoids producing a thick continuous mucus layer, whereas mucus secretion from small intestine enteroids is poorly defined (Liu *et al.* 2020; Sontheimer-Phelps *et al.* 2020). From the limited data available, it is unclear whether secreted mucus is localised to goblet cells or distributed as a thin layer covering the epithelial surface (Fofanova *et al.* 2019; Roodsant *et al.* 2020). Apart from epithelial complexity, other factors have been implicated in MUC2 secretion. Innovative studies by Navabi *et al.* (2013) demonstrated that reduced medium volume and shear stress related to intestinal peristalsis enhanced the formation of a mucus layer in Caco-2/HT29-MTX co-cultures (Navabi, McGuckin, and Lindén 2013). Application of these conditions with T84/LS epithelia may result in a continuous mucus layer.

In conclusion, our studies indicate a secreted discontinuous mucus layer in T84/LS epithelia with mucus localisation to goblet-like LS174T cells. This pattern of MUC2 production agrees with the Caco-2/HT29-MTX model, as well as certain studies on murine biopsies and human-derived small intestine enteroids. Nevertheless, other studies have produced conflicting data showing a thin, yet continuous mucus layer. The differences in results likely reflect the complexity and dynamics of mucus secretion in the small intestine, which is driven by a multitude of factors such as goblet cell turnover, shear stress and microbiota (Paone and Cani 2020). In addition, differences in cell and tissue handling, as well as staining techniques are also likely to contribute to inter-study variability (Herath *et al.* 2020; Lim *et al.* 2013a). Regardless of whether the T84/LS epithelia developed here are representative of native tissue, the fact that secreted mucus is present in a low-oxygen environment facilitates the study of host-commensal-pathogen interactions in an environment that more accurately represents the human intestinal mucosa than conventional cell culture models.

It is important to stress that other culture systems have been developed attempting to mimic the low oxygen conditions and mucus secretion seen in the intestine. Several low oxygen systems capable of culturing anaerobic bacteria have been produced. These include interconnecting bioreactors that mimic oxygen levels, pH, bile salt and digestive enzyme levels of the various regions of the GIT (von Martels *et al.* 2017). These bioreactor models have been applied to culture complex microbiotas derived from human faecal samples, although they lack the presence of a host epithelium (Aguirre *et al.* 2014; Kovatcheva-Datchary *et al.* 2009; Terpend *et al.* 2013). Cells can be introduced into these systems through the use of a Host-Microbiota Interaction (HMI™) module, which contains IEC monolayers separated from the bioreactor effluent by a semi-permeable membrane. This system allows the study of interactions between bacteria and the host via secreted metabolites. The Human-oxygen Bacteria-anaerobic (HoxBan) model enables culture of strictly anaerobic *Faecalibacterium prausnitzii* in an agar column overlaid by IECs grown on coverslips. (Sadabad *et al.* 2015). Culture of *Bacteroides caccae* has also been demonstrated in the human–microbial crosstalk (HuMiX) microfluidics model, which is composed of a microbial and host cell chamber separated by a nanoporous membrane (Shah *et al.* 2016). More recent microfluidic models, such as the gut microbiome (GuMI) physiome platform and the gut-on-a-chip system have demonstrated culture of obligate anaerobes (*F. prausnitzii* and *Bacteroides fragilis*) with direct contact to host cells (Jalili-Firoozinezhad *et al.* 2019; Zhang *et al.* 2020). Additional culture experiments with the intestine-on-a-chip system highlighted culture of a human faecal microbiota containing *Ruminococcus* sp., although species classification was not performed (Jalili-Firoozinezhad *et al.* 2019). A recent study introduced intestinal organoids into the intestine-on-a-chip model, demonstrating the production of a secreted mucus layer inhabited by *Bacteroides fragilis* (Jalili-Firoozinezhad *et al.* 2019). This is similar to a study

utilising an anaerobic adaption to the Transwell system, which supported organoids with a mucus layer ~20 µm thick (Sasaki *et al.* 2020). This system facilitated culture of the obligate anaerobe *Akkermansia municiphila*, which colonised and degraded the secreted mucus.

The system developed here supports the culture of obligate anaerobic bacteria alongside a mucus-secreting intestinal epithelium using human-derived cell lines and will be used to gain insight into host-microbe interactions.

Chapter Four - Investigating host-microbe interactions in a mucus-secreting epithelium

4.1 Introduction

Intestinal mucus provides a habitat for the microbiota whilst restricting access to the underlying intestinal epithelium. In both the small and large intestines, MUC2 is the major glycoprotein in secreted mucus (Johansson, Larsson, and Hansson 2011). The range and breadth of glycosylation patterns observed on MUC2 glycoproteins (built from 47 potential O-linked and 30 N-linked glycans) yields a diverse set of colonisation sites and nutrients for the intestinal microbiota (Arike, Holmen-Larsson, and Hansson 2017). In the large intestine, this results in colonisation by a complex microbiota that are crucial in metabolism and maintaining gut homeostasis. The relationship between the small intestine mucus and the microbiota is more complex, due to the need to retain permeability for nutrient absorption but to restrict exposure of the epithelium to luminal bacteria. To overcome this, small intestine mucus contains antimicrobial compounds (such as defensins and lectins) that are produced at higher concentrations close to the epithelium, but decrease towards the intestinal lumen, maintaining a bacteria-free region proximal to the host cells whilst minimising impact on viability of luminal bacteria (Dupont *et al.* 2014; Meyer-Hoffert *et al.* 2008; Vaishnava *et al.* 2011). In addition to secreted mucus, intestinal epithelial cells are covered by a thin layer of transmembrane mucins known as the glycocalyx (Hattrup and Gendler 2008). The glycocalyx provides the second line of defence if the mucus layer is breached, re-enforcing antagonist activities through anti-microbial peptides, decoy binding sites and immunomodulation upon microbial detection (Johansson and Hansson 2011; McGuckin *et al.* 2011).

As a pathogen that targets the small intestinal epithelium, EPEC must penetrate both the mucus layer and glycocalyx to initiate the intimate adherence required for pathogenicity. Characterisation of carcinoembryonic antigen-related cell adhesion molecules (CEACAMs), an essential component of the glycocalyx, production in polarised IECs revealed extracellular CEACAMs present on the apical surface of T84 cells, suggesting the presence of a glycocalyx. As EPEC adherence, T3S and stimulation of the host-inflammatory response have been studied using T84 monolayers, it is presumed that EPEC can subvert the glycocalyx to infect the host cells (McNamara *et al.* 2001; Zhou *et al.* 2003). Nevertheless, as T84 cells do not secrete much mucus, the interactions between EPEC and a mucus-secreting epithelium are poorly understood. Even within the limited studies investigating the role of mucus in EPEC adherence, the evidence is conflicting. Studies adding purified mucins from cell lines or rabbit small intestine tissue to non-mucus secreting HT-29 or Hep-2 cells demonstrated reduced EPEC adherence when mucins were present (Mack *et al.* 1999; Smith, Kaper, and Mack 1995). On the contrary, EPEC adherence to mucus-producing LS174T cells was 10-fold higher compared with mucus-deficient HT-29 cells (Walsham *et al.* 2016). Regardless, it is clear that EPEC adherence is affected by

the presence of mucus and more insight can gained from studies using mucus-producing model systems. Although studies identifying mucus adhesins from EPEC are scarce, flagellin may well be a mediator. Purified H6 flagellin from EPEC strain E2348/69 has been shown to bind to crude bovine mucus, and a corresponding EPEC *fliC* mutant exhibited reduced colonisation of bovine intestinal tissue (Erdem *et al.* 2007).

In addition to being a potential mediator of mucus adhesion, flagellin also triggers the host innate immune response. Activation of TLR5 on the surface of IECs by EPEC flagellin causes the release of IL-8, the predominant cytokine release during EPEC infection of human intestinal cell lines and biopsy tissue (Ruchaud-Sparagano, Maresca, and Kenny 2007; Schuller *et al.* 2009; Sharma *et al.* 2006). IL-8 acts as a chemoattractant to recruit circulating neutrophils into the mucosa, which form the primary defence to EPEC by releasing anti-bacterial enzymes and phagocytosis (Hammond *et al.* 1995). Studies evaluating the IL-8 response to EPEC using epithelial models with and without goblet cells are lacking, and cross-study comparisons are challenging due to differences in protocols (e.g. infection times, MOI, DMEM priming). As such the contribution of goblet cells to the innate immune response to EPEC remains unknown.

Strong adhesion to the mucus layer by lactobacilli is crucial to efficient colonisation due to the shear stress generated by strong peristaltic waves in the small intestine. To aid mucosal colonisation, *L. reuteri* expresses several surface adhesins which interact with mucus *in vitro*, including MUB, CmbA and MapA (Etzold *et al.* 2014; Jensen *et al.* 2014; Roos and Jonsson 2002; Miyoshi *et al.* 2006). Mutant strains lacking these adhesins exhibit significantly reduced adhesion to Caco-2 and LS174T cells, highlighting their importance in intestinal colonisation. Interestingly, studies investigating *L. reuteri* adhesion to human biopsy tissues indicated that the bacteria were found exclusively in the mucus layer, likely due to an inability to penetrate it and reach the epithelial surface (Ouwehand *et al.* 2002; Walsham *et al.* 2016). Whilst the application of biopsies has advanced understanding of how *L. reuteri* interacts with native intestinal tissue, the high oxygen levels required to maintain biopsy tissue prohibits application of microaerobic conditions relevant to the intestinal environment.

R. gnavus is a prominent member of the gut microbiota exhibiting mucin-degradation activities that are strain-specific (Crost *et al.* 2013). Strain ATCC 35913 encodes a range of glycoside hydrolases (GHs) to liberate complex mucin-derived oligosaccharides and convert them to smaller sugars for catabolism (Crost *et al.* 2013). Of the proteins with GH activity, NanH, a protein with the capacity to release sialic acid from mucins, also contains the carbohydrate binding module (CBM) 40. Out of a variety of mucin sources, CBM40 exhibited highest affinity to mucus purified from LS174T cells likely due to the considerable degree of sialylation (91% compared to 8% for other sources) (Owen *et al.* 2017).

Nevertheless, the contribution of secreted mucus glycoproteins to *R. gnavus* growth and adherence has not yet been studied with human epithelial cells, due to the oxygen sensitivity of *R. gnavus* and its incompatibility with conventional cell culture.

In this section we sought to determine the influence of mucus secreting LS174T cells on the growth and adherence of EPEC E2348/69, *L. reuteri* PTA 6475 and *R. gnavus* ATCC 35913. In addition, the inflammatory IL-8 response to EPEC infection was assessed.

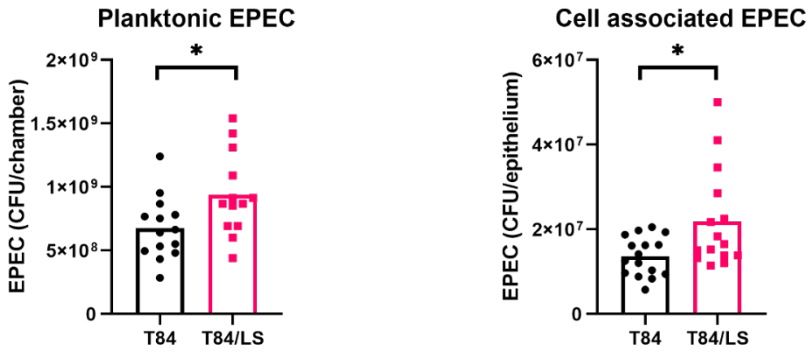
4.2 Results

4.2.1 LS174T cells enhance EPEC growth and epithelial adherence

In order to determine the effect of LS174T cells on EPEC E2348/69 proliferation and epithelial binding, T84 monolayers and T84/LS co-cultures were infected with EPEC in the VDC. After 4 hours, viable planktonic and adherent bacteria were quantified in apical media and cell lysates, respectively, by plating on LB agar and counting CFUs. In addition, infected epithelia were stained for MUC2 and LPS (EPEC) to assess mucus production and bacterial adherence.

Data presented in **Fig. 4.1** reveals significantly enhanced numbers of planktonic and adherent EPEC in T84/LS compared to T84 epithelia. Immunofluorescence images showed MUC2 production by LS cells and EPEC adherence to both T84 and T84/LS epithelia. Interestingly, cross-reaction between the EPEC LPS and MUC2 antibody was observed.

A



B

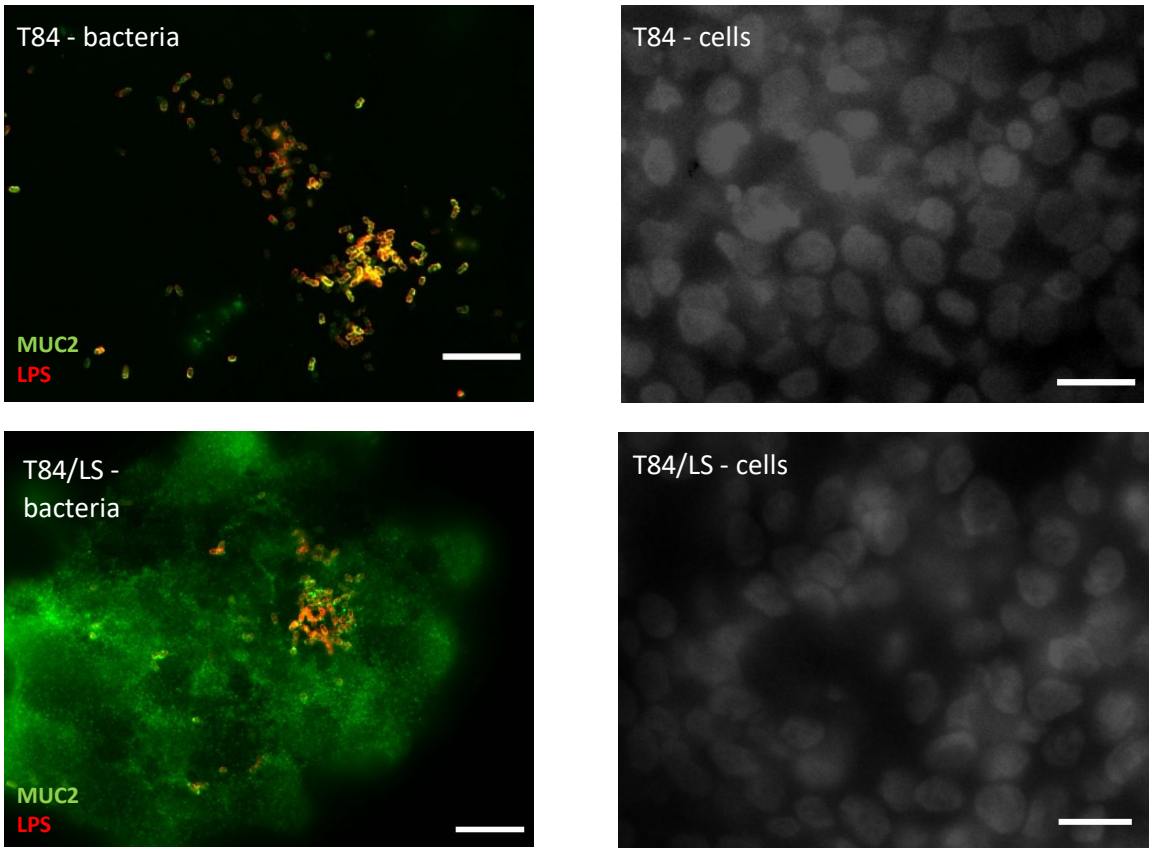


Figure 4.1 The presence of LS174T cells increases numbers of planktonic and adherent EPEC. T84 and T84/LS epithelia were infected with EPEC for 4 hours in the VDC. (**A**) Planktonic and adherent EPEC were quantified as CFUs. The graphs show data collected from four independent experiments performed in triplicate (n = 12). **P* < 0.05. (**B**) Intestinal epithelia were stained for MUC2 (green), EPEC LPS (red) and cell nuclei (grey, right panels). As bacteria and cell nuclei were localised in different focal planes, two images of the same field of view were taken in each plane. Scale bar = 10 µm. Images are representative of two independent experiments performed in triplicate (n = 6).

To determine whether the enhanced EPEC growth observed with T84/LS was dependent on secreted mucus, the growth of EPEC with purified porcine gastric mucin (PGM) was assessed. Growth curves of EPEC in DMEM/F-12 supplemented with PGM revealed enhanced growth in response to higher concentrations of PGM, suggesting utilisation of mucin-derived sugars for growth **(Fig. 4.2**).

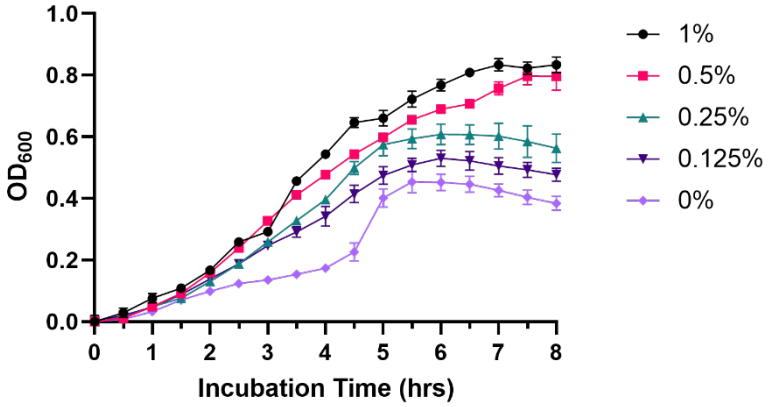
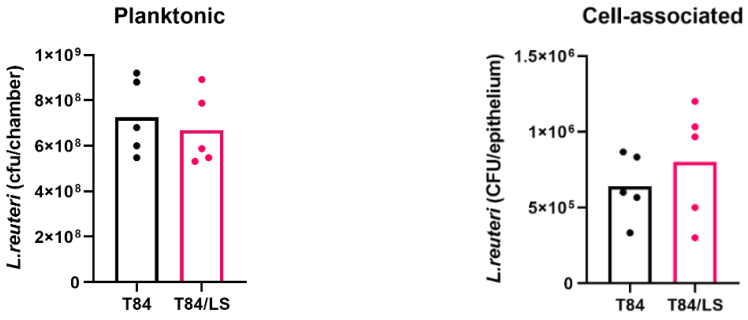


Figure 4.2 PGM enhances the growth of EPEC. EPEC was cultured in DMEM/F-12 supplemented with 0.125, 0.25, 0.5 or 1% (w/v) PGM or plain medium at 37 °C in air/5% CO_2. OD_{600} was monitored every 30 minutes. Data shown as mean value ± SEM of a single experiment performed in triplicate (n = 3).

4.2.2 Viability and adherence of *L. reuteri* are unaffected by the presence of LS174T cells

To investigate how mucus producing LS174T cells influence *L. reuteri* PTA 6475 growth and adherence, T84 and T84/LS epithelia were incubated with *L. reuteri* for 4 hours in the VDC. Viable bacteria were quantified from apical media and cell lysates (planktonic and adherent *L. reuteri*, respectively) by plate counting on MRS agar. In addition, bacterial adherence was evaluated by immunofluorescence staining using an antibody against the *L. reuteri* adhesin CmbA. Colony counts revealed no significant difference in numbers of adherent or planktonic *L. reuteri* in T84 and T84/LS epithelia **(Fig. 4.3A**). Interestingly, planktonic growth of *L. reuteri* in the VDC was minimal, indicating only a 2.5-fold increase of the inoculum after 4 hours of incubation. In agreement with colony counts, immunofluorescence images demonstrated no discernible difference in numbers of adherent lactobacilli between the two different epithelia (**Fig. 4.3B**). Notably, *L. reuteri* was mainly localised to LS174T cells in mixed epithelia, suggesting a preferential adherence to this cell line in co-cultures. Notably, images with 63x magnification revealed co-localisation of the CmbA and MUC2 antibodies.

A



B

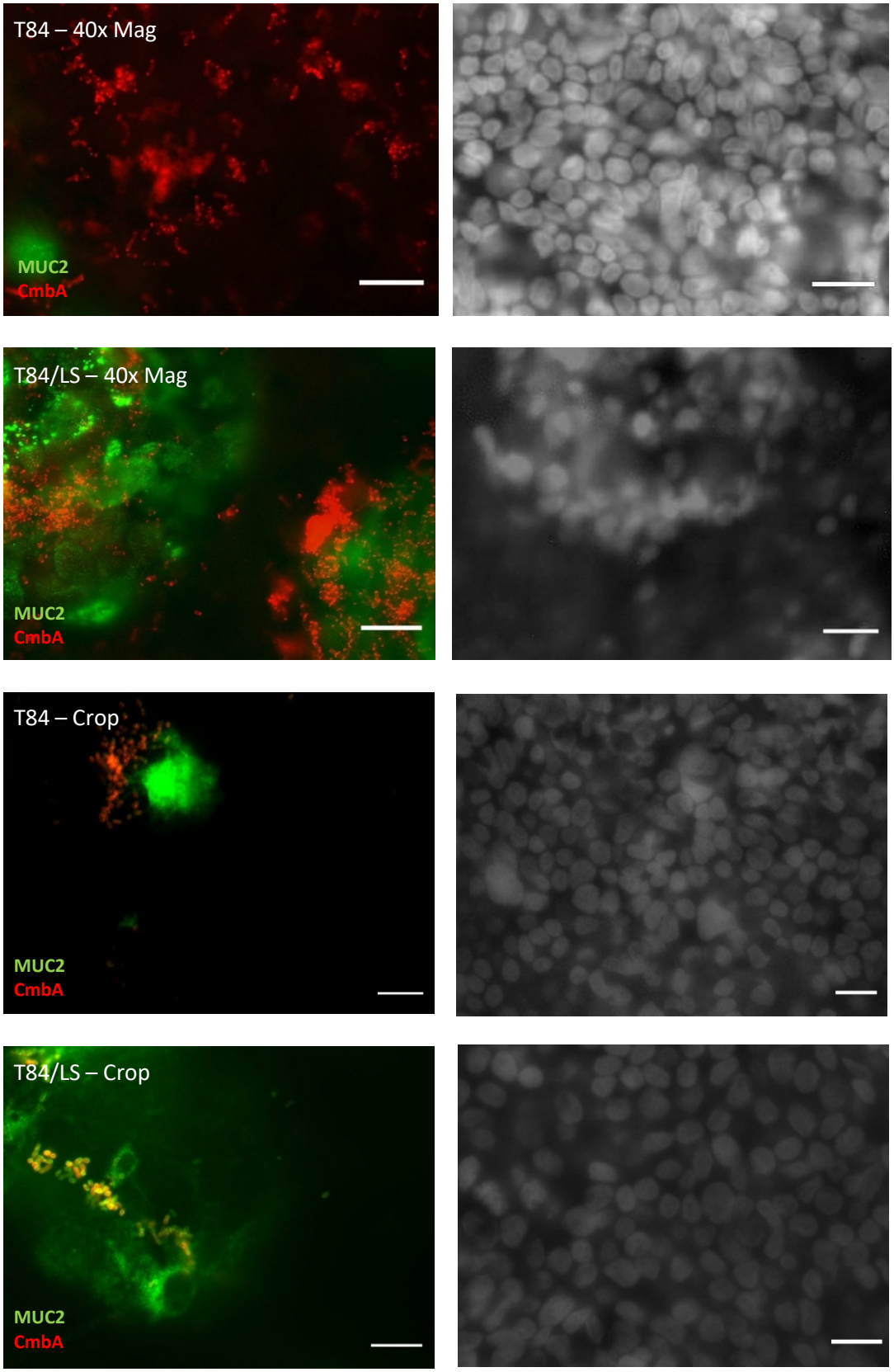

T84 – 40x Mag
MUC2
CmbA
T84/LS – 40x Mag
MUC2
CmbA
T84 – Crop
MUC2
CmbA
T84/LS – Crop
MUC2
CmbA

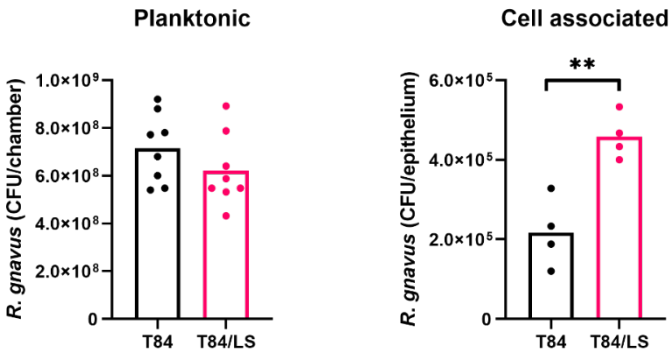
Figure 4.3 LS174T cells do not affect amount of planktonic or adherent *L. reuteri*. T84 and T84/LS epithelia were incubated with *L. reuteri* for 4 hours in the VDC. (**A**) Planktonic and cell-associated bacteria were quantified by plate counting. The graphs show data collected from five replicates collected over two independent experiments (n = 6). (**B**) Intestinal epithelia were stained for MUC2 (green), *L. reuteri* CmbA (red) and cell nuclei (grey, right panels). Cropped images are also shown. Scale bar = 25 µm or 10 µm for cropped images. Images are representative of six replicates collected from two independent experiments (n = 6).

4.2.3 Epithelial colonisation by *R. gnavus* is enhanced in T84/ LS174T epithelia, whilst planktonic growth remains unaffected

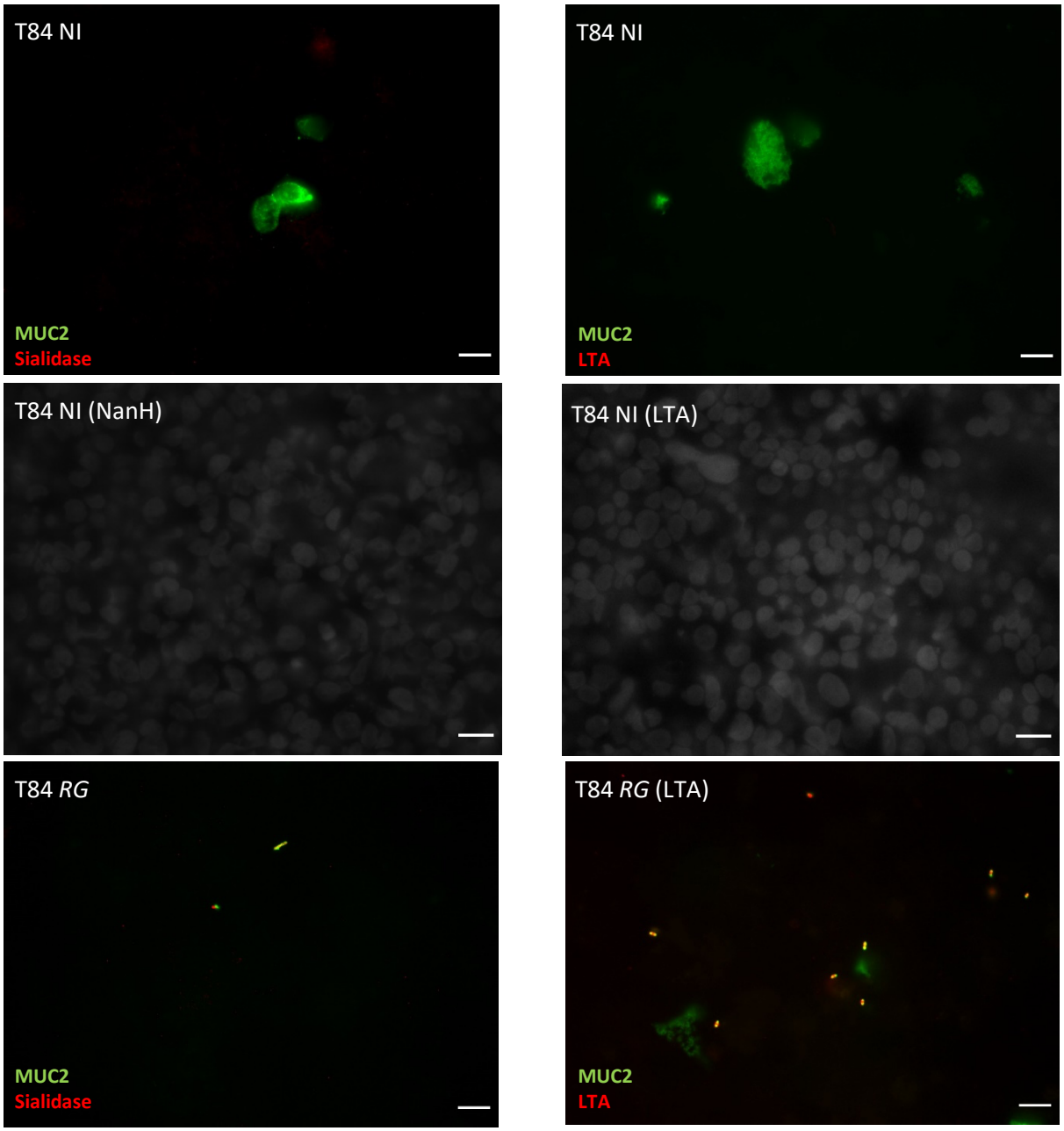
To determine the influence of LS174T cells on *R. gnavus* ATCC 35913 growth and adherence, VDC incubations of *R. gnavus* with T84 and T84/LS epithelia were performed, and numbers of planktonic and adherent bacteria were quantified as described above using BHI-YH agar plates. Initial experiments revealed that *R. gnavus* was sensitive to 1% Triton X-100 routinely used to prepare cell lysates to quantify adherent bacteria. Therefore, cells and associated bacteria were homogenised by vigorous pipetting in PBS in subsequent experiments. In addition to determining CFUs, *R. gnavus* association with T84 and mixed epithelia was investigated by immunostaining with antibodies targeting the *R. gnavus* sialidase NanH or LTA, a cell wall component of Gram-positive bacteria. The LTA antibody was included as NanH expression is dependent on mucin and might not be evident in T84 cells.

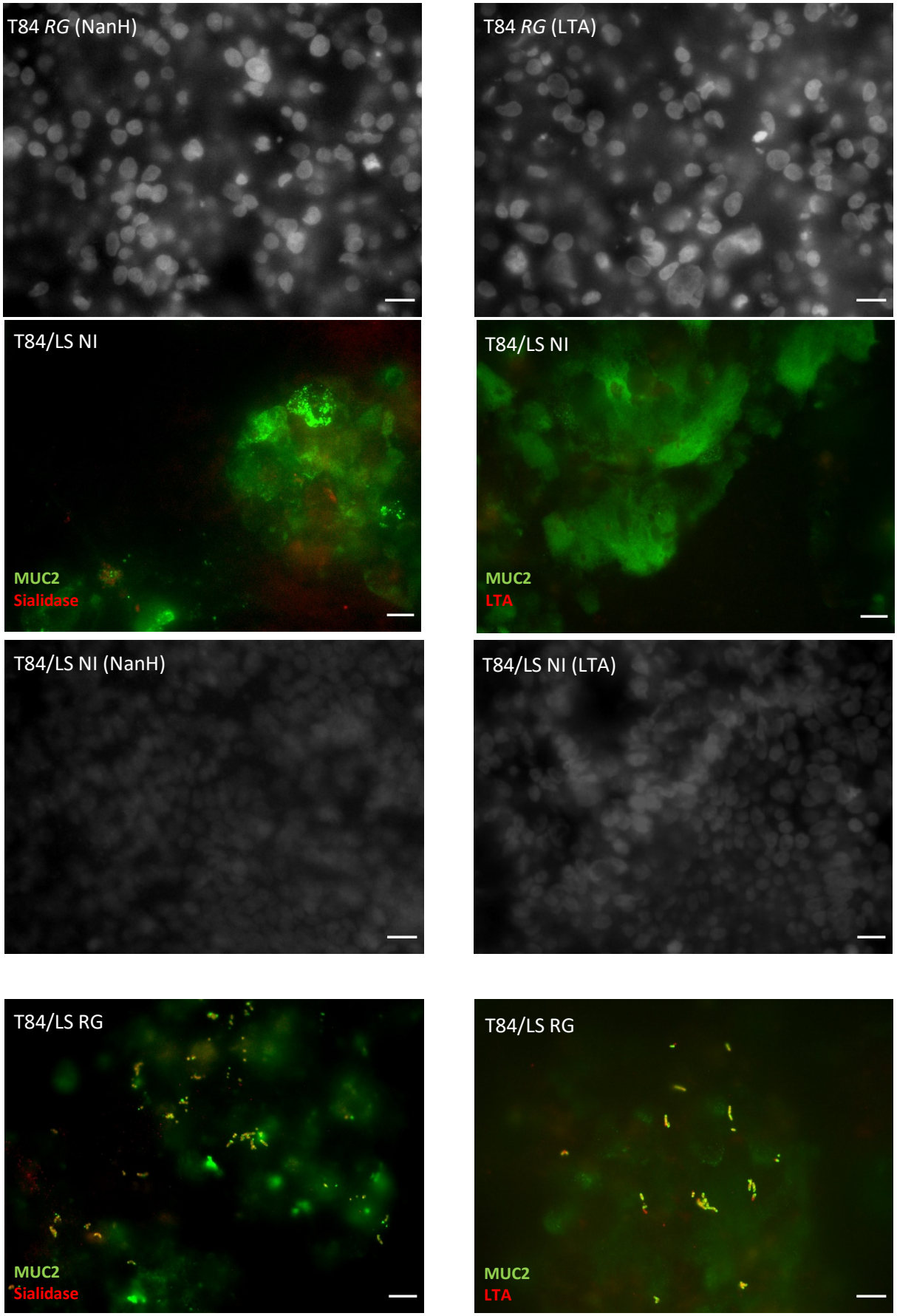
As shown in **Fig. 4.4A**, similar numbers of *R. gnavus* were quantified from the apical media of T84 and T84/LS epithelia, indicating that the presence of LS174T cells did not affect planktonic growth. However, *R. gnavus* adherence to T84/LS epithelia was increased more than 2-fold compared to T84 cultures. This was confirmed by immunofluorescence staining which showed higher numbers of *R. gnavus* on T84/LS versus T84 epithelia, with bacteria localised to mucus secreting LS174T cells **(Fig. 4.4B**). These observations were similar for antibodies targeting NanH and LTA, suggesting that potential differences in NanH expression between T84 and T84/LS epithelia did not notably influence estimates of bacterial numbers. Interestingly, *R. gnavus* bacteria were also stained with anti-MUC2.

A


Planktonic
Cell associated
R. gnavus (CFU/chamber)
1.0×10⁹
8.0×10⁸
6.0×10⁸
4.0×10⁸
2.0×10⁸
0
T84
T84/LS
R. gnavus (CFU/epithelium)
6.0×10⁵
4.0×10⁵
2.0×10⁵
0
**
T84
T84/LS

B


T84 NI
MUC2
Sialidase
T84 NI
MUC2
LTA
T84 NI (NanH)
T84 NI (LTA)
T84 RG
MUC2
Sialidase
T84 RG (LTA)
MUC2
LTA


T84 *RG* (NanH)
T84 *RG* (LTA)
T84/LS NI
MUC2
Sialidase
T84/LS NI
MUC2
LTA
T84/LS NI (NanH)
T84/LS NI (LTA)
T84/LS RG
MUC2
Sialidase
T84/LS RG
MUC2
LTA

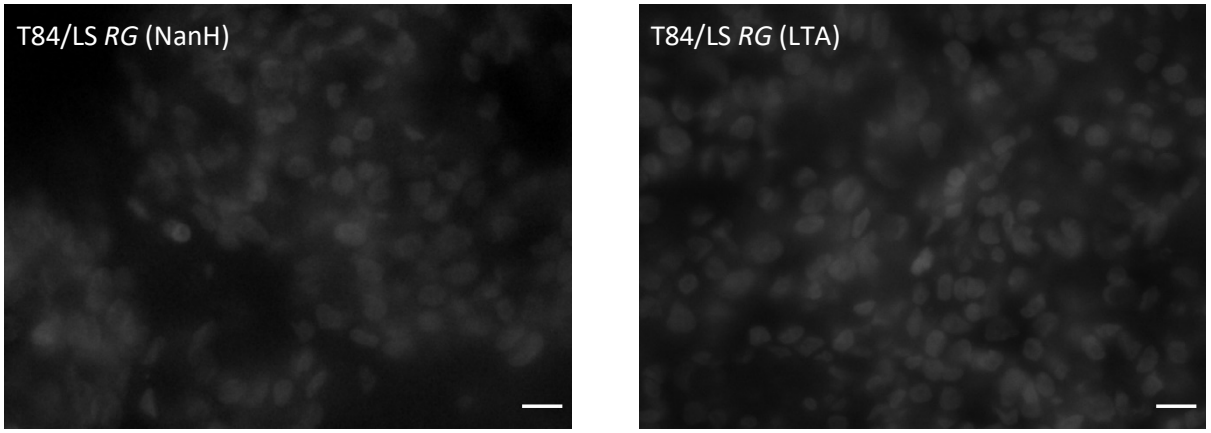


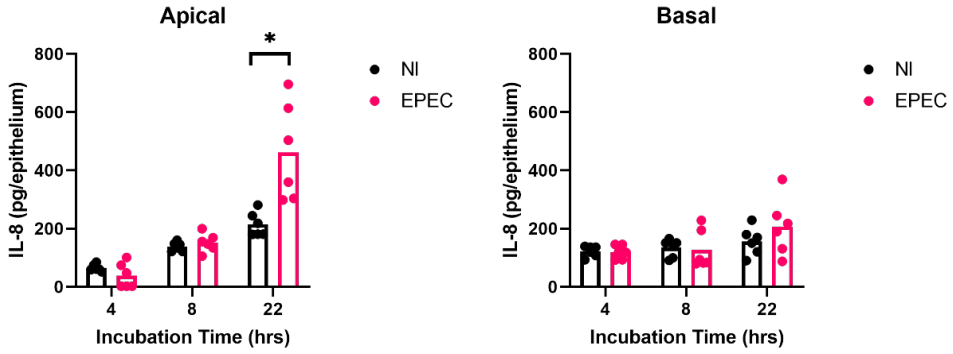
Figure 4.4 LS174T cells enhance *R. gnavus* colonisation, but not planktonic growth. T84 and T84/LS epithelia were incubated with *R. gnavus* (*RG*) for 4 hours in the VDC. (**A**) Planktonic and adherent bacteria were quantified by plate counting. The graphs show data from eight replicates collected over four independent experiments (Planktonic *RG*, n = 8) or four replicates from two independent experiments (Cell-associated *RG*, n = 4). $^{**}P < 0.01$. (**B**) Epithelia were stained for MUC2 (green), *R. gnavus* NanH or LTA (red) and cell nuclei (grey, below panels). Images are representative of six replicates collected over two independent experiments (n = 6).

4.2.4 EPEC infection of T84 monolayers results in predominantly apical IL-8 secretion

The objective of these experiments was to develop a protocol for detecting IL-8 secretion from T84 epithelia during EPEC E2348/69 infection in the VDC. Two variables were selected for optimisation including length of incubation time and direction of IL-8 release. To this aim, T84 monolayers were infected with EPEC or left non-infected as a control, and apical and basal supernatants were sampled after 4, 8 and 22 hours of infection. To prevent bacterial overgrowth, apical medium was exchanged for fresh medium containing gentamicin (50 µg/mL) at 4 hours post-infection. Utilisation of gentamicin for this purpose is common in published literature and has been employed previously to assess the IL-8 response of T84 monolayers to EPEC and STEC (Lewis *et al.* 2015; Ruchaud-Sparagano, Maresca, and Kenny 2007). Experimental timepoints were selected on the basis that 4 hours represents the infection time required for EPEC to initiate effector translocation and actin pedestal formation under these conditions. In addition, other studies have shown maximal IL-8 secretion after 8 to 24 hours of incubation, therefore samples were also collected at these timepoints (Lewis *et al.* 2016; Ruchaud-Sparagano, Maresca, and Kenny 2007). After sample collection, IL-8 was quantified by sandwich ELISA. Furthermore, epithelial barrier function was assessed by measuring TEER after 22 hours to identify any changes in epithelial permeability in cells infected with EPEC. This was important to ascertain as,

whilst gentamicin removes extracellular bacteria, translocated effectors are likely to still influence host cell processes.

A



B

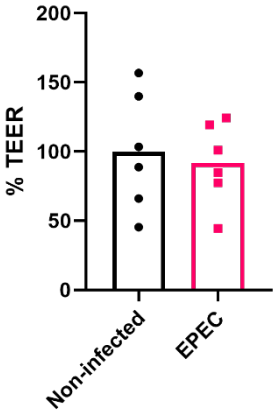


Figure 4.5 EPEC infection of polarised T84 cells stimulates apical IL-8 release while maintaining barrier function. T84 monolayers were infected with EPEC or left non-infected (NI) for 4 hours and treated with 50 µg/mL gentamicin for 18 hours to inhibit bacterial overgrowth. (**A**) IL-8 was quantified in apical and basal supernatants at 4-, 8- and 22-hours post-infection by ELISA. (**B**) TEER was assessed after 22 hours. TEER is expressed as percentage relative to barrier function before incubation. The graphs show data from two independent experiments performed in triplicate (n = 6). $P^* < 0.05$.

IL-8 release into the apical compartment by EPEC-infected T84 cells increased with incubation time, with maximal secretion at 22 hours post-infection (**Fig. 4.5A**). In contrast, IL-8 secretion into the basal compartment was similar between EPEC-infected and non-infected epithelia, suggesting that IL-8 is secreted exclusively into the apical media in EPEC-infected T84 cells.

TEER measurements indicated that T84 monolayer integrity and barrier function are not affected by extended incubation with EPEC (**Fig. 4.5B**). This indicates that either the pathogen does not impact epithelial permeability during a 4-hour infection or that the cells recover from damage over the 18-hour incubation once EPEC growth has been inhibited.

4.2.5 EPEC infection elicits a stronger IL-8 response in T84/LS versus T84 epithelia

To determine the influence of mucus-secreting LS174T cells on EPEC-induced IL-8 production, T84 and T84/LS epithelia were infected with EPEC E2348/69 or left non-infected for 4 hours, followed by gentamicin treatment and a further 18-hour incubation. Apical and basal media were sampled, and IL-8 quantified by sandwich ELISA.

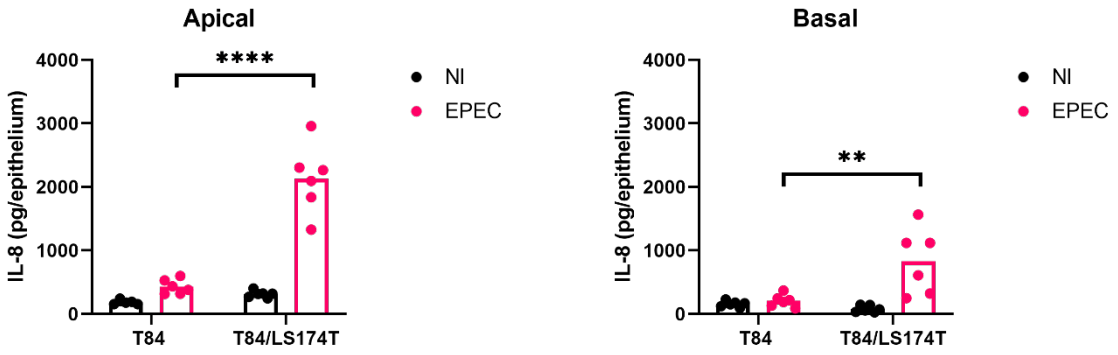


Figure 4.6 IL-8 release by T84 and T84/LS epithelia infected with EPEC. Epithelia were infected with EPEC or left non-infected (NI) for 4 hours, followed by an 18-hour incubation in gentamicin-containing medium. IL-8 from apical and basal supernatants was quantified by ELISA. The graphs data from two independent experiments performed in triplicate (n = 6). $^{**}P < 0.01$, $^{****}P < 0.001$.

As **Fig. 4.6** shows, the IL-8 response to EPEC was significantly higher in T84/LS epithelia with ~5-fold more IL-8 compared to T84 cells only (2132 pg/epithelium and 423 pg/epithelium, respectively). Similar to T84 monolayers, infected T84/LS epithelia secreted IL-8 predominantly in the apical compartment (2131 pg/mL and 831 pg/mL in the apical and basal compartments, respectively). These data suggest that the presence of LS174T cells enhances the pro-inflammatory response of epithelia during EPEC infection.

4.2.6 Mucolytic reagents do not remove mucus secreted by T84/LS epithelia

Given the differences in bacterial growth, adhesion and IL-8 response between T84 and T84/LS epithelia, the question arises whether these are mediated by mucus or other properties inherent to each specific cell line. Interestingly, transcriptomic analysis of 1290 colorectal cancer tumours identified effective clustering to 5 distinct subtypes, including goblet-like (e.g. LS174T) and enterocyte-like (e.g. T84) which exhibited differential gene expression in several phenotypes such as mucus-associated genes, apoptosis, and pathogen invasion (Sadanandam *et al.* 2013). Given the lack of MUC2-deficient LS174T cell derivatives, removal of secreted mucus could provide a suitable control to determine its influence on host-pathogen-commensal interactions.

Several reagents have demonstrated mucolytic activity *in vitro*, including N-acetylcysteine (NAC) and dithiothreitol (DTT). Notably, previous studies showed that LS174T cells incubated for 48 hours with 5 mM NAC exhibited reduced MUC2 staining compared to non-treated cells, and complete loss of MUC2 signal with 10 mM NAC (Amini *et al.* 2015). Therefore, a similar protocol was applied to LS174T cells grown on Snapwell membranes, and quantitative assessment was performed by integrated density. Nevertheless, MUC2 staining was similar between cells treated with up to 10 mM NAC compared with non-treated controls (**Fig. 4.7A).**

Other publications have utilised DTT (5-10 mM) to remove mucus from intestinal biopsy tissue (Goodyear *et al.* 2014; Miyazaki *et al.* 2019). In these experiments, apical medium of polarised LS174T cells were treated with 10-100 mM DTT for 15 minutes and MUC2 production was assessed by integrated density measurements of IFS images. However, these data indicated no difference in MUC2 staining between LS174T cells incubated with up to 100 mM DTT and non-treated controls (**Fig. 4.7B**), indicating a lack of mucolytic activity under the conditions in this study.

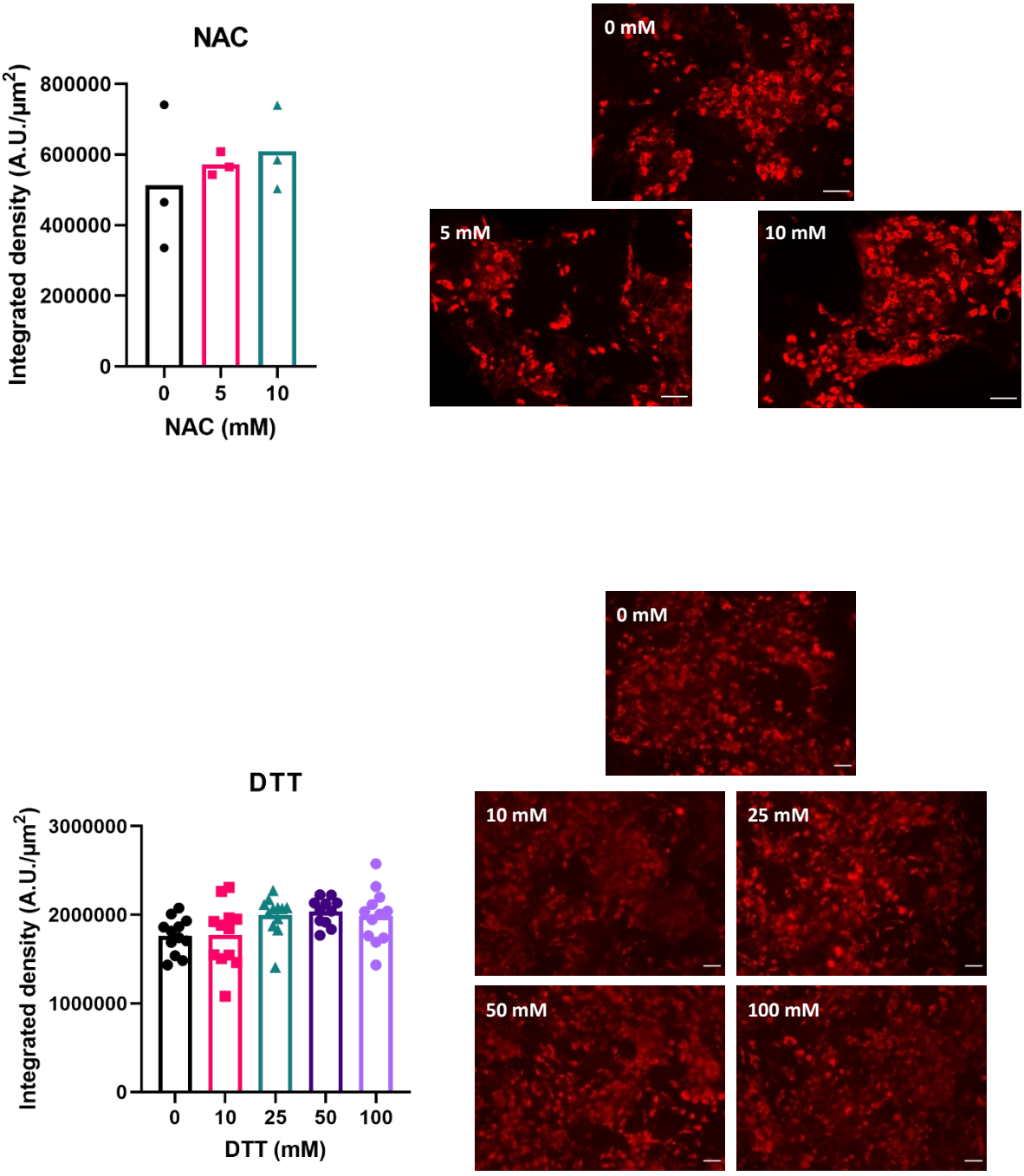


Figure 4.7 MUC2 production by LS174T cells is unaffected by NAC or DTT. LS174T cells were cultured on Snapwell filters and treated apically with either 5- or 10 mM NAC for 48 hours, or 10 to 100 mM DTT for 15 minutes. Non-treated controls were included. Cells were stained for MUC2, and signal intensity was assessed by integrated density analysis. Data shown from three replicates collected from one experiment (NAC) or twelve replicates collected from three independent experiments (DTT). Scale bar = 25 µm.

4.3 Discussion

There is growing recognition of the role secreted mucus has in facilitating host-microbe interactions at the intestinal epithelium, with implications for both commensal and pathogenic organisms. Not only does the mucus layer provide nutrients and colonisation sites, but it also acts to protect the epithelial cells from immune stimulation by bacterial proteins. A lack of mucus-producing model systems supporting epithelial viability and polarisation in a low oxygen environment has hampered the study of interactions between intestinal epithelia and anaerobic gut commensals. Here, we have cultured human mucus-producing (T84/LS) and mucus-deficient epithelia (T84) with the human gut pathogen EPEC E2348/69, probiotic *L. reuteri* PTA 6475*,* or the oxygen-sensitive gut symbiont *R. gnavus* ATCC 35913. and determined the influence of LS174T cells on bacterial growth and host cell binding and IL-8 release during EPEC infection. (see **section 3.2.4**).

4.3.1 Influence of LS174T cells on EPEC growth and colonisation

In these experiments, we sought to determine how mucus producing LS174T cells influenced planktonic growth and mucosal colonisation by the gut pathogen EPEC E2348/69. Our results indicated increased numbers of planktonic and adherent EPEC when LS174T cells were present, suggesting that introduction of goblet-like cells to the intestinal epithelium of the VDC model is beneficial for planktonic growth and mucosal colonisation by EPEC. In line with our results, previous studies comparing EPEC adherence to mucus-deficient HT-29 and mucus-producing LS174T cells have demonstrated increased adherence to the latter (Walsham *et al.* 2016). A similar trend was seen for STEC strains which exhibited enhanced colonisation of LS174T cells compared to mucus-deficient HT-29 and Caco-2 cell lines (Hews *et al.* 2017). Although these findings might be due to different host receptors on each cell line, it is possible that mucus plays a role in enhanced bacterial binding. Interestingly, numerous studies suggest that mucus protects from epithelial colonisation by EPEC and closely related pathogens, appearing to contradict the findings made here using mucus secreting LS174T cells. (Mack *et al.* 1999; Smith, Kaper, and Mack 1995). However, these studies hypothesise that mucin-derived glycans may mimic binding receptors on the surface of epithelial cells, promoting adherence to purified mucus which is removed following preparation for downstream analysis. In contrast, mucus produced by LS174T cells forms a thin layer close to epithelial surface, resembling that of the tightly adherent inner mucus layer which is difficult to remove by aspiration (Johansson, Larsson, and Hansson 2011; Walsham *et al.* 2016). The interactions between EPEC and intestinal mucus are poorly understood as much of the knowledge on EPEC colonisation has been acquired using non-mucus secreting cell lines. There is some evidence that flagellin mediates interactions with bovine

mucus as purified flagellin from EPEC and STEC binds to crude bovine mucus and *fliC* mutants exhibit attenuated adherence to bovine intestinal tissue (Erdem *et al.* 2007). More recent work indicated a decrease in EPEC adherence to HT-29 cells after silencing the gene required for core-2 *O*-glycan biosynthesis, demonstrating the importance of MUC2 glycosylation to mucosal colonisation by EPEC (Ye *et al.* 2015).

Our results show that the presence of mucus secreting LS174T cells also promotes EPEC growth in the apical medium. Utilisation of secreted mucus appears to be a feasible explanation for these results. WGS of EPEC has identified several GHs degrading mucus-derived mono- and disaccharides, including the enzymes GalK, ManA and NagE, which degrade galactose, mannose and N-acetyl glucosamine, respectively (Conway and Cohen 2015). In STEC, utilisation of these sugars as a sole carbon source has been demonstrated *in vitro*, and colonisation of mice by mutant strains lacking *galK*, *manA* and *nagE* was significantly reduced compared to the WT, suggesting that utilisation of these mucin-derived sugars is crucial for proliferation *in vivo* (Bertin *et al.* 2013; Fabich *et al.* 2008). In addition, STEC demonstrated growth on the sialic acid N-acetylneuraminic acid (Neu5Ac) commonly found in intestinal mucin (Fabich *et al.* 2008; Shen *et al.* 2004). NanS is a sialate O-acetylesterase responsible for the utilisation of sialic acid from mucin glycoproteins by *E. coli* (Schauer, Reuter, and Stoll 1988). WGS of EPEC E2348/69 has identified 4 distinct *nanS* copies, present on both the EPEC chromosome and inserted prophage (Vimr 2013). Whilst characterisation of EPEC NanS enzymes has not been performed, release of Neu5Ac and its derivatives was identified by mass spectrometry after incubation of bovine mucin with purified STEC NanS protein (Feuerbaum *et al.* 2018). Utilisation of mucin-derived sialic acid by EPEC could be crucial in the system developed here, as MUC2 secreted by LS174T cells is composed predominantly of sialylated structures (Etzold *et al.* 2014).

Nevertheless, there is still some dispute over the capacity of *E. coli* to break down complex polysaccharides found in intestinal mucus (Iguchi *et al.* 2009; Fabich *et al.* 2008; Hoskins *et al.* 1985). One study assessing mucin degradation of PGM by bacteria isolated from human faecal matter highlighted absence of mucolytic activity in 5 distinct *E. coli* strains (Hoskins *et al.* 1985). As growth of *E. coli* on mucus-derived mono- and disaccharides have been routinely demonstrated *in vitro*, it is thought that they rely on release of these sugars by neighbouring bacteria specialised in mucus degradation for growth *in vivo* (Fabich *et al.* 2008; Conway and Cohen 2015). Despite this, there is mounting evidence for mucinase activity in pathogenic *E. coli*. Mucin-degrading enzymes have been identified in STEC (StcE), ExPEC and ETEC (SslE), and appear to be crucial in increasing migration through the mucus layer to enhance epithelial colonisation (Hews *et al.* 2017; Nesta *et al.* 2014; Valeri *et al.* 2015). Infection of small intestinal biopsies with EPEC E2348/69 revealed the presence of

bacteria in both the mucus layer and on the epithelium, suggesting mucus penetration (Walsham *et al.* 2016). In parallel experiments with *L. reuteri* PTA 6475 the commensal was confined to the mucus layer, indicating restriction of epithelial access to bacteria by the mucus and suggesting that penetration by EPEC is an active process likely requiring a mucinase. Interestingly, Valeri *et al.* (2015) highlighted enhanced growth of ExPEC in M9 minimal medium supplemented with mucus from HT29-MTX cells, which was abolished in an isogenic Δ*sslE* strain (Valeri *et al.* 2015). This suggests that the role of mucin-degrading enzymes may extend beyond penetration of the intestinal mucus to liberation of mucus-derived sugars that can be utilised for growth. Whilst EPEC expresses SslE as part of the type II secretion system operon, studies have focused on its role in biofilm formation rather than potential mucinase activity (Baldi *et al.* 2012; Yang *et al.* 2007). The observation that pathogenic *E. coli* do not degrade mucus may stem from the fact that Hoskins *et al.* (1985) used bacteria isolated from healthy participants for assessment of mucinase activity. Indeed, both StcE and SslE have functions that are linked to pathogenesis, and infection of rabbits with a Δ*sslE* REPEC strain completely abolished mortality (Baldi *et al.* 2012). As such, degradation of mucus may be a crucial virulence trait that is generally absent in commensal *E. coli*. The results shown here are in line with this hypothesis, suggesting that EPEC can utilise mucus for growth. Not only was enhanced EPEC growth seen in the presence of mucus-secreting LS174T cells, improved growth in DMEM/F-12 medium was also observed after supplementation with PGM. Further work is required to identify and characterise proteins with mucinase activity from EPEC and assess their role in pathogenesis.

Taken together, the results here suggest that the presence of goblet cells might enhance EPEC pathogenicity, as seen by increases in the key virulence determinants planktonic growth and epithelial colonisation.

4.3.2 Impact of mucus secreting LS174T cells on *L. reuteri* growth and adherence

Our data indicated no difference in numbers of planktonic and adherent *L. reuteri* PTA 6475 between T84 and T84/LS epithelia. There is very little data investigating the influence of mucus on *L. reuteri* growth, and mucus-degrading GHs have not been identified. In contrast, adherence of *L. reuteri* to IECs and intestinal mucus has been well studied. *L. reuteri* expresses several surface proteins including MUB and CmbA which bind murine and porcine intestinal mucin *in vitro* (Etzold *et al.* 2014; Jensen *et al.* 2014; Roos and Jonsson 2002; Walsham *et al.* 2016; Sequeira *et al.* 2018). In addition, *L. reuteri* MUB and CmbA deletion mutants exhibited attenuated adherence to human IECs relative to their wild types (Jensen *et al.* 2014; Walsham *et al.* 2016). Unexpectedly, similar levels of reduced adhesion were

observed in cell lines with high (LS174T) and low (HT-29, Caco-2) mucus production, suggesting that these adhesins mediate attachment to the epithelial surface as well as to mucus.

Similar to our approach, Walsham *et al.* employed LS174T cells as a mucus-secreting model to assess *L. reuteri* adherence and identified a 30-fold increase in adherence compared to non-mucus secreting HT-29 cells (0.5% and 15%, respectively). Although the mucus-producing epithelia used in our study consisted only of 10% LS174T cells (see **section 3.2.4**), we expected the mucus-secretion of T84/LS epithelia to be sufficient to observe an enhancement of *L. reuteri* adherence. Despite this, similar levels of adherent *L. reuteri* were observed in T84 and T84/LS epithelia. These results indicate that T84 epithelia offer an abundance of binding sites for *L. reuteri* to interact with over the course of the incubation, and that introduction of secreted mucus by LS174T cells does not influence epithelial colonisation. The disparity between results seen here and those in Walsham *et al.* could be due to the use of different cell models (T84 and T84/LS versus HT-29 and LS174T) or the difference in incubation times. It is possible that *L. reuteri* adhesins have higher affinity for surface proteins of T84 compared to HT-29 cells, but there is little data investigating *L. reuteri* adherence to T84 cells.

Another explanation is differential mucus production in the respective comparators for each study. Walsham *et al.* utilised LS174T cells exclusively which, as we showed previously, produce more MUC2 than T84/LS epithelia (see **Fig. 3.6**). As such, the contribution of mucus from T84/LS epithelia to *L. reuteri* adherence may be negligible. An important distinction between both studies is the timeframe of the experiments: Walsham *et al.* used a much shorter incubation time than that used here (1 hour vs 4 hours). Differences in adhesin binding affinities would be emphasised over shorter incubation times, with longer times facilitating saturation of all binding sites (mucus- and cell-derived). Whilst *L. reuteri* produces adhesins, such as MapA, the affinities of these proteins for their ligands and how this compares to mucus adhesins are unknown (Miyoshi *et al.* 2006; Ouwehand *et al.* 2002; Walsham *et al.* 2016).

A further explanation for these results could be the presence of transmembrane mucins in T84 epithelia. The small intestinal epithelium is characterised by a surface-associated glycocalyx composed of transmembrane mucins (MUC3, MUC4, MUC12, MUC13 and MUC17) (Johansson and Hansson 2016). Polarised T84 cells have been shown to form a glycocalyx, exhibiting similar expression levels of glycocalyx components to healthy intestinal epithelial cells (Ou *et al.* 2009). Furthermore, expression of MUC3, the most abundant transmembrane mucin of the human duodenal glycocalyx, is induced in T84 cells incubated in hypoxic conditions (Louis *et al.* 2006; Johansson and Hansson 2016). Consequently, under the microaerobic conditions of the VDC utilised here, T84 epithelia may highly express MUC3, providing a similar number of mucin-binding sites to *L. reuteri* compared with the

mucus-secreting T84/LS epithelia. Interestingly, kinetic analysis of mucus-binding of four *Lactobacillus* species demonstrated a greater affinity for MUC3 compared to other mucins, including MUC2 (Das *et al.* 2016). Therefore, expression of MUC3 by polarised T84 cells, particularly under low oxygen conditions, should be determined in future studies. Antibody-based assays, such as immunofluorescence staining and western blotting of host membrane fractions, could achieve this given the availability of efficacious antibodies.

With regards to utilisation of mucin-derived sugars as a nutrient source by lactobacilli, growth assays in mucin-supplemented medium are scarce. However, genomics has revealed the presence of a gene in *L. sakei*, *asnA*, which encodes a putative glycoside asparaginase potentially capable of liberating GlcNAc from asparagine found in N-glycoproteins, although a lack of functional analyses means this hypothesis remains unexplored (Hüfner *et al.* 2007).

In conclusion, the data collected here indicate that *L. reuteri* is capable of adhering to secreted mucus and the epithelial surface of T84 and LS174T cells over 4 hours. Further work using mutant strains could elucidate the role of adhesins in mediating cell and mucus attachment.

4.3.3 Effect of mucin producing LS174T cells on *R. gnavus* growth and adherence

Here, we have cultured *R. gnavus* alongside human intestinal epithelia for the first time, by harnessing the physiological properties offered by the VDC. Experiments with purified mucins have demonstrated the ability of *R. gnavus* to grow on a range of glycans through the activity of several glycoside hydrolases (GHs) encoded by the bacteria (Bell *et al.* 2019; Crost *et al.* 2013; Crost *et al.* 2016). Of these GHs, genes encoding utilisation of sialylated glycans are most strongly upregulated in the presence of PGM. For ATCC 35913, the strain used in this study, GH33 expression increased 7-fold, and was shown to have 100% sequence homology to an intramolecular *trans*-sialidase characterised in ATCC 29149 (IT-sialidase, NanH) (Bell *et al.* 2019; Crost *et al.* 2013). This is pertinent to the use of LS174T cells in our system, which produce heavily sialylated MUC2 (up to 91% sialylated glycans) relative to other mucin sources such as PGM and mouse intestinal mucus (2-8% sialylation) (Owen *et al.* 2017). Consequently, it is surprising that we did not observe an increase in *R. gnavus* growth during culture with T84/LS epithelia compared to non-mucus secreting T84 monolayers. This lack of effect might be due to the use of F12/BHI-YH medium. BHI is a nutrient-rich medium including animal products and glucose as sources of nitrogen and carbon, that can support fastidious microbes. As a result, *R. gnavus* may be utilising these more readily available nutrient sources over those offered by mucus secreted by LS174T cells. In line with this, mucus utilisation studies with *R. gnavus* were performed in yeast casitone fatty acids (YCFA) minimal medium supplemented with carbon sources to

facilitate growth (Crost *et al.* 2013; Crost *et al.* 2016). Utilisation of an apical medium lacking a carbon source may have revealed a growth benefit of *R. gnavus* in the presence of mucus but would also have impacted growth of the other organisms used in this study.

Interestingly, viable counts in cell lysates indicated greater numbers of *R. gnavus* associated with T84/LS versus T84 epithelia, which was confirmed by immunofluorescence staining. While no mucus-binding proteins have been characterised so far in *R. gnavus*, a carbohydrate binding module of the IT-sialidase NanH (CBM40) has been identified which could enable enhanced bacterial binding to sialylated glycans (Owen *et al.* 2017). Notably, the presence of NanH in adherent *R. gnavus* was shown by IFS in this study. Whilst it is tempting to speculate that co-localisation of NanH and MUC2 staining indicates interaction between the two molecules, MUC2 staining was also observed at the surface of adhering EPEC and *L. reuteri*, suggesting cross-reactivity of the MUC2 antibody. Despite this potential role for CBM40, the primary function of the IT-sialidase is the utilisation of sialylated glycans as a source of nutrients (Bell *et al.* 2019; Crost *et al.* 2016). As a result, the increase in cell-associated *R. gnavus* in T84/LS epithelia may well reflect increased bacterial growth at the mucosal surface as well as mucosal adherence. Nevertheless, the role of alternative, yet uncharacterised adhesins cannot be ignored as similar levels of adherence were determined for WT *R. gnavus* and an isogenic *nan* cluster mutant in GF mice (Bell *et al.* 2019). Intriguingly, when cultured together, the WT strain outcompeted the *nan* mutant, resulting in undetectable levels of mucosal colonisation by the mutant. Investigations into the location of these strains within the mucus layer revealed deeper penetration of the mucus layer by the WT compared to the *nan* mutant. From these results, the authors concluded that other mediators of mucosal adherence were present in *R. gnavus,* although they were more likely to lead to loss of adherence through renewal of mucus than *nan*-encoded adhesins. Adherence assays with the *nan* cluster mutant in the system developed here could elucidate the role of *nanH* in mucosal adhesion and highlight other mucus adhesins (Robbe *et al.* 2003).

4.3.4 Influence of LS174T cells on the inflammatory response to EPEC

To characterise host-pathogen interactions in the optimised VDC system, it was important to assess the immune response of host cells to EPEC. Cytokine ELISAs performed here demonstrated that apical infection of polarised T84 epithelia by EPEC resulted in a significant IL-8 response. IL-8 secretion during EPEC infection has been shown by various cell lines (e.g. Caco-2 and HT-29) but studies in T84 cells are scarce (Khan *et al.* 2008; Ruchaud-Sparagano, Maresca, and Kenny 2007; Sharma *et al.* 2006; Zhou *et al.* 2003; Czerucka *et al.* 2001).

IL-8 is the predominant cytokine released by IECs following activation of TLR5 by EPEC flagellin. During polarisation, access of molecules (such as PAMPs) is restricted to the apical surface and differential expression of immune response receptors (such as TLRs) is exhibited on apical and basal membranes (Chabot *et al.* 2006; Lee, Mo, Katakura, Alkalay, Rucker, Liu, Lee, Shen, Cojocaru, Shenouda, *et al.* 2006). In line with this, immunofluorescence staining of polarised T84 monolayers revealed predominant localisation of TLR5 to the basal membrane (Gewirtz *et al.* 2001). This finding is significant, as recognition of EPEC flagellin by host TLR5 is critical to instigate an IL-8 response (Khan *et al.* 2008; Sharma *et al.* 2006; Zhou *et al.* 2003). Consequently, the poor apical expression of TLR5 by polarised T84 cells may explain the modest IL-8 response measured here. More direct evidence of this was shown by Ruchaud-Sparagano *et al.* (2007), who observed considerably stronger IL-8 secretion from polarised T84 epithelia after basal exposure to EPEC flagella compared apical (Ruchaud-Sparagano, Maresca, and Kenny 2007). Interestingly, this study also demonstrated that the IL-8 response to apical EPEC infection by Caco-2 cells was seen mainly in the basal compartment. This is in direct opposition to the results here, where IL-8 secretion by infected T84 epithelia into the apical compartment was notably higher than basal secretion. Our findings contradict the hypothesis that IL-8 is predominantly secreted into the lamina propria during infection, which has been corroborated by observations that apically applied EPEC and STEC flagellin stimulated predominantly basal IL-8 secretion by polarised Caco-2 cells (Ruchaud-Sparagano, Maresca, and Kenny 2007; Berin *et al.* 2002). Despite this, more recent work indicated that IL-8 secretion in polarised T84 cells is vectoral depending on the side of stimulation, as apical exposure to STEC or flagellin resulted in mainly apical IL-8 release (Lewis *et al.* 2015). This is substantiated by the observation that both IL-8 and neutrophils have been detected in luminal contents of patients suffering with inflammatory bowel disease (Fournier and Parkos 2012; Keshavarzian *et al.* 1999). *In vivo* infection of mice with A/E lesion forming pathogen *C. rodentium* showed influx of neutrophils into colon tissue, but apical translocation into stool was not assessed (Kim *et al.* 2011).

Interestingly, our results demonstrated significantly increased IL-8 secretion in EPEC-infected T84/LS compared to T84 monolayers. This indicates that LS174T cells can detect EPEC and mount an inflammatory response. Indeed, LS174T cells express TLR5 and secrete IL-8 after exposure to purified flagellin from *Salmonella enterica* serovar Typhimurium (Croix, Bhatia, and Gaskins 2011). Although infection with *Entamoeba histolytica* and *Bacteroides fragilis* has been shown to stimulate IL-8 secretion by LS174T cells, studies with more conventional diarrhoeagenic pathogens are scarce (Cuiv *et al.* 2017; Yu and Chadee 1997). The contribution of secreted mucus to the differential IL-8 response to EPEC by T84 and T84/LS epithelia is unclear. As one of the functions of mucus *in vivo* is to restrict exposure of epithelial cells to bacteria, reduced activation of cell surface TLR5 by EPEC would be

expected in the presence of secreted mucus. This is further corroborated by the observation of reduced mucus thickness observed in colonic biopsies of patients with chronic inflammatory bowel disease (Swidsinski *et al.* 2007). Another study, investigating the mechanism behind chronic intestinal inflammation induced by dextran sulphate salt (DSS) in mice, revealed a reduction in mucus thickness as evidenced by enhanced epithelial access of fluorescent beads (Johansson *et al.* 2010). Hence, the observation that IL-8 secretion is enhanced with mucus secreting LS174T cells suggests adequate access of EPEC MAMPs to the epithelial surface.

Furthermore flagellin, which is the main stimulator of IL-8 secretion in EPEC infection, potentially mediates mucus adhesion (Erdem *et al.* 2007; Schuller *et al.* 2009; Zhou *et al.* 2003). Interestingly, exposure of LS174T cells to recombinant *S.* Typhimurium flagellin resulted in enhanced *MUC2* and *TLR5* expression, suggesting a link between flagellin exposure, mucus production and host inflammatory response in LS174T cells (Croix, Bhatia, and Gaskins 2011). Furthermore, the bacterial peptide formyl-methionyl-leucyl-phenylalanine stimulated IL-8 and MUC2 secretion from LS174T cells, mirroring the results seen with flagellin (Leiper *et al.* 2001). However, until the link between mucus production and enhanced inflammation is confirmed by the use of MUC2-deficient LS174T cells, other differences between the T84 and LS174T cell line cannot be ruled out. It is highly likely that the relatively poor barrier function of T84/LS (TEER measured at 30-80 Ω x cm^2 compared to 1000 Ω x cm^2 for T84 epithelia) contributed to an elevated IL-8 response to EPEC. Poor barrier function is indicative of increased intestinal permeability, which would result in exposure of the basal PRRs to EPEC MAMPs. This would also explain why a basal IL-8 response was seen for T84/LS but not T84 epithelia. In addition to barrier function and secreted mucus, expression and localisation of TLR5 receptors, sensitivity to pro-inflammatory mediators or induction of the EPEC T3SS could all contribute to the elevated IL-8 response by T84/LS epithelia.

4.3.5 Assessing activity of mucolytic reagents

Whilst T84 epithelia provide a convenient comparator to investigate how mucus secreted by T84/LS epithelia influences host-microbe interactions, there are several limitations. Secondly, genetic differences between LS174T and T84 cells are likely due to their distinct lineages (goblet and enterocyte, respectively) and the individuality of tumour progression. Consequently, we attempted to produce an isogenic comparator by removing the secreted mucus from T84/LS epithelia using either NAC or DTT, both of which have demonstrated mucolytic activity with IECs (Amini *et al.* 2015; Goodyear *et al.* 2014; Miyazaki *et al.* 2019). The results shown here indicated no mucolytic activity of either NAC or DTT in LS174T cells. This is particularly surprising for NAC, as Amini *et al.* showed reduced

intracellular and secreted MUC2 production by LS174T cells following the same protocol used here. Whilst IFS images showed an apparent decrease in MUC2 staining after NAC treatment, no densitometric assessment was presented and secreted MUC2 was quantified by ELISA. This difference in methodology might account for the contrasting results. Similarly, our studies failed to remove mucus from LS174T cells by treatment with up to 100 mM DTT. This was unexpected as DTT is used routinely to breakdown the globular structure of MUC proteins prior to SDS-PAGE, remove mucus from sputum or enhance the access of stem cells from intestinal biopsies (Johansson 2012; Johansson *et al.* 2008; Sandberg *et al.* 1994; Woolhouse, Bayley, and Stockley 2002). In line with this, a study using rat intestinal segments has demonstrated increased luminal MUC2 after exposure to 10 mM DTT, seen by Alcian blue staining and western blotting of the luminal components, suggesting mucolytic activity (Miyazaki *et al.* 2019). Once again, the disparity with the results collected here may be because cell proximal mucus production was not studied. It is possible that DTT liberates MUC2 at a rate that does not cause disruption of the layer. A previous study investigating optimal conditions for stem cell isolation from colonic biopsies revealed enhanced cell viability when DTT was used at 37ºC compared to room temperature, suggesting enhanced mucolytic activity (Goodyear *et al.* 2014). It is possible that DTT may have been more effective at removing mucus from T84/LS epithelia by increasing the incubation temperature.

Taken together these results indicate that neither NAC nor DTT are capable of effectively removing mucus produced by T84/LS epithelia. Consequently, T84 monolayers will continue to be used as a low mucus-producing control to assess the influence of mucus on colonisation resistance by the commensal organisms.

Chapter Five – Influence of *L. reuteri* and *R. gnavus* on EPEC pathogenesis

5.1 Introduction

Colonisation resistance refers to the ability of the microbiota to protect the host from colonisation by pathogens. The phenomenon was first discovered in antibiotic-treated mice, which exhibited enhanced colonisation by *Salmonella* Typhimurium compared to non-treated controls (Bohnhoff, Drake, and Miller 1954). The importance of the intestinal microbiota was further substantiated in a study by van der Waaij *et al.* in 1971, which also coined the term "colonisation resistance" (van der Waaij, Berghuis-de Vries, and Lekkerkerk 1971). This pioneering work not only showed enhanced susceptibility of antibiotic-treated mice to pathogenic *E. coli*, but also that microbiota transplantation from healthy mice into germ-free counterparts bestowed protection against *E. coli infection*. Since then, the interactions between diarrhoeagenic *E. coli* and the intestinal microbiota has been studied in more detail, with the aim of identifying protective commensal species for alternative therapies. Many of these studies have focused on *E. coli* Nissle 1917, which reduced EPEC adherence to porcine intestinal IPEC-J2 cells and protected polarised T84 cells from barrier disruption during EPEC infection (Alvarez *et al.* 2019; Kleta *et al.* 2006; Zyrek *et al.* 2007a).

In addition, lactobacilli have been subject to intensive study, and reduction of EPEC adherence to Caco-2 cells has been demonstrated by *L. acidophilus* LB, *L. johnsonii* La1 and *L. casei rhamnosus* Lcr35 (Bernet-Camard *et al.* 1997; Coconnier *et al.* 1993; Forestier *et al.* 2001). Despite this, clinical studies have been unable to consistently show a benefit of *L. rhamnosus* or *L. acidophilus* in the treatment of infantile diarrhea. Whilst some studies showed reduction in the duration of diarrhea after probiotic treatment, others revealed no difference compared to placebo (Costa-Ribeiro *et al.* 2003; Simakachorn *et al.* 2000; Szymański *et al.* 2006; Vivatvakin and Kowitdamrong 2006). As the etiological agent is seldom identified in clinical studies, it is difficult to ascertain the prevalence of EPEC and whether a disparity between clinical and *in vitro* efficacy exists. In contrast, *L. reuteri* has exhibited some efficacy at the clinical level. A systematic review which collated data from five RCTs indicated that treatment of acute diarrhoea in children with *L. reuteri* DSM 17938 and ATCC 55730 significantly reduced the duration of diarrhoea, although the aetiological agent(s) were not confirmed (Szajewska *et al.* 2014). In a subsequent study, pre-treatment with *L. reuteri* DSM 17938 reduced the prevalence of EPEC in patients hospitalised with diarrhoea (Savino *et al.* 2015). In addition to clinical data, *in vitro* studies have demonstrated reduced EPEC adherence to HT-29 cells and human duodenal biopsies when cultured with *L. reuteri* PTA 6475 or ATCC 53608 (Walsham *et al.* 2016). Nevertheless, the mechanism(s) by which *L. reuteri* protect from EPEC adherence require further study.

In addition to antagonising pathogen colonisation, *L. reuteri* may also contribute to colonisation resistance by reducing inflammation. A prevailing hypothesis is that enhanced oxygen content in the

inflamed gut microenvironment may shift the balance of the microbiota from strictly anaerobic Bacteroidetes to facultative anaerobes of the Proteobacteria phyla, resulting in dysbiosis that may then promote pathogen colonisation (Henson and Phalak 2017; Rigottier-Gois 2013). Interestingly, infection with the EPEC-related mouse pathogen *Citrobacter rodentium* causes a shift in microbiota composition that is similar to chemically induced inflammation (Lupp *et al.* 2007). As *L. reuteri* PTA 6475 has been shown to suppress MAPK and NF-κB activation required for IL-8 release during EPEC infection, it may augment resistance to dysbiosis caused by EPEC-induced inflammation (Lin *et al.* 2008; Liu *et al.* 2010).

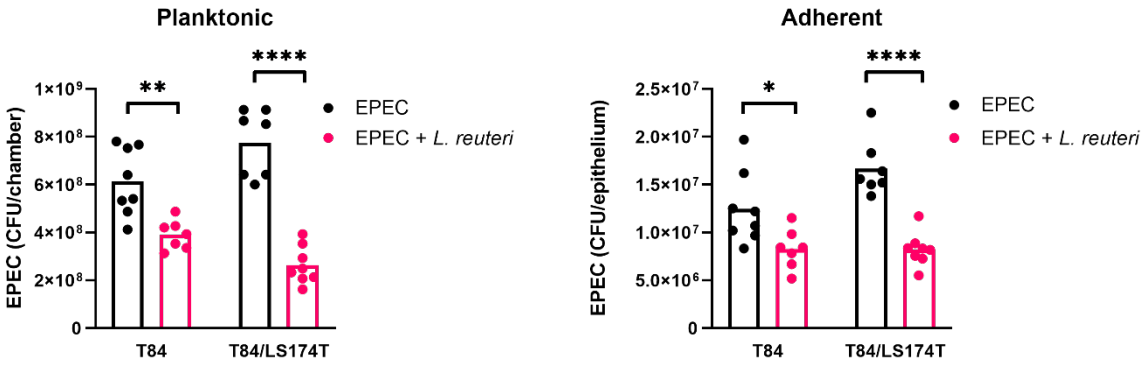
In contrast to *L. reuteri*, very little is known about the role of *R. gnavus* in colonisation resistance despite its prevalence in the human microbiota (Qin *et al.* 2010). *R. gnavus* is highly adapted to the intestinal mucosa, utilising the mucus layer for colonisation and as a carbon source (Bell *et al.* 2019; Crost *et al.* 2013; Crost *et al.* 2016). In addition, *R. gnavus* is present in the fecal microbiota of over 90% of healthy adults, suggesting that it carries out functions essential for gut homeostasis (Qin *et al.* 2010). Consequently, *R. gnavus* could play a vital role in colonisation resistance through competition for mucosal nutrients and colonisation sites.

5.2. Results

5.2.1. *L. reuteri* reduces numbers of planktonic and adherent EPEC during co-culture

In order to assess the effect of *L. reuteri* on EPEC infection, T84/LS epithelia were incubated with EPEC E2348/69 and *L. reuteri* PTA 6475 or EPEC alone in the VDC. After 4 hours, planktonic and adherent EPEC were quantified by plating on LB agar and incubation under aerobic conditions, which is selective for EPEC growth. Interestingly, co-culture with *L. reuteri* resulted in a significant decrease in numbers of planktonic and adherent EPEC compared with EPEC controls (**Fig. 5.1A**). Regression analysis of data collected from EPEC and *L. reuteri* co-cultures showed no correlation ($P = 0.86$, $R^2 < 0.01$ for T84 and $P = 0.99$, $R^2 < 0.01$ T84/LS) between planktonic and adherent EPEC (**Fig. 5.1B**). Even spread of the residuals indicated that the models used for T84 and T84/LS were appropriate.

A



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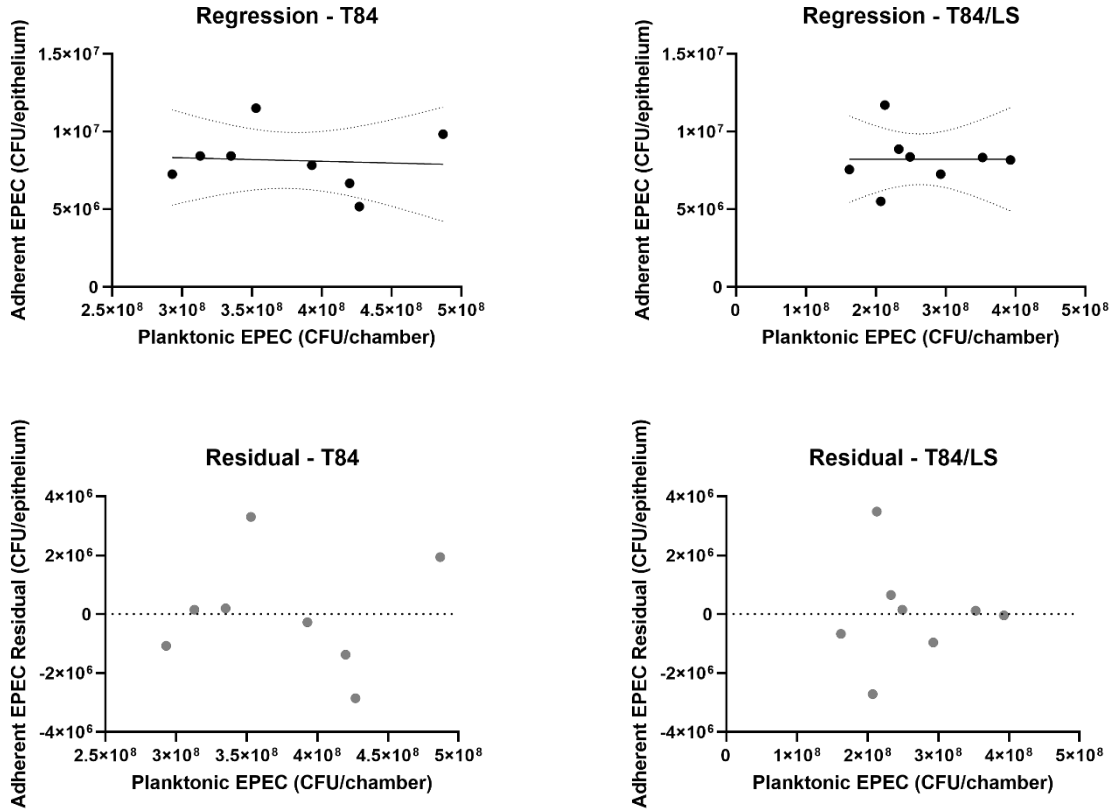


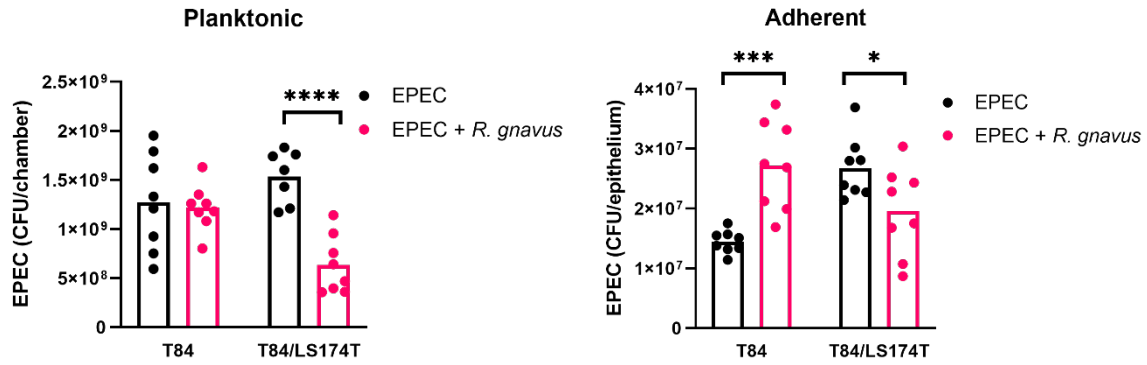
Figure 5.1 EPEC proliferation and adherence was reduced during co-culture with *L. reuteri*. T84 or T84/LS epithelia were incubated EPEC and *L. reuteri* or EPEC alone for 4 hours in the VDC. Planktonic and adherent EPEC were quantified in apical media and cell lysates, respectively, by plate counting. (**A**) Mean values for each condition compared by two-way ANOVA. (**B**) Regression analysis performed by plotting planktonic (x-axis) and adherent EPEC (y-axis) values following co-culture with *L. reuteri*. Dotted lines indicate 95% confidence interval. Residual values for adherent EPEC after fitting to

regression model are shown in separate graphs. Data shows eight (Planktonic and Adherent EPEC EPEC with T84 epithelia, n = 8) or seven (all other conditions, n = 7) replicates collected from three independent experiments. $^{**}P < 0.01$, $^{****}P < 0.0001$.

5.2.2. Culture with *R. gnavus* reduced EPEC growth and adherence in T84/LS but not T84 epithelia

Despite the prevalence and considerable host adaption of *R. gnavus* to the human intestine, its role in colonisation resistance remains largely unknown. Here, the influence of *R. gnavus* ATCC 35913 on EPEC growth and adherence was assessed as described for *L. reuteri* above. As shown in **Fig. 5.2A**, reduced numbers of planktonic and adherent EPEC were observed during co-culture with *R. gnavus* in the presence of T84/LS epithelia. In contrast, co-culture with *R. gnavus* increased EPEC adherence but did not affect planktonic growth in T84 epithelia. Regression analysis of data collected from EPEC and *R. gnavus* co-cultures showed no correlation ($P = 0.87$, $R^2 < 0.01$) between planktonic and adherent EPEC with T84 epithelia **(Fig. 5.1B**). Corresponding data with T84/LS epithelia revealed a positive correlation ($P = 0.02$, $R^2 = 0.65$), indicating that changes in planktonic EPEC are a factor affecting changes in adherent EPEC. Even spread of the residuals indicated that the models used for T84 and T84/LS were appropriate.

A



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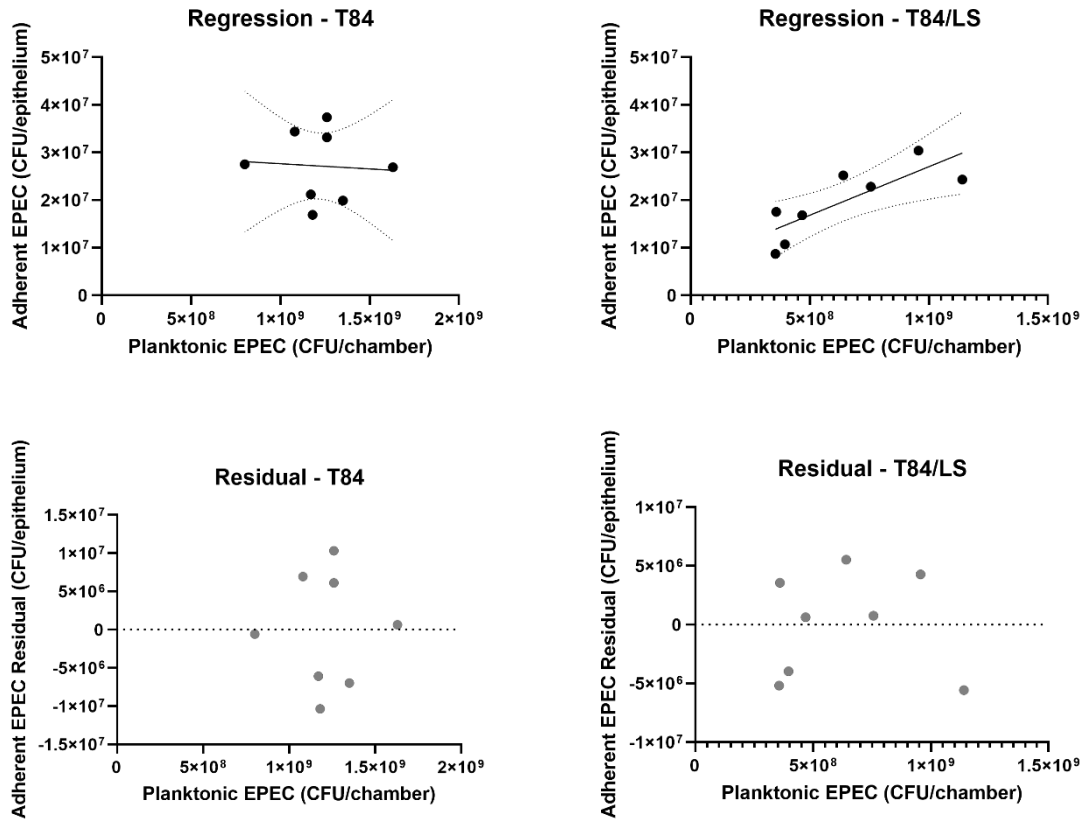


Figure 5.2 Co-culture with *R. gnavus* decreased EPEC growth and adhesion in T84/LS but not T84 epithelia. T84 or T84/LS epithelia were incubated EPEC and *R. gnavus* or EPEC alone for 4 hours in the VDC. Planktonic and adherent EPEC were quantified in apical media and cell lysates, respectively, by plate counting. (**A**) Mean values for each condition compared by two-way ANOVA. (**B**) Regression analysis performed by plotting planktonic (x-axis) and adherent EPEC (y-axis) values following co-culture with *R. gnavus*. Dotted lines indicate 95% confidence interval. Residual values for adherent EPEC after fitting to regression model are shown in separate graphs. Data shows eight replicates collected from three independent experiments (n = 8). **P* < 0.05, ****P* < 0.001, *****P* < 0.0001.

5.2.3 Cell-free supernatants of *L. reuteri* and *R. gnavus* cultures do not influence EPEC growth

Previous work has shown that reuterin and the organic acids lactate and acetate, which are secreted metabolites produced during fermentative growth of *L. reuteri* can inhibit microbial growth (Cadieux *et al.* 2009; Spinler *et al.* 2008). To determine whether reduced EPEC growth in the presence of *L. reuteri* PTA 6475 or *R. gnavus* ATCC 35913 was due to secreted metabolites, cell-free supernatants (CFS) was sampled from *L. reuteri* and *R. gnavus* cultured in DMEM/F-12 + BHI-YH in the VDC for 4

hours. In addition, medium was incubated without bacteria as NI control. EPEC was then cultured in the VDC (without host cells) in CFS diluted 1:1 with DMEM/F-12 + BHI-YH for 4 hours, and bacteria were enumerated by colony counts on LB agar. Cultures of EPEC alone and with *L. reuteri* or *R. gnavus* were included as controls.

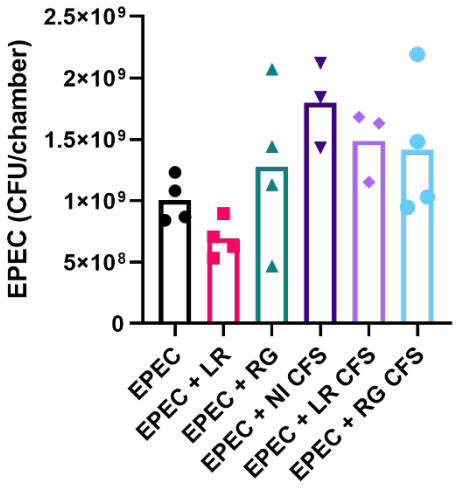


Figure 5.3 *L. reuteri* and *R. gnavus* CFS does not affect EPEC growth. EPEC was grown for 4 hours in the VDC without host cells on its own or in the presence of *L. reuteri* (EPEC + LR), *R. gnavus* (EPEC + RG), or CFS from NI (EPEC + NI CFS), *L. reuteri* (EPEC + LR CFS) or *R. gnavus* (EPEC + RG CFS) VDC cultures. EPEC CFUs were then quantified by plate counting. Data shown from three (EPEC + RG, EPEC + NI CFS and EPEC + LR CFS, n = 3) or four (all other conditions, n = 4) replicates collected from two independent experiments.

Fig. 5.3 demonstrates enhanced EPEC growth with CFS from all cultures (1.4-1.7 x 10^9 CFU compared to 1 x 10^9 CFU for EPEC only). In contrast, co-culture of EPEC with *L. reuteri* bacteria decreased EPEC growth (6.3 x 10^8), whereas co-culture with *R. gnavus* did not influence EPEC proliferation. None of these differences reached statistical significance.

5.2.4. *L. reuteri* and *R. gnavus* do not affect EPEC-induced IL-8 secretion during 4 hours of co-culture EPEC infection

To assess the influence of *L. reuteri* PTA 6475 and *R. gnavus* ATCC 35913 on EPEC-induced IL-8 secretion in intestinal epithelia, T84/LS cells were incubated with EPEC E2348/69 or commensal strains alone, EPEC and commensal or left un-treated. After 4 hours, media were replaced with fresh DMEM/F-12 + BHI-YH containing 50 µg/mL gentamicin to inhibit bacterial overgrowth, and chambers were incubated for a further 18 hours. IL-8 from apical supernatants was then quantified by sandwich ELISA.

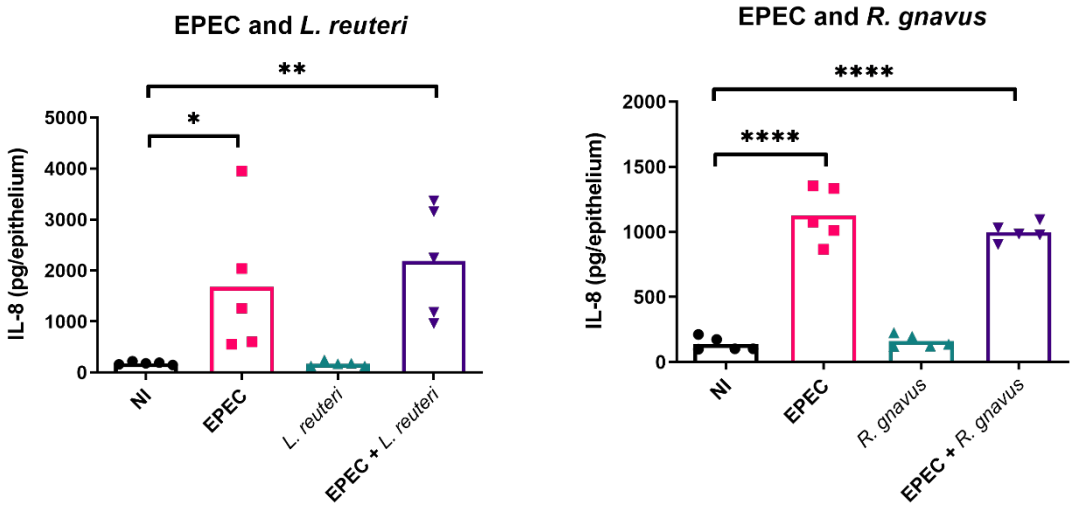


Figure 5.4 Co-culture with *L. reuteri* or *R. gnavus* did not affect EPEC-induced IL-8 secretion by T84/LS epithelia. EPEC and *L. reuteri* or *R. gnavus* were cultured with T84/LS epithelia alone or in co-culture for 4 hours in the VDC. Apical medium was then replaced with medium containing 50 µg/mL gentamicin and epithelia were incubated for a further 18 hours. Subsequently, IL-8 was quantified from apical supernatants by ELISA. Data is shown from five replicates collected in two independent experiments (n = 5). *P < 0.05, **P < 0.01, ****P < 0.0001.

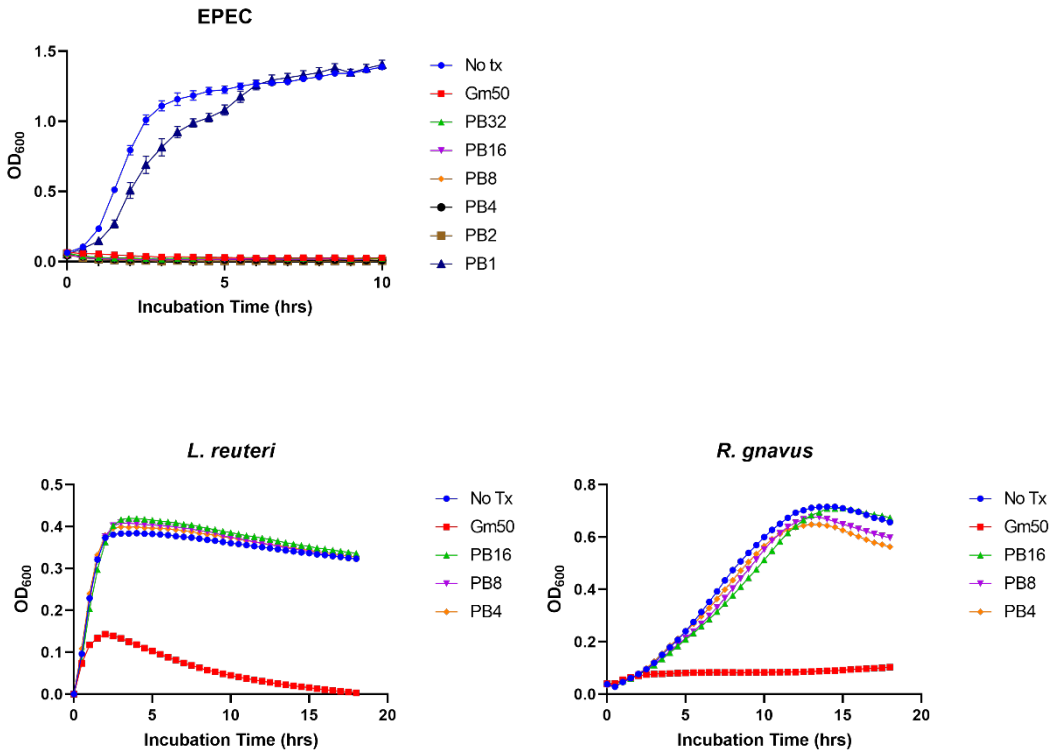
As shown in **Fig. 5.4**, neither *L. reuteri* nor *R. gnavus* evoked an IL-8 response from T84/LS epithelia. Despite the observations that both commensal organisms reduced planktonic and adherent EPEC, no significant effect on IL-8 secretion in EPEC-infected T84/LS cells was observed.

5.2.5. *L. reuteri* decreased the IL-8 response to EPEC after extended co-culture

Previous studies demonstrating anti-inflammatory effects of *L. reuteri* in intestinal epithelia employed incubation periods of at least 10 hours (Karimi *et al.* 2009; Liu *et al.* 2010). To allow extended replication of *L. reuteri* while preventing EPEC overgrowth the antimicrobial polymyxin B, which specifically targets Gram-negative bacteria such as EPEC, was used instead of the broad-spectrum antibiotic gentamicin (Charteris *et al.* 1998; Stille and Shah 1973; Zhou *et al.* 2005). To determine the effect of polymyxin B on bacterial growth, EPEC E2348/69, *L. reuteri* PTA 6475 and *R. gnavus* ATCC 35913 were cultured in 96-well plates in BHI-YH containing 1-32 µg/mL polymyxin B or no antibiotic as a positive control. In addition, bacteria were incubated in media with 50 µg/mL gentamicin. After 18-hours of culture in either aerobic (EPEC) or anaerobic (*L. reuteri, R. gnavus*) conditions, optical

density (OD_{600}) was determined. These measurements revealed complete inhibition of EPEC growth at polymyxin B concentrations of 4 µg/mL and above, whilst no effect on Gram-positive *L. reuteri* or *R. gnavus* proliferation was observed in the presence of up to 16 µg/mL polymyxin B. growth **(Fig. 5.5**). According to these results, the protocol for IL-8 detection was modified and 16 µg/mL polymyxin B was used instead of gentamicin. This change allowed extended growth of *L. reuteri and R. gnavus* whilst inhibiting EPEC replication in the VDC as confirmed by OD_{600} (1.8 and 0.03 for *L. reuteri* and EPEC, respectively). As shown in **Fig. 5.5,** prolonged culture of *L. reuteri* did not stimulate IL-8 secretion by T84/LS epithelia but significantly reduced cytokine release during EPEC infection.

A



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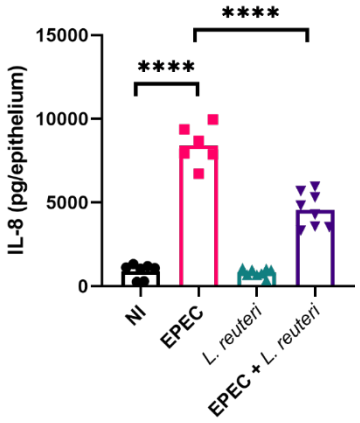


Figure 5.5 Co-culture with *L. reuteri* for 22 h reduced IL-8 secretion in EPEC-infected T84/LS epithelia. (**A**) EPEC, *L. reuteri and R. gnavus* were cultured for 18 hours in 96-well plates containing BHI-YH medium with 50 µg/mL gentamicin (Gm50), 1-32 µg/mL polymyxin B (PB) or without antibiotics (No Tx). Growth was assessed by optical density (OD_{600}). Data shown is from a single experiment performed in triplicate (n = 3). (**B**) T84/LS epithelia were incubated with EPEC, *L. reuteri*, EPEC and *L. reuteri*, or without bacteria (NI) for 4 hours. Medium was exchanged for medium containing 16 µg/mL polymyxin B and incubation was continued for 18 h IL-8 levels in apical media were quantified by ELISA. Data shown is from six (EPEC, n = 6) or eight (all other conditions, n = 8) replicates collected from four independent experiments. ****P < 0.0001.

5.2.6 Influence of *L. reuteri* CFS on EPEC-induced IL-8 release

Immunomodulation by secreted metabolites of the gut microbiota is well documented. Short-chain fatty acids (SCFAs), histamine and lactocepin are examples of metabolites secreted by lactobacilli with demonstrated anti-inflammatory activity, as evidenced by a decrease in pro-inflammatory cytokine production by stimulated IECs (Gao *et al.* 2015b; Moens *et al.* 2019; Parada Venegas *et al.* 2019; Thomas *et al.* 2012; von Schillde *et al.* 2012). To determine whether secreted metabolites contributed to the anti-inflammatory activity of *L. reuteri* PTA 6475 on EPEC-infected epithelia, CFS of *L. reuteri* PTA 6475 cultured with T84/LS epithelia for 22 h (4h incubation followed by 18 h in the presence of 16 µg/mL polymyxin B) were collected. The CFS was mixed 1:1 with fresh DMEM/BHI-YH medium containing 32 µg/mL polymyxin B, which was used to replace the spent apical medium following a 4-hour EPEC infection.

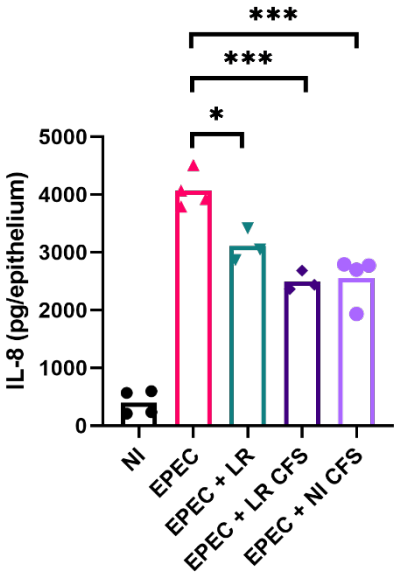


Figure 5.6 CFS from *L. reuteri* and non-infected epithelia reduced IL-8 secreted by EPEC-infected T84/LS cells. T84/LS epithelia were infected with EPEC for 4 hours, followed by replacement of the apical medium with CFS from *L. reuteri* grown in the VDC for 22 hours with host epithelia (LR CFS) or from non-infected controls (NI CFS) and incubation for a further 18 hours. T84/LS epithelia incubated with EPEC alone or EPEC and *L. reuteri* (EPEC + *L. reuteri*) were also included. IL-8 was quantified from apical suspensions by ELISA. Data shown of three (EPEC + LR and EPEC + LR CFS, n = 3) or four (all other conditions, n = 4) replicates collected from two independent experiments. *P < 0.05, ***P < 0.001.

As demonstrated before, IL-8 was secreted by T84/LS cells in response to EPEC infection, and co-culture with *L. reuteri* significantly attenuated this response (**Fig. 5.6**). Whilst introduction of CFS from *L. reuteri* culture decreased IL-8 secretion by infected epithelia even further, use of CFS from NI epithelia resulted in a similar reduction. These data suggest that there is some component(s) of the apical medium after 18 hours culture with T84/LS epithelia that has anti-inflammatory activity. Due to this, it is not possible to ascertain whether anti-inflammatory activity of whole PTA 6475 is dependent on a secreted metabolite.

5.2.7 *R. gnavus* decreased the IL-8 response to EPEC during extended culture

Previous experiments with *L. reuteri* highlighted the requirement of extended culture to elicit immunomodulatory activity on T84/LS epithelia infected with EPEC. Whilst the interactions between *R. gnavus* and mucosal immunity are less defined than for *L. reuteri*, it is possible that extended culture could facilitate expression and production of inflammatory mediators, such as propionate or glucorhamnan (D'Souza *et al.* 2017; Henke *et al.* 2019). To investigate this, T84/LS epithelia were incubated with EPEC E2348/69 and *R. gnavus* ATCC 35913 alone or cultured together for 4 hours, followed by apical medium replacement with fresh medium containing 16 µg/mL polymyxin B and further culture for 18 hours. IL-8 was then quantified from the apical medium by ELISA.

While culture with *R. gnavus* alone did not stimulate any IL-8 secretion, the IL-8 response in EPEC-infected epithelia was significantly decreased in the presence of *R. gnavus*, suggesting that the production of anti-inflammatory mediators during extended culture **(Fig. 5.7**).

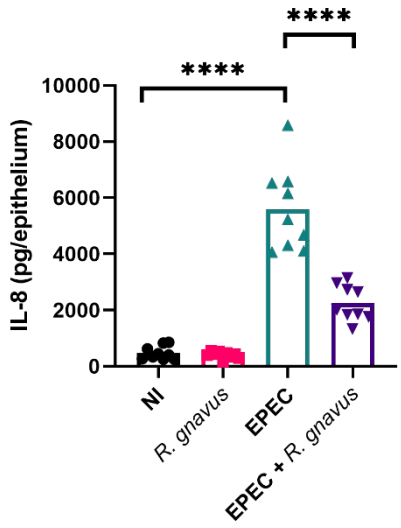


Fig. 5.7 *R. gnavus* reduced IL-8 secretion from T84/LS epithelia infected with EPEC. T84/LS epithelia were incubated with EPEC, *R. gnavus*, EPEC and *R. gnavus*, or without bacteria (NI) for 4 hours. Medium was exchanged for medium containing 16 µg/mL polymyxin B and incubation was continued for 18 hours before IL-8 levels in apical suspensions were quantified by ELISA. Data shown is from nine replicates collected over three independent experiments (n = 9). ****P < 0.0001.

5.2.8 EPEC T3SS activity is not affected by co-culture with *L. reuteri* or *R. gnavus*

EPEC pathogenesis is underpinned by effector protein translocation into the host cell via the T3SS. Recent publications indicated that T3SS subunits are targeted by proteases released by the gut bacteria *Enterococcus faecalis* and *Bacteroides thetaoitamicron* (Cameron *et al.* 2018; Cameron, Sperandio, and Dunny 2019). Western blot analyses of STEC co-cultured with either *E. faecalis* or *B. thetaoitamicron* revealed an atypical form of EspB, the T3SS translocon pore protein, which had a lower molecular weight than native EspB. Whilst proteolysis of EspB was associated with enhanced T3SS activity in both cases (seen by increased Tir translocation and actin pedestal formation), these studies highlight that EspB can be targeted by exogenous proteases resulting in a direct impact on virulence. To determine if reduced EPEC adhesion in the presence of *L. reuteri* or *R. gnavus* was mediated by inhibition of the T3SS, T84 and T84/LS epithelia were incubated with EPEC E2348/69 alone, EPEC and PTA 6475, or left non-infected for 4 hours. In addition, a T3SS-deficient EPEC mutant (Δ*escN*) was included as control. Epithelia were lysed using Triton-X100, and EPEC-associated and translocated EspB was detected by western blotting of Triton-insoluble and -soluble fractions, respectively. A lysate of a concentrated EPEC ONC was run as a positive control and blotting with an EspB-specific antiserum demonstrated three bands of approximately 34 kDa, due to protein degradation during storage **(Fig. 5.8)**.

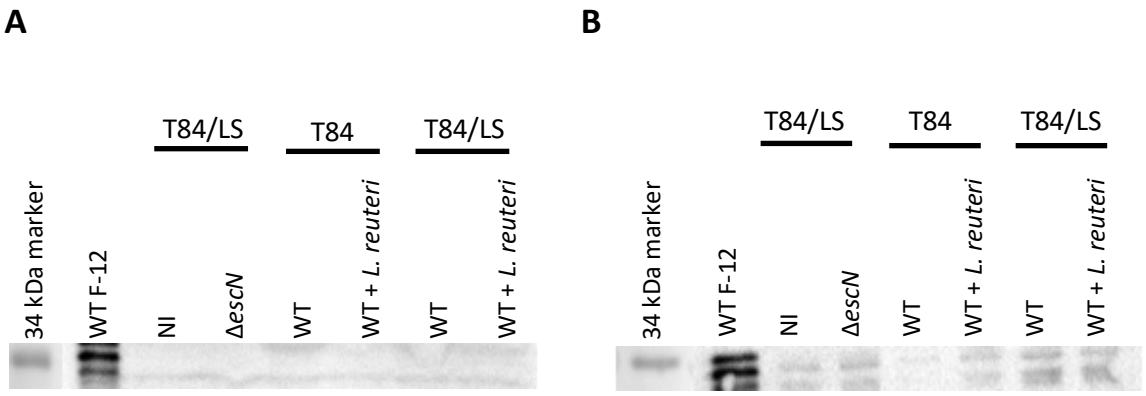
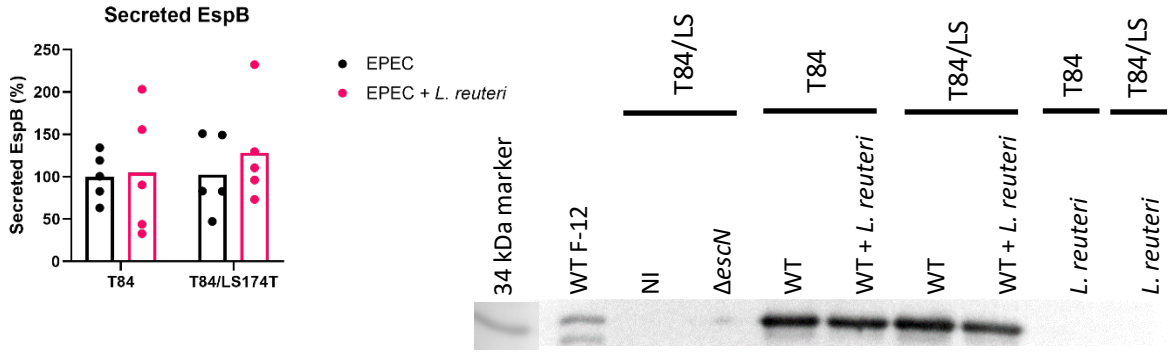


Fig. 5.8 EspB detected from all triton-insoluble fractions but not in triton-soluble fractions. T84 and T84/LS epithelia were incubated with EPEC E2348/69 wild-type (WT) or a T3SS mutant (Δ*escN*), EPEC and *L. reuteri* (WT + *L. reuteri*) or left non-infected (NI) for 4 hours. Cell lysates were prepared, and bacteria-associated and translocated EspB was detected in triton-insoluble (**A**) and -soluble fractions (**B**), by western blotting. A lysate from an EPEC ONC in DMEM/F-12 was included as a positive control, which yielded three distinct bands due to protein degradation during storage (WT F-12). Blots are representative of three independent experiments (n = 3).

As the lower two bands were also present in lysates from NI samples, this indicates cross-reaction of the antibody with host cell components. In contrast, the upper band was not detected in any Triton-insoluble or -soluble fraction indicating that amounts of EspB in adherent bacteria or translocated into the host cell membrane were too low for detection by Western blotting. Therefore, EspB secretion was determined in apical media as these contain higher bacterial numbers. After the 4-hour incubation, bacteria were harvested from apical suspensions by centrifugation, and supernatants containing secreted proteins were concentrated by ultrafiltration. EspB was then detected from bacterial and supernatant fractions by Western blotting. To normalise for equal amounts of EPEC, blots were also probed for the stable *E. coli* housekeeping protein GroEL and analysed by densitometric analyses (Rudolph *et al.* 2010).



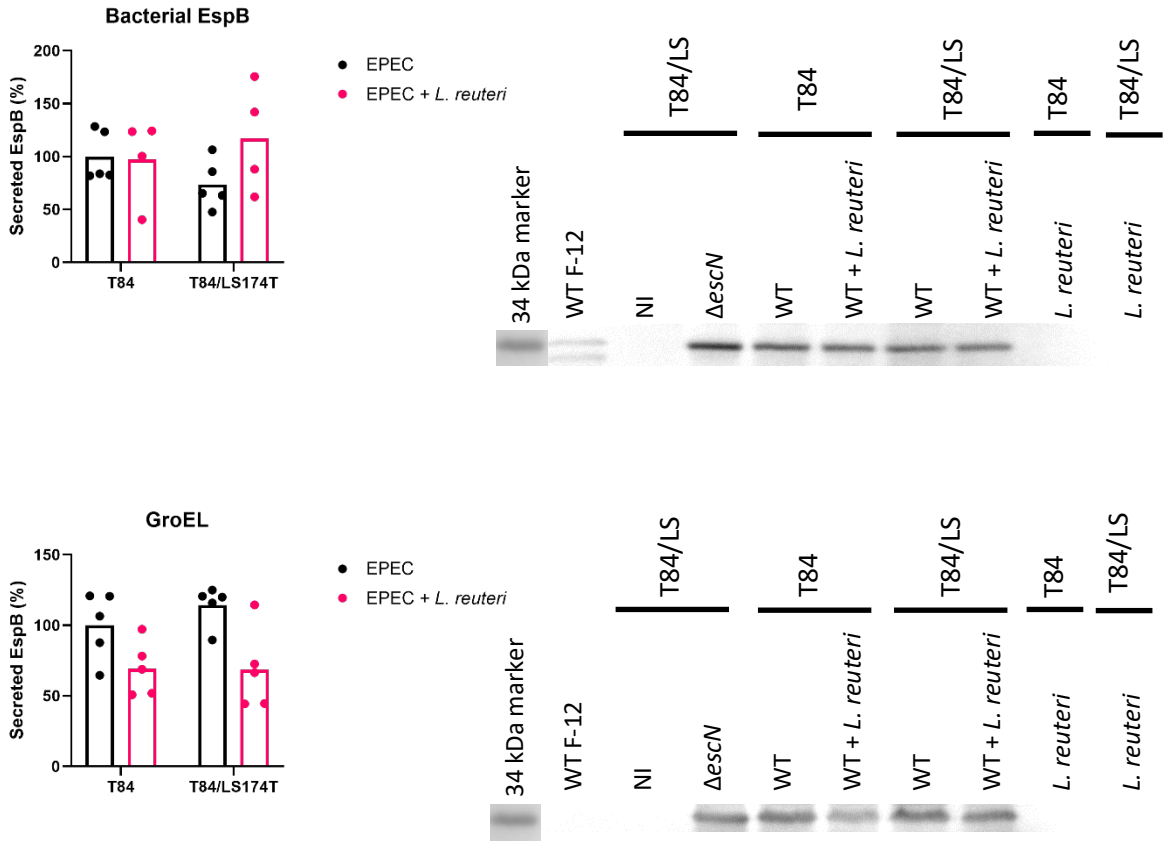


Figure 5.9 *L. reuteri* does not influence EPEC EspB secretion of EspB into apical medium. T84 and T84/LS epithelia were incubated with EPEC E2348/69 (WT) alone or with *L. reuteri* (WT + *L. reuteri*) for 4 hours and EspB detected from secreted and bacterial fractions of apical suspensions by western blotting. T84/LS epithelia were also infected with an isogenic T3SS EPEC mutant (Δ*escN*) or incubated without bacteria (NI). EPEC overnight culture in DMEM/F-12 was included as a positive control (WT F-12). Blots with bacterial proteins were re-probed with an antibody targeting the GroEL *E. coli* HK protein. Densitometric analyses of the bands were performed, and the values were first normalised to the GroEL housekeeper and then expressed as a % of the values for the WT T84 condition. Data shown are representative of three independent experiments (n = 5).

The experiments were subsequently repeated using *R. gnavus* instead of *L. reuteri*. Data analysis was performed as outlined for experiments with *L. reuteri*.

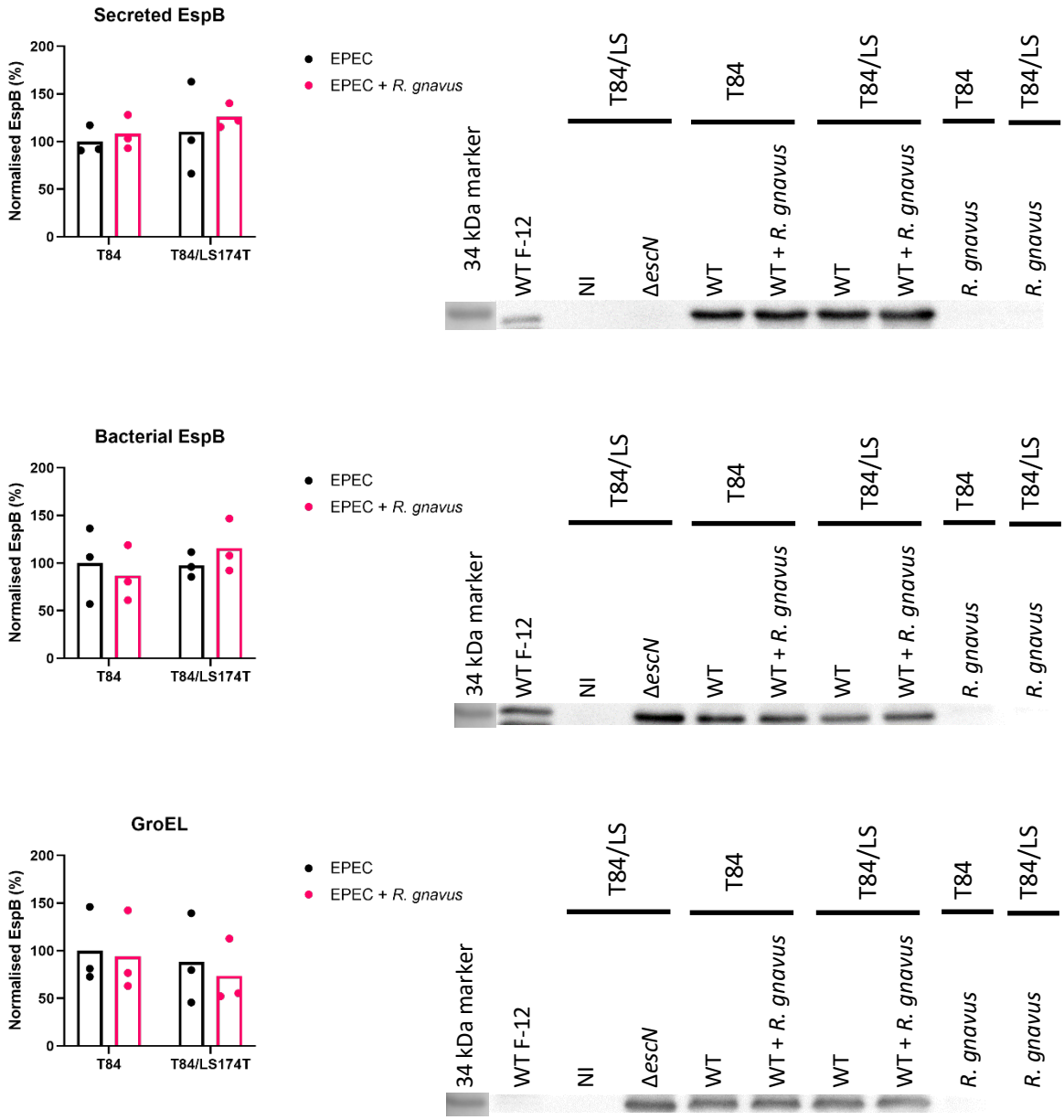


Figure 5.10 *R. gnavus* did not influence T3SS secretion of EspB into apical medium by EPEC. T84 and T84/LS epithelia were incubated with EPEC E2348/69 (WT) alone or with *R. gnavus* (WT + *R. gnavus*) for 4 hours and EspB detected from secreted and bacterial fractions of apical suspensions by western blotting. T84/LS epithelia were also infected with an isogenic T3SS EPEC mutant (Δ*escN*) or incubated without bacteria (NI). EPEC overnight culture in DMEM/F-12 was included as a positive control (WT F-12). Blots with bacterial proteins were then re-probed with an antibody targeting the GroEL *E. coli* HK protein. Densitometric analyses of the bands were performed, and the values were first normalised to the GroEL housekeeper and then expressed as a % of the values for the WT T84 condition. Data shown are representative of three independent experiments (n = 3).

As shown in **Fig. 5.9** and **Fig. 5.10**, EspB was detected in secreted and bacterial fractions of apical VDC suspensions during infection with WT EPEC. No bands were observed in samples from NI epithelia demonstrating the specificity of the EspB antiserum. In addition, EspB was present in bacterial but not secreted fractions of *ΔescN* samples, thereby validating the experimental EspB detection protocol. For GroEL, no signals were observed in VDC samples from incubations with *L. reuteri* or *R. gnavus* only, which confirms the specificity of anti-GroEL for EPEC. Densitometric analyses and normalisation showed no significant influence of *L. reuteri* and *R. gnavus* on EPEC EspB production and secretion into the medium.

5.3 Discussion

Since the discovery of colonisation resistance in the 1950s, much research has been conducted into the underlying mechanisms and how this information can be applied to create novel therapeutics for enteric infections. Due to a lack of suitable experimental model systems, microbe-host interactions have remained unstudied for most of the intestinal microbiota which are sensitive to oxygen. In addition to the incompatibility of conventional cell culture models with a low oxygen environment, most intestinal epithelial cell lines do not produce a mucus layer. which represents the primary interface of host-microbiota interactions by providing nutrients and colonisation sites for commensal bacteria. Competition for these mucosal niches is thought to provide the foundations for colonisation resistance (Sorbara and Pamer 2019).

In this part of the project, we employed the VDC system developed in Chapter Three to investigate colonisation resistance by *L. reuteri* and *R. gnavus* in EPEC infection. EPEC pathogenesis was assessed by the following criteria: bacterial growth, host cell adherence, secretion of the T3SS pore protein EspB and stimulation of host IL-8 secretion.

5.3.1 Colonisation resistance mediated by *L. reuteri*

Here, we have shown that *L. reuteri* PTA 6475 reduces planktonic EPEC growth in the presence of T84 or T84/LS epithelia. This agrees with previous studies which have revealed growth inhibition of enteric pathogens during co-culture with lactobacilli. *Lactobacillus delbrueckii subsp. lactis* at an excess of 100-1000 fold reduced growth of STEC O157:H7 over 1000 fold after 7 days of culture (Brashears, Reilly, and Gilliland 1998). Co-culture of *L. casei* or *L. acidophilus* with *S. sonnei* even resulted in complete removal of the pathogen at 24 hours post-inoculation (Apella *et al.* 1992). Similar results

were observed in a separate study where decreased STEC growth was observed after only 2 hours of anaerobic culture with a 100 fold excess of *L. casei* or *L. acidophilus* (Ogawa, Shimizu, Nomoto, Tanaka, *et al.* 2001). This antagonistic activity was correlated to lactic acid production, as growth of STEC in TSB supplemented with lactic acid revealed bactericidal effects at concentrations similar to that produced by both LAB species after 2 hours growth in tryptic soy broth (TSB).

Production of secondary metabolites inhibitory to bacterial growth is commonly documented for LAB. Organic acids (lactic acid and acetic acid) and bacteriocins (e.g. reuterin) are often implicated, and several studies have investigated their influence on the growth of enteric pathogens in greater detail. One study utilising agar well diffusion assays showed increased zones of EPEC E2348/69 inhibition in wells containing CFS from several LAB isolated from fecal samples of healthy infants (Davoodabadi *et al.* 2015). Heat-treatment of the CFS had no influence on pathogen inhibition, whereas introduction of catalase abolished antagonistic activity, suggesting that the secreted agent(s) was not proteinaceous and dependent on oxidative stress. Lactic acid has demonstrated growth inhibition against a range of enteric pathogens, including Gram-positive (*L. monocytogenes*, *Brochothrix thermosphacta*) and Gram-negative (STEC, *Salmonella*) bacteria (de Haan *et al.* 2021; Khatun *et al.* 2016; Talarico and Dobrogosz 1989). The bactericidal activity of lactic acid is thought to be due to membrane permeability of undissociated acid coupled with impermeability of dissociated acid, which causes high intracellular concentrations of protons and the conjugate anions (Brocklehurst and Lund 1990). Several suggestions have been put forward to explain how increased levels of intracellular dissociated acid can impact survival including disruption of the cellular membrane, loss of critical enzymatic reactions by fluctuating redox potential, uncoupling of the proton motive force and dysregulation of osmotic homeostasis (Lu, Breidt, Perez-Diaz, *et al.* 2011). The role of organic acids such as lactic acid in colonisation resistance is unclear, as several acid response systems have been characterised in *Salmonella*, *Shigella*, *Listeria* and *E. coli* species (Davis, Coote, and O'Byrne 1996; Foster and Hall 1990; Lin *et al.* 1995; Xu *et al.* 2020). This extends to EPEC, which exhibited recovery of growth after exposure to extremely low pH (1.5-4.5) in simulated gastric medium for up to 3 hours. Whilst this recovery was perturbed at the pH 1.5 and 2.5, no difference was noticed between EPEC exposed to pH 3.5 and above and non-exposed controls (Pienaar, Singh, and Barnard 2019). Consequently, high concentrations of lactic acid (60-100 mM) combined with a medium pH of 2-4 are often required to detect antagonistic effects on pathogen growth (Lu, Breidt, Pérez-Díaz, *et al.* 2011; Ogawa, Shimizu, Nomoto, Tanaka, *et al.* 2001).

In addition to lactic acid, *L. reuteri* produces reuterin, an anti-bacterial metabolite that exerts bactericidal activity against *E. coli* at a low minimum inhibitory concentration of 1 mM (Talarico *et al.*

1988; Talarico and Dobrogosz 1989). The contribution of reuterin to colonisation resistance mediated by *L. reuteri* is unclear. Whilst the small intestine is the site for breakdown of dietary triglycerides into glycerol, the concentration of glycerol in the healthy human gut is unknown (Khatun *et al.* 2016). Interestingly, results presented here revealed unperturbed growth of EPEC in CFS of *L. reuteri* PTA 6475. indicating that secreted metabolites such as reuterin and lactic acid were not the cause of the antagonism observed here. This is unsurprising, as reuterin is produced during fermentation of glycerol, which is not present in DMEM/F-12 + BHI-YH medium, and no acidification of the medium was observed even during extended culture of *L. reuteri* PTA 6475.

Consequently, it is more likely that antagonism of EPEC growth by *L. reuteri* is mediated by competition for nutrients. The nutritional composition of DMEM/F-12 + BHI-YH is very unlikely to recreate the complexity of the small intestinal chyme, which contains an array of dietary macronutrients, lytic enzymes, bile salts and other host-derived compounds (de Haan *et al.* 2021). Nevertheless, there may be some similarities between the two. The small intestine is the site for breakdown and absorption of most macronutrients, particularly simple carbohydrates, fats and proteins (Kastl *et al.* 2020). In DMEM/F-12 + BHI-YH, D-glucose is provided via DMEM/F-12, as well as a comprehensive range of amino acids either in purified form (DMEM/F-12) or via degraded meat products (BHI-YH). Therefore, competition between *L. reuteri* PTA 6475 and EPEC for the nutrients in DMEM/F-12 + BHI-YH observed here could occur *in vivo* and may well be an important mechanism underpinning colonisation resistance.

In addition to reduced proliferation, co-culture with *L. reuteri* PTA 6475 also resulted in diminished EPEC adhesion to T84 and T84/LS epithelia. Previous studies have demonstrated attenuation of EPEC adherence to Caco-2 cells by *L. acidophilus* LB, *L. johnsonii* La1 and *L. rhamnosus* Lcr35 (Bernet-Camard *et al.* 1997; Coconnier *et al.* 1993; Forestier *et al.* 2001). Reduction of EPEC adherence to T84 cells was also observed after co-culture with *L. acidophilus* R0052 or *L. rhamnosus* R0011 (Sherman *et al.* 2005). With regards to *L. reuteri*, Walsham *et al.* indicated a significant reduction in EPEC adherence to HT-29 cells and polarised human small intestinal biopsies during co-culture with either *L. reuteri* PTA 6475 or ATCC 53608 (Sherman *et al.* 2005; Walsham *et al.* 2016). Interestingly, SEMs revealed that the probiotic was confined to the mucus layer of the biopsy tissue suggesting blockage of EPEC mucus binding sites or hindrance of mucosal penetration as mechanism underpinning colonisation resistance. *L. reuteri* expresses several surface proteins (CmbA, MUB and MapA) which have been shown to mediate adhesion to both mucus and epithelial surfaces and may facilitate competition with EPEC for host binding receptors (Etzold *et al.* 2014; Miyoshi *et al.* 2006; Roos and Jonsson 2002; Walsham *et al.* 2016).

In most studies highlighting colonisation resistance, lactobacilli are pre-incubated with host cells in the absence of the pathogen for several hours to allow non-competitive adhesion. The high efficacy of pre-incubation suggests that, once attached to the host, displacement of lactobacilli by pathogens is extremely challenging (Lebeer, Vanderleyden, and De Keersmaecker 2008). Here, EPEC and *L. reuteri* PTA 6475 were inoculated simultaneously, allowing access of both bacteria to adhesion sites on the host surface. Hence, the antagonism shown by *L. reuteri* relies on mechanisms that actively impact EPEC adherence in real time. One possible explanation is that the kinetics of ligand-host receptor interactions are similar between EPEC and *L. reuteri* PTA 6475. It is important to note that Sherman *et al.* (2005) did not observe a reduction in EPEC adherence to polarised T84 cells after co-incubation with *L. acidophilus* R0052 or *L. rhamnosus* R0011 for 3 hours (Sherman *et al.* 2005). Therefore, *L. reuteri* PTA 6475 likely must expresses adhesins with higher affinity, or apical conditions (e.g. low oxygen or DMEM/F-12 + BHI-YH) must enhance receptor binding of ligands shared with *L. acidophilus* and *L. rhamnosus*. Further studies on LAB and EPEC ligand-receptor kinetics under more physiologically relevant conditions are required.

An alternative explanation is steric hindrance. Steric hindrance was implicated in the reduction of EPEC adherence to HT-29 by *L. reuteri* PTA 6475 seen by Walsham *et al.* (Walsham *et al.* 2016). Removal of non-adherent *L. reuteri* from HT-29 cells after a 2-hour pre-incubation abolished the reduction in EPEC adherence seen when this step was not performed. This result highlights a role for non-adherent *L. reuteri* in antagonising EPEC adherence, possibly by limiting pathogen access to host receptors. This may also explain why excessive LAB doses (relative to EPEC), such as that used here (560x excess), are often required to see reductions in pathogen adherence. Indeed, EPEC adherence to T84 epithelia was unaffected by *L. acidophilus* R0052 or *L. rhamnosus* R0011 when equivalent inoculums were used (Sherman *et al.* 2005). As co-culture with *L. reuteri* resulted in diminished EPEC growth, reduced adhesion might represent a direct consequence of this. However, regression analyses indicated poor correlation between EPEC proliferation and adhesion suggesting involvement of other factors ($R^2 <$ 0.01 for T84 and T84/LS).

As EPEC adheres to intestinal epithelium by forming A/E lesions which are mediated by a T3SS, reduced adherence might be caused by impaired functionality of the T3SS by commensal bacteria. This would also impact on translocation of effector proteins and host cell signal transduction (Croxen and Finlay 2010). Intimate adherence and pathogenesis of EPEC is dependent on the translocation of effector proteins into the host cells. Mutant EPEC strains deficient in T3SS activity lose the ability to form A/E lesions required for intimate adherence to the intestinal epithelium (Cepeda-Molero *et al.* 2017; Lai *et al.* 1997). Furthermore, transforming non-pathogenic *E. coli* DH5α with a cosmid

containing the LEE pathogenicity island from EPEC (which encodes the T3SS machinery and some effectors) resulted in EspB secretion and A/E lesion formation on HEp-2 cells, seen by FAS staining (McDaniel and Kaper 1997). Mouse models of EPEC infection using the A/E lesion forming murine pathogen *C. rodentium* revealed abolished virulence after infection with T3SS mutant strains (Δ*ler*, Δ*tir*, Δ*eae*), seen by loss of bacteria in the colon, minimal tissue damage (colonic hyperplasia) and 100% survival of mice up to 24 days post-infection (Deng *et al.* 2004). Notably, previous studies have shown enhanced STEC T3SS activity during co-culture with the gut commensals *Enterococcus faecalis* and *Bacteroides thetaoitamicron* (Cameron *et al.* 2018; Cameron, Sperandio, and Dunny 2019). This was mediated by commensal proteases cleaving EspB (Cameron, Sperandio, and Dunny 2019).

However, our studies did not detect any significant influence of *L. reuteri* on EPEC EspB secretion into the medium. This does not rule out other potential effects of *L. reuteri* on the T3SS including host cell translocation of effector proteins which could be assessed by β-lactamase reporter assay which has been widely used to characterise EPEC effector translocation. In this system, β-lactamase is fused to the C-terminus of the respective effector protein and translocation into the cytoplasm is quantified by liberation of fluorescein (Charpentier and Oswald 2004; Mills *et al.* 2008; Runte *et al.* 2018; Yerushalmi *et al.* 2014). Nevertheless, quantification of fluorescence in polarised epithelial cells grown on permeable membranes is problematic due to high background from filter membranes.

In addition to targeting pathogen growth and adherence, immunomodulation by commensal organisms has been suggested as a mechanism of colonisation resistance (Plaza-Diaz *et al.* 2019; Preidis *et al.* 2011). Access of gut microbes to the intestinal epithelium is restricted by secreted mucus which is thought to prevent stimulation of the immune response by MAMPs. Evidence for this has been shown in MUC2-deficient *Winnie* mice, where expression of pro-inflammatory cytokine genes (*Ifn-γ*, *Il-1β*, *Il-17, Mip*-2) was reduced in germ-free counterparts (Wang, Moniruzzaman, *et al.* 2021). No difference was observed in WT mice, suggesting that an inflammatory response to the gut microbiota is only mounted in the absence of secreted mucus. As the model used here does not produce a continuous mucus layer, it was important to ascertain whether *L. reuteri* PTA 6475 alone stimulated an IL-8 response in T84/LS epithelia. No IL-8 induction was observed in T84/LS cells during culture with *L. reuteri* PTA 6475 for up to 22 hours. Aside from TLR5, which recognises bacterial flagellin, IL-8 release has been demonstrated as a response to activation of TLR2 and 4 (Bhattacharyya *et al.* 2008; Kurt-Jones *et al.* 2002; Re and Strominger 2001; Valenty *et al.* 2017). As TLR4 and 5 respond to LPS and flagellin, both of which are not present in lactobacilli, activation of these is unlikely. In contrast, TLR2 recognises teichoic acids present on the cell wall of Gram-positive bacteria including lactobacilli. Whilst apical TLR2 expression in polarised T84 cells has been demonstrated, minimal NF-

κB activation was observed from monolayers exposed to LTA from *S. aureus* (Melmed *et al.* 2003). Poor TLR2 responsiveness was associated with low levels of surface expression and enhanced levels of Tollip, an inhibitor of downstream inflammatory signalling pathways. Unlike T84 cells, very little is known is known regarding TLR2 expression and subsequent microbial recognition in LS174T cells. Poor responsiveness of T84/LS epithelia to TLR2 ligands may explain the lack of IL-8 secretion during 18-hour culture with *L. reuteri*. In line with these results, incubation of the probiotic strain with rat intestinal cells or tissues did not elicit IL-8 secretion (Liu *et al.* 2010). Notably, intestinal tolerance to *L. reuteri* PTA 6475 was also noted *in vivo* as small intestinal expression of IL-1β and IFN-γ in mice treated with PTA 6475 was similar to non-infected controls (McCabe *et al.* 2013). Interestingly, TNF-α expression in treated mice was reduced, indicating anti-inflammatory activity of *L. reuteri* in the small intestine epithelium *in vivo*. Contrary to the lack of inflammatory response seen *in vivo*, IL-8 secretion was observed from porcine intestinal epithelial IPEC-J2 cells following incubation with *L. reuteri* PTA 6475, 4659 and 5289, although the cause of this was not elucidated (Liu *et al.* 2010).

Studies investigating the inflammatory response to *L. reuteri* PTA 6475 show mixed results. Human THP-1 monocytes or monocyte-derived macrophages do not produce TNF-α during culture with *L. reuteri* PTA 6475 (Lin *et al.* 2008). In contrast, pro-inflammatory responses by human DCs to PTA 6475 have been reported. Bene *et al.* demonstrated elevated secretion of pro-inflammatory TNF-α, IL-1β and IL-6 by DCs during culture with *L. reuteri* PTA 6475 and ATCC 53608 (Bene *et al.* 2017). This was dependent on binding of the mucus adhesins CmbA and MUB to the C-lectin receptors DC-SIGN and Dectin2 on host cells. As DC-SIGN and Dectin2 are expressed exclusively by DCs, pro-inflammatory activity of *L. reuteri* may only occur during inflammation when direct contact between microbes and immune cells occurs. Due to the conflicting results seen with both immune cells and IECs, more insight is required to conclude the inflammatory potential of *L. reuteri* PTA 6475.

While not affecting IL-8 secretion when incubated with T84/LS cells alone, *L. reuteri* attenuated the IL-8 response to EPEC infection during extended incubations of 22 hours. The immunosuppressive activity of *L. reuteri* PTA 6475 has been documented before, and IL-8 secretion by porcine IECs stimulated with LPS was attenuated during culture with PTA 6475 (Liu *et al.* 2010). This indicates the complex relationship between *L. reuteri* and the inflammatory response of porcine IPEC-J2 cells; whilst *L. reuteri* stimulates IL-8 production in non-stimulated cells, immunosuppressive activity is observed after stimulation with LPS. Further experiments in rat pups exposed to LPS demonstrated decreased production of KC (neutrophil chemoattractant equivalent to IL-8) from ileal sections of rats treated with PTA 6475. This anti-inflammatory activity also extended to mice as secretion of pro-inflammatory IL-1β, MIP-1α and IFN-γ during rotavirus infection of murine ileal biopsies was reduced by PTA 6475

(Preidis *et al.* 2012). As bacterial access to the intestinal epithelium is normally restricted *in vivo*, these results suggest that the anti-inflammatory activity is either mediated by transient contact with the epithelium or by secreted factors that can diffuse across the mucus.

There is considerable evidence for immunomodulatory metabolites secreted by PTA 6475. Multiple studies have shown attenuated TNF-α production by LPS-stimulated THP-1 monocytes incubated with PTA 6475 CFS (Jones and Versalovic 2009; Lin *et al.* 2008; Thomas *et al.* 2012). This observation extended to LPS-stimulated monocyte-derived macrophages from children with Crohn's disease indicating consistent anti-inflammatory activity of PTA 6475 in human monocyte cell lines and primary cells (Lin *et al.* 2008). Reduction of TNF-a release is partially dependent on histamine which is produced by *L. reuteri* and associated with bacterial cells and supernatants (Thomas *et al.* 2012). In monocytes, histamine binds and activates the H2 receptor but its expression in IECs still needs to be elucidated (Gao *et al.* 2015a). Interestingly, a recent publication highlighted a role for extracellular vesicles (EVs) in the anti-inflammatory activity of *L. reuteri* BBC3 (Hu *et al.* 2021). Incubation of LPS-stimulated jejunal chicken explants with *L. reuteri* EVs resulted in a similar reduction of *Tnfα*, *Il-8*, *Il-1β* and *Il-6* gene expression as whole bacterial culture.

Furthermore, several LAB species have demonstrated immunomodulation via surface-layer proteins (SLPs). SLPs are glycoproteins that form an outer coating of the PG and have been implicated in an array of functions including host cell adhesion, biofilm formation and immunomodulation (Fagan and Fairweather 2014). SLPs isolated from *L. rhamnosus* GG reduced *Il-6* and *Tnfα* expression in porcine IPEC-J2 cells stimulated with LPS (Gao *et al.* 2017). Similar results were obtained using Slps from *L. crispatus* JCM 2009, which reduced production of the pro-inflammatory mediator prostaglandin E_2 from LPS-stimulated murine macrophages (Wang, Zhang, *et al.* 2021). Whilst corresponding investigations have not been performed with *L. reuteri* PTA 6475, Walsham (2016) showed attenuation of EPEC-induced human intestinal IL-8 secretion by whole bacteria but not CFS, suggesting a role for bacterial surface proteins. Whilst we did not delineate whether anti-inflammatory activity of PTA 6475 on EPEC-infected T84/LS epithelia was due to secreted or contact-dependent factors, it is evident that extended culture of the commensal is required which could allow accumulation of secreted anti-inflammatory metabolites or EVs or may support prolonged contact time of bacterial-bound mediators with host cells. Surprisingly, CFS from non-infected T84/LS cells attenuated IL-8 production by EPEC-infected T84/LS epithelia. This suggests that culture of host cells without bacteria modifies composition of the apical medium, either through depletion of nutrients or secretion of host metabolites, resulting in anti-inflammatory activity. Very little is known about metabolite secretion by T84 and LS174T cells, although factors such as hormones or proteases may be involved in mediating

permeability of the epithelium to MAMPs (Lewis, Berg, and Kleine 1995). Differences in the nutrient composition of CFS and fresh apical medium, such as decreased glucose or breakdown of toxic BHI-YH components, may reduce epithelial permeability to MAMPs (Suzuki 2020). Nevertheless the relationship between nutrient access and the innate response is poorly understood and is does not sufficiently support this hypothesis.

Whilst the mediator(s) of the anti-inflammatory activity of PTA 6475 seen here remains unknown, the target for their activity is likely the inflammatory transcriptional regulators NF-κB and AP-1. NF-κB and AP-1 are crucial to the innate immune response to pathogens, translocating into the nucleus and transcribing genes encoding pro-inflammatory cytokines following activation by stimulated TLRs (Wullaert, Bonnet, and Pasparakis 2011; Liu *et al.* 2009). Notably, activation of both transcriptional regulators has been demonstrated during EPEC and STEC infection of T84 cells, resulting in IL-8 release (Hecht and Savkovic 1997; Dahan *et al.* 2002). *L. reuteri* has been shown to inhibit NF-κB activation in LPS-stimulated rat ileal biopsies, likely by protecting the NF-κB inhibitor IκB from degradation (Liu *et al.* 2012). In addition to targeting NF-κB, PTA 6475 can interfere with AP-1 activity by suppressing the upstream mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway (Thomas *et al.* 2012). Taken together, these data indicate robust suppression of inflammation by PTA 6475 in stimulated monocytes, macrophages and IECs. The data collected here using T84/LS intestinal epithelia agree with these studies, although anti-inflammatory mediators were not elucidated.

In conclusion, *L. reuteri* reduced numbers of planktonic and adherent EPEC during infection of T84/LS epithelia, and attenuated the host inflammatory response. The colonisation resistance activities of *L. reuteri* PTA 6475 may help to explain the beneficial effects in treating infant diarrhoea seen in clinical trials (Dinleyici *et al.* 2015; Gutierrez-Castrellon *et al.* 2014; Pernica *et al.* 2017; Savino *et al.* 2015; Shornikova *et al.* 1997). This would allow future studies to understand what features of *L. reuteri* PTA 6475 are required to protect the host from EPEC infection, permitting a more targeted approach to identifying a successful probiotic treatment.

5.3.2 Colonisation resistance by *R. gnavus*

Here, we have demonstrated previously undiscovered colonisation resistance activity of *R. gnavus* against EPEC. Our results show that culture with *R. gnavus* ATCC 35913 reduced proliferation of planktonic EPEC, but this effect was only observed with T84/LS and not T84 epithelia. This suggests that growth inhibition by *R. gnavus* is not solely due to competition for nutrients in DMEM/F-12 + BHI-YH medium, and that LS174T cell-derived factors mediate antagonism of EPEC growth. This is

surprising as *R. gnavus* did not demonstrate enhanced apical growth with T84/LS compared to T84 epithelia when cultured alone, suggesting that *R. gnavus* does not utilise LS174T cell-derived factors for growth in DMEM/F-12 + BHI-YH. However, co-culture with EPEC might enhance competition for nutrients and promote the use of LS174T-derived factors for *R. gnavus* growth, although we did not determine this experimentally. Mucus-derived sugars are a likely nutrient source from LS174T cells, as *R. gnavus* can grow in minimal medium supplemented with mucus-derived sugars (Crost *et al.* 2016). Interestingly, gene expression analysis has revealed upregulation of several GHs during *R. gnavus* growth with PGM as the sole carbon source (Crost *et al.* 2016). It is unclear whether GH genes are upregulated exclusively is response to mucins, or if a lack of alternative nutrients is also required. If the latter is true, GH might not be produced in nutrient rich DMEM/F-12 + BHI-YH medium but activated in a nutrient-depleted environment during co-culture with EPEC. Interestingly, experiments in mice revealed enhanced colonisation by *R. gnavus* ATCC 29149 compared to an isogenic mutant lacking a functioning *nan* cluster (crucial to sialic acid utilisation from intestinal mucus), but only during competition of both strains (Bell *et al.* 2019). Whilst bacterial growth was not assessed, these results indicate that genes involved in utilising mucus-derived sugars may be activated during culture with other bacteria to provide a competitive advantage.

Interestingly, sialic acid utilisation by *R. gnavus* ATCC 29149 can occur via a non-canonical pathway dependent on the intramolecular trans-sialidase NanH, generating an anhydrous form of Neu5Ac which the authors hypothesised would be inaccessible to bacterial competitors at the intestinal mucosa (Bell *et al.* 2019). Despite a follow-up study revealing the prevalence of homologous proteins with similar activity in *E. coli*, encoded on the *yjhBC* operon, these genes are absent in EPEC E2348/69 (Bell *et al.* 2020; Iguchi *et al.* 2009). Consequently, by utilising anhydrous Neu5Ac from LS174T cells, which is inaccessible to EPEC E2348/69, *R. gnavus* may have a competitive advantage over the pathogen.

Apart from potential competition for LS174T cell-derived nutrient sources, *R. gnavus* might produce metabolites in the presence of T84/LS epithelia that inhibit EPEC growth. So far, two bacteriocins have been characterised from *R. gnavus strain* E1. While Ruminococcin A and C exhibited growth inhibition of Clostridia and other Gram-positive gut bacteria, Gram-negative *Bacteroides* spp. and *Salmonella* Typhimurium were resistant to the antimicrobials (Crost *et al.* 2011; Dabard *et al.* 2001). Thus, ruminococcins are unlikely to cause growth inhibition of EPEC.

Similar to results regarding EPEC growth, *R. gnavus* ATCC 35913 reduced numbers of adherent EPEC but only with T84/LS epithelia. It is likely that the reduction in planktonic EPEC by *R. gnavus* ATCC 35913 could directly result in reduced adherent EPEC as there are fewer bacteria to colonise the

epithelium. Contrary to *L. reuteri*, regression analysis of planktonic and adherent EPEC CFU during co-culture with *R. gnavus* revealed that planktonic EPEC did significantly influence adherent EPEC, accounting for ~65% of the variation seen in adherent numbers (R^2 = 0.65). Nevertheless, the remaining 35% may be due to other factors. Enhanced competition for mucus or epithelial binding sites on LS174T cells is a feasible explanation. Indeed, both EPEC and *R. gnavus* colonised T84/LS cells more effectively than T84 epithelia in monoculture (**Fig. 4.6**). Whilst the amount of adherent *R. gnavus* in monoculture is much lower than that of EPEC (4 x 10^5 and 1.5 x 10^7 CFU, respectively) after 4 hours, the commensal is present in excess at the start of the incubation. Therefore, *R. gnavus* may be able to compete with EPEC for binding sites at earlier stages of the incubation. Host cell binding of *R. gnavus* might be facilitated by the carbohydrate binding module (CBM40) of NanH (discussed in **section 4.3.4**). As discussed above, competition for LS174T cell-derived nutrients could also influence proliferation at the epithelial surface resulting in decreased numbers of adherent EPEC. Another cause of reduced EPEC adhesion could be inhibition of T3SS activity by *R. gnavus*. Although no influence has been detected on EspB secretion into the medium, other effector proteins could be affected as discussed in **section 5.3.1**.

Finally, we investigated the host IL-8 response to *R. gnavus* and whether the commensal affected IL-8 secretion by EPEC-infected T84/LS epithelia. The data shown here indicate no IL-8 response during exposure to *R. gnavus* ATCC 35913 for up to 22 hours. Historically, *R. gnavus* has been associated with inflammation at the intestinal mucosa. A year-long study tracking the fecal microbiome of individuals with IBD found frequent "blooms" of *R. gnavus* and a relative abundance of 41.6% compared to only 2.44% in healthy participants (Hall *et al.* 2017a). Phylogenetic analysis of genes specific to *R. gnavus* revealed two distinct clades: one which was associated with healthy microbiomes (Clade 1) and the other with IBD (Clade 2). Cataloguing reference genomes placed ATCC 35913 in Clade 1, suggesting it may not play a role in IBD. Nevertheless, a recent study has reported TNF-α secretion from DCs in response to *R. gnavus* strain ATCC 35913 which is likely mediated by glucorhamnan activation of TLR4 (Henke *et al.* 2019). As DCs are not present in our epithelial model and minimal TLR4 expression has been detected in T84 and LS cells, it is unlikely that strain ATCC 35913 would evoke an inflammatory response in intestinal epithelia (Abreu *et al.* 2001; Hsu *et al.* 2011).

Importantly, our results revealed an anti-inflammatory activity of *R. gnavus* ATCC 35913 on T84/LS epithelia infected with EPEC, as evidenced by attenuation of IL-8 secretion. As extended culture of 22 hours was required for this effect, the antagonistic activity of *R. gnavus* on EPEC growth and adherence during the initial 4-hour period is unlikely to be the cause. A previous study has indicated an anti-inflammatory activity of *R. gnavus in vivo* as repopulation of antibiotic-treated chronically inflamed

IL10-/- mice with *R. gnavus* attenuated TNF, IL-6 and Il-1β gene expression. This was accompanied by increased cecal propionate levels which might suppress inflammatory gene expression. A role of propionate has also been shown in IL-8 secretion as propionate pre-treatment of Caco-2 cells stimulated with flagellin resulted in decreased *IL-8* gene expression (Iraporda *et al.* 2015). The mechanism driving anti-inflammatory activity of propionate is poorly understood, requiring further work to identify crucial intracellular signaling pathways targeted by the SCFA (Tedelind *et al.* 2007). Notably, propionate production by *R. gnavus* ATCC 29149 during anaerobic culture has been confirmed by NMR analysis (Crost *et al.* 2013). While there is evidence of *R. gnavus*-mediated immunomodulation, further work is required to elucidate specific MAMPs and respective host cell signaling pathways at the intestinal epithelium.

Taken together, the results shown here demonstrate previously unrecognised colonisation resistance activity of *R. gnavus* ATCC 35913 in EPEC infection, the discovery of which has been enabled by the microaerobic conditions in the VDC model. This activity was dependent on the presence of LS174T cells, highlighting the importance of goblet-like cells when assessing gut commensal-pathogen interactions. Characterising the roles of mucus-degrading anaerobes in colonisation resistance using microaerobic mucus-producing epithelial models can progress our understanding of the healthy human microbiota and lead to novel therapeutic discoveries for enteric infections.

Chapter Six – Conclusions

This study aimed to assess the ability of the gut commensals *Limosilactobacillus reuteri* PTA 6475 and *Ruminococcus gnavus* ATCC 35913 to reduce enteropathogenic *E. coli* pathogenesis. To do this we utilised the microaerobic conditions generated by the Vertical Diffusion Chamber (VDC) and established a mucus-secreting epithelium by co-culturing enterocyte (T84) and goblet-like (LS174T) cell lines. The influence of commensals on pathogen growth, adherence and host innate immune response was assessed using mucus- and non-mucus secreting epithelia (T84/LS174T mixed culture and T84 monolayers, respectively).

In the first part of this study, we generated a microaerobic model system of the intestinal epithelium that supported culture of all three microbes and a host epithelium using the VDC. To do this, we identified an optimal apical medium (DMEM/F-12 + BHI-YH) that supported growth of the bacteria, EPEC pathogenesis, as well as cell polaristaion and epithelial barrier function. We also generated a mucus-secreting epithelium by culturing goblet-like LS174T alongside T84 enterocytic cell lines (T84/LS). Our results showed that mucus secretion by T84/LS epithelia was localised to the goblet-like cells, which is similar to the commonly used Caco-2/HT29-MTX cell culture model but disagrees with the continuous layer observed in mammalian small intestinal biopsies. This suggests that mucus-producing intestinal epithelia derived from cell lines are lacking features that promote mucus secretion. This may be overcome by utilising 2D organoids derived from native intestinal tissue, which have been shown to produce a mucus layer under low oxygen conditions. A continuous mucus layer would likely have profound effects on host-bacterial interactions, as each organism used in this study has been shown to interact with mucus to mediate colonisation.

In the second part of this study, we compared host-microbe interactions in T84 and T84/LS epithelia. We demonstrated that the microaerobic VDC supported bacterial growth and epithelial adherence, which was particularly important for the oxygen-sensitive anaerobe *R. gnavus*. Furthermore, enhanced growth, adherence and interleukin-8 response to EPEC, as well as increased adhesion of *R. gnavus* was observed with T84/LS epithelia. Immunofluorescence images revealed preferential colonisation of secreted mucus over the epithelial surface for *L. reuteri* and *R. gnavus*, suggesting that mucus impacts interaction with the host. However, the role of mucus on host-bacterial interactions was difficult to confirm as T84 monolayers were used as a non-mucus secreting control. Phenotypic differences between T84 and LS cells, such as barrier function, secreted metabolites, and expression of TLRs and microbial binding receptors may have impacted interactions with the bacteria. We attempted to overcome this by removing secreted mucus from T84/LS epithelia using mucolytic reagents, however this was unsuccessful. Another approach would be to silence *MUC2* gene

expression in LS174T cells using siRNAs or CRISPR-Cas9 technology, which have been applied routinely to mammalian cell lines.

In the final section, we assessed the ability of *L. reuteri* and *R. gnavus* to reduce EPEC pathogenesis in T84 and T84/LS epithelia using the VDC model developed previously. Our results revealed reduced pathogen growth and adherence by *L. reuteri* with both T84 and T84/LS epithelia, whereas pathogen antagonism by *R. gnavus* was only evident with T84/LS epithelia. We attempted to identify whether growth inhibition of EPEC by commensal strains was mediated by a secreted factor by assessing the inhibitory activity of cell-free supernatants (CFS) from *L. reuteri* and *R. gnavus* grown without host cells. These results showed that commensal CFS did not inhibit affect EPEC growth, suggesting that growth inhibition requires whole bacteria or that secreted metabolites are only produced during culture with EPEC and/or host cells. Similar results were observed in experiments assessing the influence of commensals on the IL-8 response by EPEC-infected T84/LS epithelia, where the commensals reduced IL-8 secretion via a mechanism independent of bacterial secreted factors. To identify potential mediators involved in reducing EPEC pathogenesis, differential gene expression of *L. reuteri* and *R. gnavus* during culture with and without EPEC could be performed. Genes which are significantly up- or down-regulated during co-culture with the pathogen could highlight proteins or metabolic pathways involved in antagonising EPEC.

Investigations into the effect of commensals on IL-8 secretion by EPEC-infected epithelia demonstrated that extended culture (22 h vs 4 h) resulted in anti-inflammatory activity of LR and RG. This indicates that prolonged host-microbe interaction might be required to detect changes in epithelial response. Therefore, the introduction of a perfusion system which continuously replenishes nutrients and dilutes bacteria and their by-products might be beneficial.

We assessed the influence of commensals on the activity of the EPEC type III secretion system using semi-quantitative western blotting for secreted effector EspB. From these experiments we concluded that neither culture with *L. reuteri* nor *R. gnavus* impacted EPEC T3SS activity. This suggested that reduction of EPEC adherence by *L. reuteri* and *R. gnavus* was independent of intimate adherence. Reduced pathogen adherence may be driven by competition for binding sites on the host surface, such as N-acetyllactosamine. Blocking these binding sites with antibodies and then assessing the influence of commensals on EPEC adherence could have elucidated their involvement.

One of the limitations of effector secretion assay was the inability to detect EspB translocated into host cells, where the effector directly influences pathogenesis. Given more time, we could have created reporter fusions to EspB and assessed translocation into the host. To gain more insight into

how commensals affect T3SS activity, this could be extended to other effector proteins, especially Tir which is crucial for intimate adherence.

The mucus secreting, microaerobic model of the intestinal epithelium developed here can be expanded to study other pathogens and commensal organisms. Both microaerobic conditions and secreted mucus have a profound effect on host interactions with gut bacteria, which could be explored in much greater detail using this system. Furthermore, the gut microbiota is composed primarily of strict anaerobes that die following exposure to oxygen, hence their interactions with human intestinal epithelia are relatively understudied. This can be rectified using the model system developed here, which supports culture of strict anaerobes and direct interaction with a viable human epithelium.

In conclusion, we demonstrate colonisation resistance activity of both *L. reuteri* PTA 6475 and *R. gnavus* ATCC 35913 against EPEC using a human intestinal epithelium. *L. reuteri* reduced pathogen growth, adherence and host innate immune response in both T84 and T84/LS epithelia, highlighting robust colonisation resistance activity. This study supplements clinical trials showing the efficacy of *L. reuteri* supplementation in infectious diarrhoea. For *R. gnavus*, colonisation resistance activity was only observed with T84/LS epithelia. Very little is known about the colonisation resistance activity of *R. gnavus*, but our results suggest that future studies aiming to gain more insight should choose model systems that include goblet-like cells.

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