Determining the protective effects of *Lactobacillus reuteri* against Enteropathogenic *Escherichia coli* infection

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Abstract

Enteropathogenic *Escherichia coli* (EPEC) are non-invasive foodborne diarrhoeal pathogens that are a leading cause of infant death in the developing world. As antibiotic resistance increases amongst pathogens, new treatments are required to reduce infant mortality. Probiotic bacteria could offer a solution, and *Lactobacillus reuteri* have been shown to alleviate diarrhoea and reduce EPEC colonisation in clinical studies. Here, we utilised mucus and non-mucus producing human intestinal epithelial cell lines as well as human duodenal biopsies to investigate the effects of *L. reuteri* ATCC PTA 6475 and ATCC 53608 on EPEC infection, with particular focus on pathogen adherence, host mucin production, and innate immune response.

Short-term protection assays demonstrated that pre-incubation with an excess of *L. reuteri* inhibited EPEC epithelial binding, independently of secreted products and probiotic epithelial adhesion. Increased pre-incubation times enhancing *L. reuteri* adhesion reduced EPEC binding to HT-29 cells by ATCC PTA 6475. While this strain did not inhibit EPEC adhesion to LS174T cells, ATCC PTA 6475 significantly reduced cell to cell spread of EPEC, a characteristic which has not previously been described.

In addition to reducing EPEC adherence, incubation with *L. reuteri* ATCC PTA 6475 increased MUC2 protein production in LS174T cells. In contrast, EPEC infection reduced MUC2 protein levels and this effect was diminished by co-incubation with ATCC PTA 6475, suggesting that *L. reuteri* protect against EPEC-mediated MUC2 degradation. *L. reuteri* also demonstrated anti-inflammatory characteristics, as ATCC PTA 6475 and ATCC 53608 inhibited EPEC-induced interleukin-8 protein expression in HT-29 cells.

Taken together, our findings suggest that *L. reuteri* protects against EPEC infection by reducing pathogen binding and modulating host mucus production and inflammation. These effects are strain-specific and dependent on the host model system used. Therefore, the probiotic potential of *L. reuteri* strains needs to be carefully evaluated in relevant systems before application in clinical practice.

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AAD – Antibiotic associated diarrhoea

A/E – Attaching and effacing

AIEC – Adherent invasive *Escherichia coli*

AP-1 – Activator protein-1

ARP 2/3 – Actin related protein 2/3

BFP – Bundle forming pilus

bp – Base pairs

cDNA – Complimentary DNA

CmbA – Cell and mucus binding protein

CD – Crohn's disease

DAEC – Diffusely adherent *Escherichia coli*

DAPI – 4',6-diamidino-2-phenylindole

ddH_2O – deionised distilled H_2O

DMEM – Dulbecco's modified Eagle's medium

DMSO – Dimethyl sulfoxide

DRA - Downregulated in adenoma

EAEC – Enteroaggregative *Escherichia coli*

EAF – EPEC adherence factor

ECACC – European collection of cell cultures

EDTA – Ethylenediaminetetraacetic acid

EGF – Epidermal growth factor

EHEC – Enterohaemorrhagic *Escherichia coli*

EIEC – Enteroinvasive *Escherichia coli*

EPEC – Enteropathogenic *Escherichia coli*

Esp – *E. coli* secreted protein

ETEC – Enterotoxigenic *Escherichia coli*

ExPEC – Extraintestinal *Escherichia coli*

FAO – Food and Agriculture Organisation

FAS – Fluorescent actin stain

FBS – Foetal bovine serum

FITC – Fluorescein isothiocyanate

FOV – Field of view

GalNAc – N-Acetylgalactosamine

GEMS – Global Enteric Multicentre Study

GI - Gastrointestinal

GIT – Gastrointestinal tract

GlcNAc – N-Acetylglucosamine

GOI – Gene of interest

GRAS – Generally regarded as safe

HIP/PAP – Hepatocarcinoma-intestine-pancreas/pancreatic associated protein (also known as REGIIIα)

IBD – Inflammatory bowel disease

IEC – Intestinal epithelial cell

IFN-γ – Interferon gamma

IKK – IκB kinase

IL - Interleukin

IRAK – Interleukin-1 receptor–associated kinase

IVOC – *In vitro* organ culture

KC - keratinocyte-derived protein chemokine

KO – Knockout

LEE – Locus of enterocyte effacement

L. reuteri – Lactobacillus reuteri

LB – Lysogeny broth

LPF – Long polar fimbriae

LPS – Lipopolysaccharide

M cells – Microfold cells

MeOH/ acetone – Methanol/ acetone

MAMPs – Microbe-associated molecular patterns

Map – Mitochondrial associated protein

MAPK – Mitogen-activated protein kinase

MBP – Mucus binding protein

MOI – Multiplicity of infection

MRS – De Man, Rogosa, Sharpe

MSD – Moderate to severe diarrhoea

MUB – Mucus binding protein (Cell surface adhesin produced by *L. reuteri* ATCC 53608)

NEC – Necrotising enterocolitis

NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells

NHE3 – Na+ –H+ -exchange protein 3

Nle – Non-LEE encoded

NLR – NOD-like receptor

NMEC – Neonatal meningitis *Escherichia coli*

NOD – Nucleotide-binding oligomerization domain

NT – Non-treated

N-WASP – neural Wiskott- Aldrich syndrome protein

OCT – Optimal cutting temperature

PAI – Pathogenicity Island

pIVOC – Polarised *in vitro* organ culture

POLR2A – RNA polymerase II

PRR – Pattern recognition receptor

PTS – Proline, Threonine, and Serine

qPCR – real time quantitative polymerase chain reaction

RPM – Rotations per minute

RPS3 – Ribosomal protein S3

RT- – Reverse transcriptase negative control

RT – Room temperature

SCFA – Short chain fatty acids

SE – Standard error of the mean

SEM – Scanning electron microscopy

SGLT1 - sodium-glucose linked transporter 1

SH-1 – Src homology region 2 domain-containing phosphatase-1

SYBR - SYBR® Green JumpStart™ *Taq* ReadyMix™

T/E – Trypsin-EDTA solution

T3SS – Type 3 secretion system

TAB – TAK binding protein

TAK – TGF-β activated kinase

TER – Transepithelial electrical resistance

Tir – Translocated intimin receptor

TJ – Tight junction

TLR – Toll-like receptor

Tm – Melting temperature

TRAF – TNF receptor associated factor

TX-100 – Triton X-100

UC – Ulcerative colitis

UPEC – Uropathogenic *Escherichia coli*

WHO – World Health Organisation

Y454 – Tyrosine 454

Y474 – Tyrosine 474

YWHAZ – Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta polypeptide

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CHAPTER ONE

Introduction

1.1. Enteropathogenic *E. coli*

Escherichia coli are rod shaped, Gram-negative, facultative anaerobes which colonise the infant gastrointestinal (GI) tract (GIT) within hours after birth (Kaper *et al.*, 2004). These microbes were originally isolated from the stools of infants by Theodor Escherich in 1885 (*Bacterium coli commune*) and were seen as harmless commensals in the GIT (Reprinted in English, Escherich (1988)). However, the identification of *Bacterium coli* var. *neapolitanum*, which would later be renamed enteropathogenic *E. coli* (EPEC), as the causative agent of "summer diarrhoea" in the UK in the 1940's demonstrated that this bacterial species could also have pathogenic tendencies (Bray, 1945; Bray & Beavan, 1948; Levine *et al.*, 1978). Since the identification of EPEC, numerous other *E. coli* pathotypes have been described, which have been broadly classified as either diarrhoeagenic or extra-intestinal pathogenic *E. coli* (ExPEC) (Figure 1.1) (Kaper *et al.*, 2004).

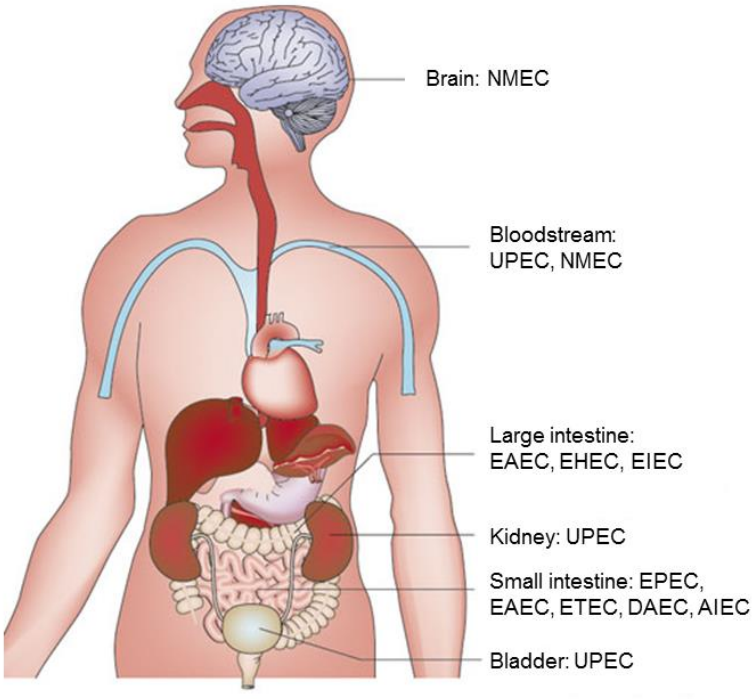

Figure 1.1: The colonisation sites of the major *E. coli* pathotypes

Modified from Croxen and Finlay (2010).

The diarrhoeagenic *E. coli* pathotypes are EPEC, enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (Croxen *et al.*, 2013). The recently described pathotype adherent invasive *E. coli* (AIEC) is also found in the GIT, where it has been linked to the development of ileal Crohn's disease. However, this strain is not specifically associated with the development of diarrhoea (Darfeuille-Michaud, 2002). The ExPEC pathotypes which have been described are uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) which colonise the bladder and brain, respectively (Croxen & Finlay, 2010). The evolution of these distinct *E. coli* pathotypes is facilitated thorough the plasticity of the *E. coli* genome, which contains a core genome (genes located in at least 95% of publically available sequenced *E. coli* genomes) of approximately 3050 genes and a pan-genome (genes located in fewer than 95% of publically available sequenced *E. coli* genomes) of over 16,300 genes (Kaas *et al.*, 2012). The expansive pan-genome contains the *E. coli* virulence repertoire which enables *E. coli* to infect the host and induce diarrhoea.

Diarrhoeal disease is currently the second largest killer of children under the age of 5, responsible for the deaths of an estimated 800,000 children per year in 2010, primarily from sub-Saharan Africa and south east Asia (Liu *et al.*, 2012a). In the developing world, socio-economic status is inversely proportional to the risk of moderate to severe diarrhoea (MSD), as children from parents with higher wealth status, greater access to clean water, and more highly educated mothers, were less likely to present with MSD than children from poorer backgrounds (Kotloff *et al.*, 2013). While there are numerous etiological factors which cause this diarrhoeal health burden, diarrhoeagenic *E. coli* are a major contributor as the pathotypes ETEC and EIEC (*Shigella*) have been identified as two of the four major causes for MSD (Kotloff *et al.*, 2013). While the incidence of EPEC infection was lower than ETEC and EIEC, the presence of EPEC in cases of MSD was significantly associated with infant death (Kotloff *et al.*, 2013). Furthermore, a systematic review of diarrhoeal mortality from 1990 to 2011 estimated that EPEC was the second major cause of death (after rotavirus) in children under 5 years of age

in 2011 (Lanata *et al.*, 2013). Crucially, a rotavirus vaccine has been developed in recent years which has shown a high clinical efficacy (Vesikari *et al.*, 2006). However, no vaccines have been developed for the prevention of EPEC infection so far.

Thus, EPEC remains an important diarrhoeal pathogen and further research into the pathogenic mechanisms is required. In this section, I will discuss the current knowledge regarding EPEC infection.

1.1.1. Epidemiology

1.1.1.1. Emergence and classification

The bacterial species *Bacterium coli* var. *neapolitanum*, later classified as EPEC serogroup O111, were first described in the United Kingdom by John Bray, who found that these bacteria were present in children with “summer diarrhoea” but were mostly absent from healthy infants (Bray, 1945; Levine, 1987). Infections mainly affected infants (average age of 6 months) and were associated with high mortality (20 deaths out of 51 reported cases) (Bray, 1945). The researchers identified these bacteria by developing an antiserum, which was used in the slide-agglutination test to differentiate EPEC from non-pathogenic *E. coli* strains (Bray & Beavan, 1948; Levine, 1987). The use of this technique for *E. coli* recognition, as well as the development of O and H antigen serotyping by Kauffman in 1947, led to the detection of *E. coli* associated with diarrhoea in children across Europe and the United States of America (Levine, 1987; Robins-Browne, 1987). This pathotype was subsequently renamed enteropathogenic *E. coli* by Neter and colleagues and further divided into the classical EPEC serotypes O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158 (Neter *et al.* (1955) and reviewed in Robins-Browne (1987), Levine (1987), Nataro and Kaper (1998), and Croxen *et al.* (2013)).

With the identification of the *E. coli* pathotypes EIEC and ETEC in the subsequent decades, popular scientific thought suggested that EPEC was a

derivative of ETEC which had lost its enterotoxin plasmid during storage (Gross *et al.*, 1976). However, the absence of toxins in diarrhoea which had an EPEC presence refuted this allegation (Gross *et al.*, 1976). Definitive confirmation of a distinct EPEC pathotype was finally achieved by volunteer studies performed by Levine and colleagues, who demonstrated that EPEC strains of the serotype O127:H6 and O142:H6, which had been stored for 7 years, were able to induce diarrhoea, but did not produce heat-stable or heat-liable enterotoxins, which were characteristic for ETEC (Levine *et al.*, 1978).

In 1995, the EPEC pathotype underwent further redefinition and now encompasses two groups, typical and atypical EPEC (aEPEC), which are characterised by the presence or absence of the bundle forming pilus (BFP) encoded on the EPEC adherence factor (EAF) plasmid, respectively (Nataro & Kaper, 1998; Trabulsi *et al.*, 2002). The EAF plasmid has an important role in the induction of diarrhoea by typical EPEC, as the loss of the EAF plasmid reduced the incidence of diarrhoea relative to the parent strain in adult volunteer studies (Levine *et al.*, 1985). While aEPEC lack the EAF plasmid, these strains may have acquired additional virulence factors which compensate for the functionality of the EAF plasmid.

While typical EPEC are a relatively homogenous group, with similar virulence factors between strains, aEPEC demonstrate greater heterogeneity and are thought to be more closely related to EHEC, with 81% of aEPEC strains not belonging to classical EPEC O-serotypes and 26.6% of aEPEC strains being “O-non-typeable” (Hernandes *et al.*, 2009). Interestingly, the aEPEC serotype O55:H7 appears to share a common ancestor with EHEC O157:H7, with divergence of these pathotypes approximately 400 years ago (Whittam *et al.*, 1993; Zhou *et al.*, 2010).

As my focus in this PhD project was on the typical EPEC strain O127:H6 E2348/69, the term EPEC will refer exclusively to typical EPEC and atypical EPEC will be referred to as aEPEC throughout this thesis.

1.1.1.2. Incidence, reservoirs, and transmission

When EPEC were initially identified, infection was widespread in developed countries (Nataro & Kaper, 1998). While infection rates have decreased in the developed world, EPEC infection has become prevalent in the developing world (Nataro & Kaper, 1998). This shift in burden has been associated with improvements in hygiene and medical treatment however, it is also important to note that EPEC-induced diarrhoea in the developed world may be underreported and thus, the incidence of EPEC infection may be underestimated (Nataro & Kaper, 1998).

The prevalence of EPEC amongst isolated diarrhoeagenic *E. coli* can vary between locations, with EPEC comprising 3.2% of diarrhoeagenic *E. coli* in Thailand (Ratchtrachenchai *et al.*, 2004) compared to 15.5% in India (Dutta *et al.*, 2013). While typical EPEC were originally thought to be more prominent in the developing world, in recent years the detection of aEPEC has increased relative to typical EPEC in children presenting with diarrhoea, as studies in India (Dutta *et al.*, 2013; Nair *et al.*, 2010), Iran (Nakhjavani *et al.*, 2013), Kuwait (Albert *et al.*, 2009), Thailand (Ratchtrachenchai *et al.*, 2004), and Vietnam (Nguyen *et al.*, 2005; Thompson *et al.*, 2015) have predominantly isolated aEPEC from stools, with a previous study estimating that 78% of isolated EPEC were attributable to the aEPEC group (Ochoa *et al.*, 2008). However, the role of aEPEC in the induction of diarrhoea is currently controversial, as the detection of aEPEC was greater in control patients than those presenting with diarrhoea (50% controls versus 18% with diarrhoea; Thompson *et al.* (2015)). In contrast, other studies have found a significant association between the presence of aEPEC and diarrhoeal symptoms (2.3% controls versus 12.8% with diarrhoea; Robins-Browne *et al.* (2004)). As the contribution of aEPEC to diarrhoea remains uncertain, further study is required to determine whether aEPEC, or other factors, are the causative agent.

A key issue when interpreting epidemiological data is the difference in protocol and inclusion criteria amongst different studies. However, the recent Global Enteric Multicentre Study (GEMS) has provided an up-to-date evaluation on the causes of MSD in children up to 5 years old in seven case-controlled

populations in sub-Saharan Africa (Kenya, Mali, Mozambique, and The Gambia) and South Asia (Bangladesh, India, and Pakistan), with standard operating procedures across all sites (Kotloff *et al.*, 2013). This landmark study analysed the role of pathogens associated with MSD in 9439 children and found that while EPEC were not a major cause of diarrhoea (with a significant association to MSD only found in Kenya), this pathogen was significantly associated with death (2.6-fold increased risk) when present in infants with MSD (Kotloff *et al.*, 2013). In contrast, aEPEC were not significantly associated with either the development of MSD or the likelihood of death when present. Risk of death from typical EPEC infection may be linked to specific allele variants, in particular *nleG*; however, while the function of this gene is known (E3 ubiquitin ligase), the specific cellular target has not yet been defined (Donnenberg *et al.*, 2015). Thus, while the incidence of typical EPEC has reduced in recent years, this pathotype still represents a serious threat to infant health.

While EPEC infection has been well documented, no specific reservoir in the environment has yet been identified. As humans are the only known natural hosts for EPEC, current thought suggests that both symptomatic and asymptomatic humans are the primary reservoirs (Croxen *et al.*, 2013; Levine & Edelman, 1984). The spread of EPEC from one host to the next occurs through faecal-oral transmission, a route shared amongst all diarrhoeagenic *E. coli*, with bacterial transfer facilitated through contact with both infected people and soiled surfaces. Additionally, contaminated water may also be a pathogen reservoir, as EPEC infection is particularly prevalent in bottle-fed infants (Levine & Edelman, 1984). While infection is predominant in children under the age of two, adult infection has been observed in volunteer studies, where a dose of 10^8 to 10^{10} EPEC were sufficient to induce diarrhoea, if the gastric acid was neutralised prior to inoculation (Levine *et al.*, 1978). However, due to the high infectious dose, and the need for gastric acid neutralisation, the study by Levine and colleagues suggested that natural adult infection was unlikely (Levine *et al.*, 1978). The dose required for the infection of infants is currently unknown, although it is thought to be much lower than the observed dose for adults (Nataro & Kaper, 1998).

1.1.2. Pathogenesis

EPEC demonstrates tropism to the duodenum, with EPEC adhesion to the intestinal epithelium being defined as a three-step process (Croxen *et al.*, 2013; Donnenberg & Kaper, 1992). Firstly, EPEC adhesins, such as BFP, bind to the epithelial surface and form an initial attachment to the mucosa. Secondly, the type 3 secretion system (T3SS) transfers translocated intimin receptor (Tir) into the host cell, which self-inserts into the host cell membrane. Finally, Tir attaches to EPEC-bound intimin, forming an intimate attachment between EPEC and the host cell. Concurrent with the transfer of Tir, further effector proteins are translocated into the host cell through the T3SS, which subsequently modify host cell processes and mediate the formation of the attaching and effacing (A/E) lesion. The attachment of EPEC to the intestinal epithelium is a key step in the development of diarrhoea. This process has been thoroughly investigated and we now have a good understanding of the individual stages required for attachment to the host.

1.1.2.1. Initial attachment

The initial attachment of EPEC to the epithelium is mediated through EPEC-bound adhesins such as BFP, a type IV pilus encoded on the EAF plasmid, which adheres to phosphatidylethanolamine (PE), a phospholipid present in the host cell membrane (Figure 1.2A) (Foster *et al.*, 1999; Giron *et al.*, 1991; Khursigara *et al.*, 2001). The role of the BFP in initial binding has been demonstrated on cancer cell lines, as an isogenic Δ*bfp* strain showed significantly reduced adhesion compared to the wildtype, when incubated on Caco-2 cells (Cleary *et al.*, 2004). Furthermore, strains which were unable to intimately adhere but produced BFP demonstrated comparable binding to the wildtype strain when incubated on Caco-2 cells (Cleary *et al.*, 2004). Additionally, the blockage of BFP with antiserum reduced EPEC binding to HeLa cells (Giron *et al.*, 1991). These cancer cell line-based studies imply an important role for BFP in epithelial binding. However, investigations with more complex model systems, such as *in vitro* organ culture (IVOC) of human

duodenal biopsies, are less conclusive, as an EPEC strain cured of the EAF plasmid was unable to adhere to the intestinal epithelium, whereas an isogenic Δ*bfp* strain demonstrated comparable binding to the wildtype when incubated on duodenal biopsies (Hicks *et al.*, 1998; Knutton *et al.*, 1987). These findings suggested that while the EAF plasmid is required for epithelial colonisation, adhesion was not mediated by BFP. In human volunteer studies, the absence of either the EAF plasmid or the *bfp* gene reduced the incidence of diarrhoea, indicating that while BFP is not essential for infection, this adhesin is associated with the onset of diarrhoea *in vivo* (Bieber *et al.*, 1998; Levine *et al.*, 1985).

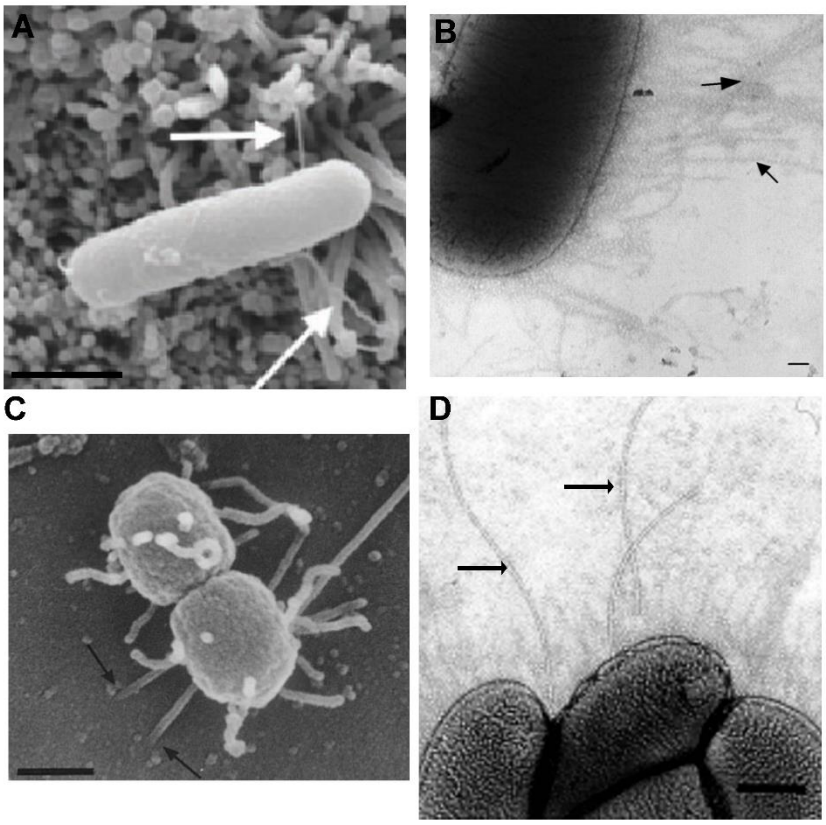

Figure 1.2: EPEC adhesins which facilitate initial contact with the host epithelium

Electron micrographs of EPEC BFP (A), ECP (B), EspA (C), and flagella (D). Arrows indicate the appendage of interest. Scale bars: 1 µm (A), 0.1 µm (B), 0.25 µm (C), and 0.7 µm (D). Images adapted from Cleary *et al.* (2004) (A), Saldana *et al.* (2009) (B), Knutton *et al.* (1998) (C), and Girón *et al.* (2002) (D).

In addition to BFP, the *E. coli* common pilus (ECP) has also been implicated in EPEC adhesion to host cells (Figure 1.2B). The ECP, which is shared amongst both pathogenic and commensal *E. coli* strains, can mediate epithelial binding, as the expression of *ecp* in the non-adherent *E. coli* strain HB101 demonstrated comparable adhesion to a HB101 strain expressing *bfp,* when incubated on HeLa and HT-29 cells (Rendón *et al.*, 2007; Saldana *et al.*, 2009). Furthermore, while the isogenic *ecp* mutant showed comparable adhesion to wildtype EPEC, the loss of both *ecp* and *espA* significantly reduced EPEC binding relative to the single gene mutant strains, suggesting an accessory role in mediating EPEC attachment to the epithelium (Saldana *et al.*, 2009). In addition to BFP and ECP, the presence of long polar fimbriae (LPF) on EPEC has been identified as a potential adhesin however, the role of these fimbriae during EPEC infection is poorly understood, with the current knowledge suggesting that LPF are not involved in EPEC binding to the intestinal epithelium (Tatsuno *et al.*, 2006). A recent study found that the production of LPF may be suppressed in EPEC, as inactivation of Histone-like nucleoid structuring (H-NS), which represses LPF expression, enhanced EPEC binding to Caco-2 cells, but did not significantly increase colonisation of murine small intestinal epithelium (Hu *et al.*, 2015). However, H-NS is a global regulator of the EPEC genome, and as such this protein may modify the expression of other adhesins which could improve EPEC binding to epithelial cells (Atlung & Ingmer, 1997; Hu *et al.*, 2015).

The translocon of the T3SS, comprised of *E. coli* secreted protein A (EspA) monomers, has also been implicated in the initial attachment of EPEC to the intestinal epithelium (Figure 1.2C) (Cleary *et al.*, 2004; Knutton *et al.*, 1998). However, the interaction of EspA with the host cell is relatively weak, as vigorous washing displaced EPEC isogenic mutant strains which lacked BFP and intimin however, the use of a gentler washing technique increased bacterial adhesion (Cleary *et al.*, 2004). These findings suggest that while the EspA translocon assists in establishing initial contact with the epithelium, this role would appear to be supportive rather than a dominant effect.

While flagella have primarily been investigated for their role in motility, this appendage can also adhere to the host epithelium, as the loss of the flagellum

reduced EPEC binding to HeLa and T84 cells (Figure 1.2D) (Girón *et al.*, 2002; Sampaio *et al.*, 2009). However, EPEC strains which were flagellated but did not produce BFP and EspA were non-adherent to Caco-2 cells, indicating that the flagellum is unable to compensate for the loss of other adhesins (Cleary *et al.*, 2004). A potential target for flagella are mucins, glycoproteins which are produced by the intestinal epithelium, as EPEC flagella adhere to bovine intestinal mucus (Erdem *et al.*, 2007). Interestingly, flagella from the probiotic *E. coli* Nissle 1917 interact with the mucus component gluconate, and this interaction mediated persistence in the host mucus (Troge *et al.*, 2012).

The cell surface protein lymphocyte inhibitory factor (LifA) is the largest known effector protein to be produced by A/E *E. coli*, with a predicted molecular mass of 366 kDa, and is thought to have a role in bacterial adhesion to the host (Deng *et al.*, 2012; Klapproth *et al.*, 2000). A similar gene has been identified in the A/E pathogens *C. rodentium* (*lifA*) and EHEC (*efa1*) where it is essential for the onset of disease in both murine and bovine models, respectively (Klapproth *et al.*, 2005; Stevens *et al.*, 2002). Interestingly, an EPEC *lifA* mutant strain demonstrated both localised adherence as well as the formation of A/E lesions when incubated with HeLa cells, implying that this protein is not essential for EPEC binding (Klapproth *et al.*, 2000). However, LifA may play an accessory role in EPEC adherence to epithelial cells, as the loss of both *lifA* and *bfp* significantly reduced adhesion relative to an EPEC strain deficient in *bfp* (Badea *et al.*, 2003).

While numerous EPEC appendages have been associated with facilitating the initial contact between EPEC and the host epithelium, the principle factor which is responsible for initial binding appears to be the BFP. However, the presence of additional adhesins supports the formation of these initial interactions and thus enhances EPEC binding to IECs. Further research is required to determine how different adhesins interact with the host, which could aid in the development of new vaccines against EPEC infection.

1.1.2.2. Formation of the A/E lesion

1.1.2.2.1. Intimate attachment

After establishing initial binding to the host cell, EPEC firmly attaches to the intestinal epithelium by forming an A/E lesion, characterised by intimate attachment, microvillous effacement, and actin pedestal formation. The development of the A/E lesion is mediated through the T3SS, which is encoded by the locus of enterocyte effacement (LEE) pathogenicity island (PAI), as the transfer of the LEE into non-pathogenic *E. coli* K12 was sufficient to enable A/E lesion formation on Caco-2 cells (McDaniel & Kaper, 1997; Pallen *et al.*, 2005). Interestingly, the LEE PAI appears to have been acquired via horizontal gene transfer, as this region has a GC content of 39%, which is substantially lower than the 51% GC content of the total *E. coli* genome (McDaniel *et al.*, 1995).

The T3SS is an approximately 3.5 MDa complex which is produced by a number of Gram-negative pathogens, including *Salmonella, Yersinia*, and EPEC (Puhar & Sansonetti, 2014). This structure spans the inner and outer bacterial membranes and facilitates the transfer of effector proteins from the bacterium into the host cell (Puhar & Sansonetti, 2014). The T3SS is composed of approximately 25 proteins, which include a cytoplasmic ATPase (EscN), a periplasmic needle-like structure (EscQ, EscJ, EscD, EscC, and EscF), a hollow filament which extrudes away from the bacterial cell (composed of EspA monomers), and a pore-forming domain at the tip of the filament (EspB and EspD) (Figure 1.3) (Pallen *et al.*, 2005; Puhar & Sansonetti, 2014). After pore-formation in the cell membrane, bacterial effector proteins are translocated from the bacterium to the host, at a rate of up to 60 molecules per second (Puhar & Sansonetti, 2014).

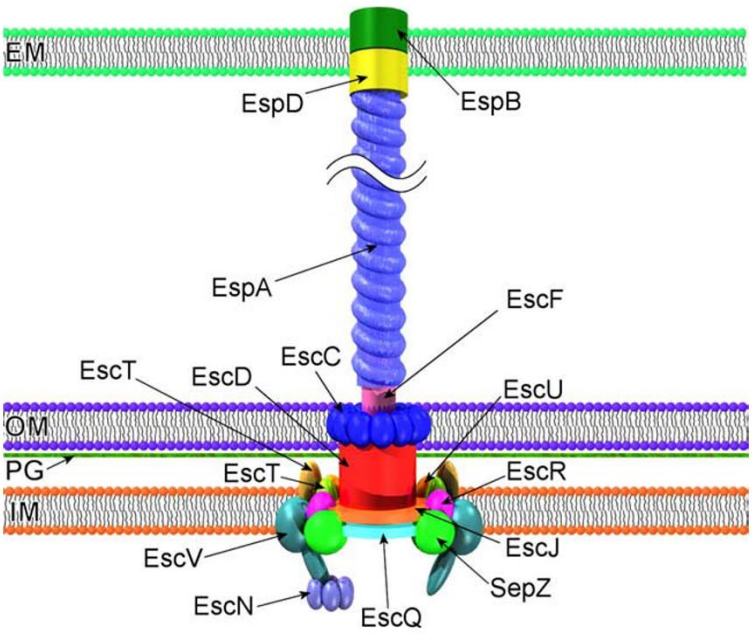

Figure 1.3: The structure of the EPEC T3SS

Taken from Pallen *et al.* (2005). IM, inner membrane; PG, peptidoglycan layer; OM, outer membrane; EM, eukaryotic membrane.

One of the first effector proteins transferred into the cell is Tir, which inserts into the host cell membrane and binds to intimin, encoded by *eaeA*, located on the bacterial cell surface (Deibel *et al.*, 1998; Donnenberg & Kaper, 1991; Kenny *et al.*, 1997; Rosenshine *et al.*, 1992; Rosenshine *et al.*, 1996). The interaction between intimin and Tir is essential for the intimate attachment of EPEC to the host cell, bringing EPEC to within 10 nm of the epithelial surface (Frankel *et al.*, 2001; Kenny *et al.*, 1997; Knutton *et al.*, 1987).

In addition to the transfer of Tir into the host cell, the EPEC T3SS delivers further effector proteins into the cell which manipulate a multitude of host processes (Dean & Kenny, 2009). These virulence factors are divided into LEE-encoded (7 confirmed, including Tir) and non-LEE-encoded (Nle) (at least 20 identified) effector proteins (Deng *et al.*, 2012). A core repertoire of 21 effector proteins, which include the 7 LEE proteins, are shared amongst all A/E pathogens and contribute to the formation of the A/E lesion, as well as having other roles in the subversion of host cell processes (effector proteins

with known functions in EPEC are shown in Table 1.1) (Wong *et al.*, 2011). Variation in the number of Nle effector proteins amongst different isolates is common, which may be a reflection of different infection strategies (Wong *et al.*, 2011).

Table 1.1: EPEC effector proteins with known functions

Effector	Function	References
LEE-encoded		
EspB	Translocation pore component, disrupt phagocytosis, microvilli effacement, inhibits myosin/actin interaction	Donnenberg *et al.* (1993a); Iizumi *et al.* (2007)
EspF	Disrupts TJ activity, inactivates SGLT-1, inactivates NHE3, disrupt aquaporins, disrupt mitochondria function, promote apoptosis, inhibit phagocytosis	Alto *et al.* (2007); Dean *et al.* (2006);; Guttman *et al.* (2007); Hodges *et al.* (2008); McNamara *et al.* (2001); Muza-Moons *et al.* (2004); Nougayrede and Donnenberg (2004); Quitard *et al.* (2006); Viswanathan *et al.* (2009)
EspG	Disrupt microtubules, disrupt DRA, disrupt aquaporins	Gill *et al.* (2007); Guttman *et al.* (2007); Tomson *et al.* (2005)
EspH	Pedestal formation, disrupt phagocytosis, promote apoptosis	Dong *et al.* (2010); Tu *et al.* (2003); Wong *et al.* (2012)
EspZ	Inhibit apoptosis, stabilise mitochondria, inhibit over-secretion of effector proteins	Berger *et al.* (2012); Roxas *et al.* (2012); Roxas *et al.* (2014); Shames *et al.* (2010); Shames *et al.* (2011)
Map	Inhibits pedestal formation, disrupt mitochondria, inactivate SGLT-1, disrupt TJ activity	Dean *et al.* (2006); Kenny *et al.* (2002); Ma *et al.* (2006); Nguyen *et al.* (2015); Thanabalasuriar *et al.* (2010)
Tir	Intimate attachment, actin pedestal formation, inhibit filopodia formation, disrupt SGLT-1, inhibit NF-κB pathway	Dean *et al.* (2006); Gruenheid *et al.* (2001); Kenny *et al.* (1997); Kenny (1999); Kenny *et al.* (2002); Wong *et al.* (2012); Ruchaud-Sparagano *et al.* (2011); Yan *et al.* (2012)
Non-LEE-encoded		
EspJ	Inhibits phagocytosis, inhibit actin pedestal	Marchès *et al.* (2008); (Young *et al.*, 2014)
NleA/ EspI	Disrupt TJ, inhibit NOD-like signalling	Thanabalasuriar *et al.* (2010); Yen *et al.* (2015)
NleB	Inhibit NF-κB pathway, inhibit apoptosis signalling	Gao *et al.* (2013); Li *et al.* (2013); Lung *et al.* (2016); Newton *et al.* (2010); Pearson *et al.* (2013)
NleC	Inhibit NF-κB pathway, inhibit MAPK pathway	Baruch *et al.* (2011); Mühlen *et al.* (2011); Pearson *et al.* (2011); Yen *et al.* (2010)
NleD	Inhibit MAPK pathway, inhibit apoptosis signalling	Baruch *et al.* (2011); Marchés *et al.* (2005)
NleE	Inhibit NF-κB pathway	Nadler *et al.* (2010); Newton *et al.* (2010); Vossenkämper *et al.* (2010)
NleF	Activates NF-κB pathway, inhibit apoptosis signalling	Blasche *et al.* (2013); Pallett *et al.* (2014)
NleH1	Inhibit NF-κB pathway, inhibit apoptosis signalling	Gao *et al.* (2009); Hemrajani *et al.* (2010); Pham *et al.* (2012)
NleH2	Activate NF-κB pathway, activate MAPK pathway, inhibit apoptosis signalling	Gao *et al.* (2009); Hemrajani *et al.* (2010); Pham *et al.* (2012)

1.1.2.2.2. Effacement of microvilli

The effacement of microvilli is a defining characteristic of the A/E lesion (an absence of microvilli underneath EPEC is shown in Figure 1.4A). Microvillous effacement is thought to contribute to the production of diarrhoea by reducing the absorptive surface available for the uptake of water and nutrients by the host (Croxen *et al.*, 2013). Microvillous effacement during EPEC infection of the small intestinal epithelium is dependent on a functional T3SS, which suggests that this process is mediated by secreted effector proteins (Shaw *et al.*, 2005). The effector protein Tir has been implicated in this process on Caco-2 cells however, Tir is not essential for the effacement of microvilli on duodenal biopsy tissue, demonstrating conflicting data dependent on the model system used (Dean *et al.*, 2006; Dean *et al.*, 2013; Shaw *et al.*, 2005). It is important to note that *tir*-deficient EPEC demonstrate reduced adhesion to the duodenal epithelium, which may inhibit Tir-associated microvillous effacement (Schuller *et al.*, 2007; Shaw *et al.*, 2005).

The effacement of microvilli has been attributed to collaborative action of the effectors EspF, Map, and Tir on Caco-2 cells, implying that the effectors involved may demonstrate functional redundancy (Dean *et al.*, 2006). Whether these proteins work in concert to efface microvilli at the duodenal epithelium remains to be determined.

1.1.2.2.3. Formation of the actin pedestal

The actin pedestal is a hallmark characteristic of A/E *E. coli* infection of epithelial cells, which has been demonstrated on both *in vitro* and *ex vivo* intestinal epithelial models (examples of *ex vivo* pedestals shown in Figure 1.4) (Knutton *et al.*, 1989). These pedestals were first identified by Knutton and colleagues, who noted that EPEC which were intimately attached to the epithelium were surrounded by “cuplike projections” and later identified that these projections contained high concentrations of filamentous actin (Knutton *et al.*, 1989; Knutton *et al.*, 1987).

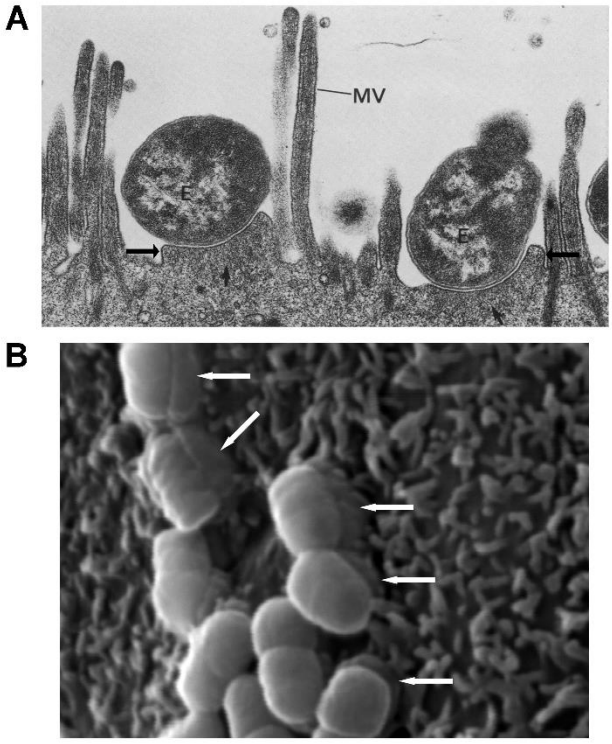

Figure 1.4: Actin pedestal formation on EPEC-infected duodenal tissue

Transmission (A) and scanning (B) electron micrographs of EPEC mounted on actin pedestals (indicated by arrows). MV = microvilli. Images were taken from Knutton *et al.* (1987) (A) and this study (B).

The formation of the actin pedestal involves substantial cytoskeletal reorganisation, as filamentous actin is recruited from the host cell cytoskeleton and transferred to the apical surface, underneath the EPEC bacterium (Knutton *et al.*, 1989). This process, which was initially documented using cancer cell lines, is instigated through the binding of intimin to Tir which causes the cytoplasmic C-terminal domain of Tir to cluster together (Campellone *et al.*, 2004a). The phosphorylation of the tyrosine 474 (Y474) residue on Tir by host cell kinases forms a binding site for the host cell adaptor protein Nck, which subsequently recruits the host protein neural Wiskott-Aldrich syndrome protein (N-WASP) to the Tir complex (Bommarius *et al.*, 2007; Gruenheid *et*

al., 2001; Phillips *et al.*, 2004; Swimm *et al.*, 2004). N-WASP activates the actin-related protein 2/3 (ARP 2/3) complex which initiates the nucleation of actin filaments underneath EPEC (Gruenheid *et al.*, 2001; Kalman *et al.*, 1999).

Interestingly, in the absence of Y474, EPEC infection still results in weak actin recruitment. This is mediated by phosphorylation of the tyrosine 454 (Y454) residue on Tir and is independent of Nck (Campellone & Leong, 2005). While these processes have been documented *in vitro*, studies using tissue explants have demonstrated that Tir was essential for epithelial colonisation, but phosphorylation of the Y454 and Y474 domains was not required for N-WASP recruitment and formation of actin pedestals, suggesting Tir-independent actin recruitment *ex vivo* (Schuller *et al.*, 2007). Furthermore, an atypical EPEC O125:H6 isolate lacking the Y474 domain on Tir demonstrated intimate adherence and microvilli effacement, despite a lack of actin pedestal formation (Bai *et al.*, 2008). This suggests an uncoupling of intimate attachment/ microvillous effacement from actin pedestal formation (Frankel & Phillips, 2008).

While Tir is the only EPEC effector protein required to form the actin pedestal *in vitro*, other effectors modulate this process. The LEE-encoded EspH protein influences actin pedestal length by promoting the recruitment of N-WASP to the site of EPEC infection (Tu *et al.*, 2003; Wong *et al.*, 2012). In contrast to EspH, effector proteins Mitochondrial associated protein (Map) and EspJ inhibit the formation of the actin pedestal. EspJ reduces actin polymerisation by inhibiting host kinases (Young *et al.*, 2014). This effector protein could prevent the formation of actin pedestals by secondary bacteria, promoting the translocation of non-adhered EPEC away from the site of infection (Marchès *et al.*, 2008; Young *et al.*, 2014). The effector Map temporarily antagonises the formation of the actin pedestal on HeLa cells however, this process is subsequently inhibited by the phosphorylation of Tir (Kenny *et al.*, 2002). Whether Map interacts with the development of the actin pedestal when using a more physiologically relevant model system remains to be investigated.

1.1.2.3. Additional mechanisms contributing to diarrhoea

1.1.2.3.1. Disruption of tight junctions

The intestinal epithelium is comprised of a single layer of enterocytes, which prevent access of intestinal contents into underlying tissue (Turner, 2009). These enterocytes are linked via tight junction (TJ) proteins, such as occludin and claudin, which regulate the access of water, ions, and nutrients into the host through paracellular channels, as well as inhibiting bacterial access to the underlying tissues (Turner, 2009). Upon infection with EPEC, permeability of the intestinal barrier increases, allowing the movement of ions and water into the lumen, instigating the production of diarrhoea (Viswanathan *et al.*, 2009).

One of the key effector proteins involved in the disruption of the TJ barrier is EspF, as the loss of this effector significantly reduced the change in transepithelial electrical resistance (TER) (a measure of the epithelial barrier integrity) of EPEC-infected T84 cells (McNamara *et al.*, 2001; Muza-Moons *et al.*, 2004; Thanabalasuriar *et al.*, 2010). TJ integrity was reduced through the inhibition of TJ protein (zonula occludens-1 (ZO-1), occludin, and claudin-1) recycling from the cytosol to the TJ, weakening the epithelial barrier (McNamara *et al.*, 2001; Muza-Moons *et al.*, 2004; Thanabalasuriar *et al.*, 2010). In addition to *in vitro* studies, the infection of mice with wildtype EPEC induced occludin relocalisation and reduced TER in the terminal ileum, whereas infection with EPEC deficient in *espF* demonstrated no effect on either of these parameters, despite comparable levels of intestinal colonisation (Shifflett *et al.*, 2005). Similarly, claudin relocalisation has been observed in mice infected with wildtype *C. rodentium,* whereas an *espF*-deficient mutant did not induce TJ protein rearrangement (Guttman *et al.*, 2006). Furthermore, the water content of faeces in the mouse colon was reduced in mice infected with the *espF* mutant relative to those infected with the wildtype, further strengthening the link between EspF-induced changes in epithelial barrier function and water secretion (Guttman *et al.*, 2006).

Other effector proteins have been associated with an increased epithelial permeability such as Map, which induced a comparable decrease in TER as

EspF when incubated on polarised Caco-2 cells (Dean & Kenny, 2004). However, these effects are disputed *in vivo,* as TJ localisation was near identical between mice infected with *map*-deficient *C. rodentium* mutant and those infected with wildtype pathogen (Guttman *et al.*, 2006). TJ protein recycling between the cytosol and the lateral surface is an important procedure in the maintenance of epithelial barrier function and thus, this process represents a key target to induce epithelial permeability (Glotfelty *et al.*, 2014a). The effector protein NleA inhibits cell protein trafficking in the host cell and as a consequence, disrupts the exchange of TJ proteins at the epithelium with those in the cytosol of the IEC (Thanabalasuriar *et al.*, 2010). A similar mechanism has been implicated for LEE-encoded EspG1 and its non-LEE-encoded homologue EspG2, which fragment microtubules in the host cell via the degradation of α-tubulin (Tomson *et al.*, 2005). The role of EspG1/2 in TJ disruption appears to be of less importance than EspF, as the deletion of EspG1/2 delayed, but did not prevent, the decrease in TER induced by EPEC infection of polarised IECs (Glotfelty *et al.*, 2014b; Matsuzawa *et al.*, 2005; Tomson *et al.*, 2005). These findings suggest that Map, NleA, and EspG1/2 may have an accessory role in altering the permeability of the epithelial barrier.

1.1.2.3.2. Ion transporter and aquaporin dysfunction

While TJ proteins control molecular access through the paracellular pathway, transporters regulate the movement of ions and nutrients through the transcellular pathway. Ion transporters such as Downregulated in adenoma (DRA), Na+ /H+ -exchange protein 3 (NHE3), and the sodium-glucose linked transporter-1 (SGLT-1) move ions from the lumen into the cell to maintain the uptake of water into the host (Viswanathan *et al.*, 2009).

DRA, an anion exchanger which removes Cl^- from the intestinal lumen by exchanging bicarbonate, has a decreased expression during EPEC infection, which has been associated with microtubule degrading effector proteins EspG1/2 (Gill *et al.*, 2007). These effectors decrease DRA levels via the simultaneous increase in DRA endocytosis from the epithelial surface

alongside the inhibition of DRA exocytosis, through the manipulation of the microtubule network (Gill *et al.*, 2007; Gujral *et al.*, 2015). Other T3S effector proteins inhibit ion transporters such as EspF, which reduced NHE3 levels in intestinal Caco-2 and PS120 fibroblast cells (Hecht *et al.*, 2004; Hodges *et al.*, 2008). Similarly, EspF, Map, and Tir work in unison to partially inactivate the SGLT-1 transporter of polarised Caco-2 cells (Dean *et al.*, 2006). As mice infected with a *C. rodentium* isogenic *espF* mutant demonstrated decreased luminal water content, this supports an *in vivo* role for EspF in ion transporter dysfunction, although it is important to note that this effect may be associated with TJ disruption (Guttman *et al.*, 2006).

In addition to the disruption of ion transporters, *C. rodentium* effectors EspF and EspG have a partial role in removing aquaporins, transporters which rapidly move water across the apical membrane (Guttman *et al.*, 2007). However, deletion of both *espF* and *espG* was not sufficient in preventing aquaporin redistribution, implying that other effector proteins may be involved, as only the infection of mice with an T3SS-deficient mutant demonstrated comparable aquaporin distribution to the non-treated controls (Guttman *et al.*, 2007). The mechanism remains to be defined however, the previously discussed roles of EspF and EspG on actin and microtubule networks, respectively, may provide a clue to the mechanisms responsible.

1.1.2.4. Clinical significance: symptoms, diagnosis, and treatment

EPEC infection is characterised by the rapid onset of acute watery diarrhoea. In addition, abdominal cramps, nausea, vomiting, and fever have been associated with EPEC infection (Donnenberg *et al.*, 1993b; Levine & Edelman, 1984; Nataro & Kaper, 1998).

As the classification of EPEC has become more complex, detection methods have adapted to distinguish between individual strains. While older diagnostic methods focused on phenotypic features of EPEC, such as O and H serotypes (which would exclude approximately a quarter of all aEPEC strains), more

modern techniques have focussed on histopathological characteristics, such as actin pedestal formation, as well as distinct genetic sequences (Croxen *et al.*, 2013). In laboratories with cell culture facilities, actin pedestals can be identified on EPEC-infected HeLa cells using the fluorescent actin stain (FAS) test, which utilises fluorescein isothiocyanate (FITC) conjugated to the phalloidin toxin to target filamentous actin (Knutton *et al.*, 1989). While the FAS test distinguishes between *E. coli* pathotypes, as only EPEC and EHEC form actin pedestals, genetic analysis is required for further identification, utilising either DNA probes or PCR screens to target *eaeA*, *bfp*, and *stx*, which encodes Shiga toxin (Croxen *et al.*, 2013). While molecular analysis of genetic sequences is the current standard for the classification of EPEC strains, the reliance on less accurate diagnostic methods in resource-poor environments, such as serotyping, may underestimate the prevalence of aEPEC in patients with diarrhoea.

As infections are generally self-limiting, current therapies are similar to most diarrhoeal treatments, with a focus on rehydration of the patient with oral rehydration therapy (Croxen *et al.*, 2013). However, oral rehydration therapy can fail in patients with severe EPEC diarrhoea (possibly due to dysfunction of the SGLT-1 transporter) and thus antibiotic treatment is occasionally required (Croxen *et al.*, 2013; Dean *et al.*, 2006). As with other pathogens, antibiotic resistance is increasing with recent clinical isolates demonstrating resistance to multiple antibiotics, particularly penicillins, quinolones, and cephalosporins (Canizalez-Roman *et al.*, 2013; Garcia *et al.*, 2011; Malvi *et al.*, 2015; Nakhjavani *et al.*, 2013; Sang *et al.*, 2012; Scaletsky *et al.*, 2010). The development of a vaccine against EPEC infection remains an area of particular interest, as none are currently available (Croxen *et al.*, 2013). Interestingly, IgA antibodies against the EPEC cell-associated proteins EspA, intimin, EspB, and BFP have been identified in the breast milk of Mexican mothers, which may represent potential targets for vaccine development (Parissi-Crivelli *et al.*, 2000).

An alternative solution could be the use of probiotics; a meta-analysis of 63 studies demonstrated that probiotics can reduce acute infectious diarrhoea (Allen *et al.*, 2010). However, these findings are limited by variable treatment

regimens and the range of probiotic species investigated thus, further study is required on specific strains to determine the mechanisms behind these anti-diarrhoeal effects (Allen *et al.*, 2010).

1.2. Probiotic *Lactobacillus*

1.2.1. The history of probiotics

The concept of probiotic bacteria was originally established by the Russian scientist Élie Metchnikoff at the start of the last century, who believed that the long life of Bulgarian peasants was associated with the consumption of fermented milk products, which contained lactic acid bacteria (Brown & Valiere, 2004; Holzapfel & Schillinger, 2002; Metchnikoff, 1907). Metchnikoff noted, “The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes”, suggesting that certain bacteria could deliver health benefits to the host (FAO/WHO, 2001; Metchnikoff, 1907). While these findings received some initial attention, the probiotic concept remained unproven and interest diminished during the following decades until the 1970’s (FAO/WHO, 2001). The term “probiotic” (meaning: for life) was conceived approximately 50 years after Metchnikoff’s observations, originally in reference to products which aid the gut microbiota, a contrast to the detrimental effects of antibiotics on microbes (Hamilton-Miller *et al.*, 2003; Holzapfel & Schillinger, 2002; Kollath, 1953; Vergin, 1954). The word “probiotic” was then adapted by Lilly and Stillwell (1965) to refer to microbial products which stimulated the growth of other microbes (Holzapfel & Schillinger, 2002).

Parker (1974) further revised the term “probiotic” to a definition similar to the modern usage, interpreting probiotics as ‘organisms and substances which contribute to intestinal microbial balance’ (Hamilton-Miller *et al.*, 2003). Probiotics were further redefined by Fuller (1989) as, “A live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance”, which referred specifically to live microbes, now an

essential component for the consideration of a product as a probiotic (FAO/WHO, 2001).

The most recent and widely used definition for probiotics was conceived by Guarner and Schaafsma (1998) and selected by the Food and Agriculture Organisation (FAO) and the World Health Organisation (WHO) expert Consultation on Health and Nutritional properties of powder milk with live lactic acid bacteria in 2001 as an appropriate definition (FAO/WHO, 2001). This definition was recently grammatically updated (Hill *et al.*, 2014) and its most current form is as follows:

"Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host".

This definition encompasses the three core components required for a product to be characterised as a probiotic, namely that bacteria in the product must be viable, that any health effect is associated with the minimum required dose, and that the minimum dose is present whenever the product is administered i.e. at the end of the products shelf life (Hill *et al.*, 2014).

Probiotic strains are generally derived from either the *Lactobacillus* or *Bifidobacterium* genera, although others such as *E. coli* Nissle 1917 and *Streptococcus salivarius ssp. thermophilus* also demonstrate probiotic characteristics (Holzapfel *et al.*, 1998). As *Lactobacillus* species are naturally present in the GIT and contribute to the maintenance of the intestinal microbiota, these bacteria are an obvious choice in the investigation for probiotics with novel characteristics (Holzapfel & Schillinger, 2002). However, before discussing probiotic effects which have been associated with specific *Lactobacillus* strains, it is important to first understand the role and function of the commensal gut microbiota.

1.2.2. The microbiota of the gastrointestinal tract

The microbiota has previously been referred to as a “virtual organ” due to its substantial metabolic activity, which plays an essential role in the normal functioning of the host (O'Hara & Shanahan, 2006). In physical terms, microbial cells far outnumber the cells of the host, with an approximate 10-fold difference between the two (O'Hara & Shanahan, 2006; Shanahan, 2002). However, at the genetic level this difference is even larger, with the combined microbial genome being over 100-fold greater than the human genome (O'Hara & Shanahan, 2006; Shanahan, 2002). The majority of these microbes are located in the GIT and are comprised of 92.9% bacteria, 5.8% viruses, and the remaining 1.3% consisting of archaea and eukaryotes (Arumugam *et al.*, 2011; Turnbaugh *et al.*, 2007). The bacterial load changes dependent on the location within the GIT, as the stomach and duodenum contain between 10^1 and 10^3 colony forming units (CFU)/ mL which increases along the intestine to between 10^{11} and 10^{12} CFU/ mL located in the adult colon (Figure 1.5) (O'Hara & Shanahan, 2006; Suau *et al.*, 1999).

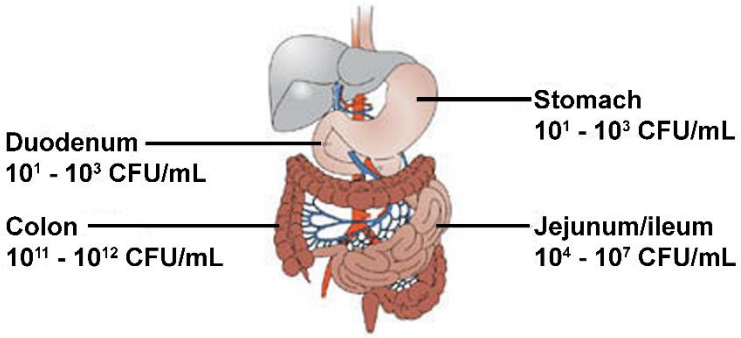

Figure 1.5: Bacterial concentration within the human GIT

The bacterial load of the GIT increases in density from the stomach and duodenum to the distal colon. Image taken from O'Hara and Shanahan (2006).

The microbiota of the GIT is unique to an individual however, species from the *Bacteroidetes* and *Firmicutes* phyla are generally predominant (Ottman *et al.*, 2012). The composition of the microbiota is influenced by numerous factors throughout the life of the host organism, including the delivery method of the neonate, diet, use of antimicrobial products, and host genetics. The general consensus on the development of the microbiota is that the infant gut *in utero* is sterile and upon birth, the neonatal GIT is colonised by pioneer species which are primarily delivered from the mother, principally through the birthing process, breast feeding, and physical contact (Palmer *et al.*, 2007). Children delivered via Caesarean section have greater numbers of *C. difficile* and *E. coli,* whereas neonates delivered via vaginal birth have a greater number of *Bacteroides fragilis* (Penders *et al.*, 2006). Similar increases in *C. difficile* and *E. coli* have also been observed in infants who are exclusively formula-fed rather than breast-fed (Penders *et al.*, 2006). As the infant continues onto solid food and grows into a toddler, the microbiota continues to develop, reaching peak complexity during adulthood wherein the microbial community stabilises until old age and diversity begins to decrease (Figure 1.6) (Ottman *et al.*, 2012; Palmer *et al.*, 2007).

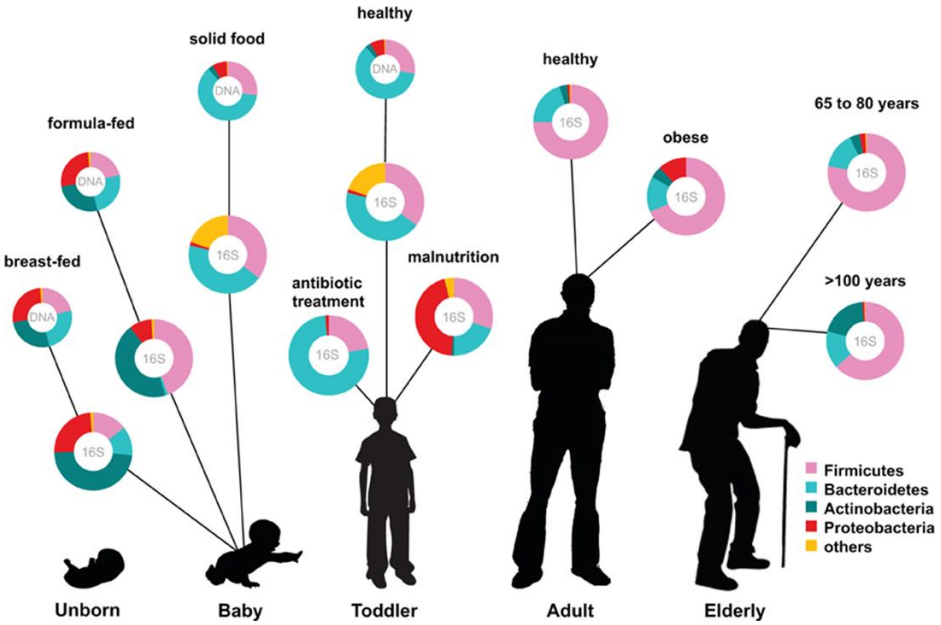

Figure 1.6: The composition of the gut microbiota develops through age and external factors

Image taken from Ottman *et al.* (2012).

The constituent members of the intestinal microbiota are unique to each individual, but key metabolic pathways are almost universally present amongst examined microbiomes, such as carbohydrate metabolism and vitamin biosynthesis (Human Microbiome Project Consortium, 2012). This demonstrates a functional redundancy amongst commensal bacterial species, where numerous bacteria possess the genes required to synthesise a particular product, for example riboflavin, and any of these bacterial species can inhabit this particular niche (Human Microbiome Project Consortium, 2012). One of the essential functions that the gut microbiota performs is the synthesis of vitamins, micronutrients used in the production of essential enzymes, as the human host lacks the biosynthetic pathways required for these processes (LeBlanc *et al.*, 2013). In addition to vitamin synthesis, the microbiota also aids in the uptake of energy by the host, as germ-free mice have a 60% increase in body fat after inoculation with a “conventional” microbiota, even if the overall calorie intake is reduced (Bäckhed *et al.*, 2004).

Alongside occupying metabolic niches, the microbiota also has an important role in the physical development of the intestinal tract, as the morphology of the intestine differs between germ-free and conventionally raised mice (Sommer & Bäckhed, 2013). Histological analysis of the GIT of germ-free mice has revealed a thinner mucus layer, thinner villi, and smaller, immature immune features such as Peyer's patches (Sommer & Bäckhed, 2013). This demonstrates that the microbiota interacts with the intestinal mucosa to develop both the physiological and immunological processes of the host. Furthermore, while the immune response can clear potential insults, the microbiota also acts as a barrier to infection, known as colonisation resistance, which can prevent the access of GI pathogens such as *Salmonella enterica* serovar Typhimurium to the epithelium (Stecher *et al.*, 2007).

1.2.2.1. Dysbiosis of the intestinal microbiota

In most individuals, the microbiota supports the host through the functions previously discussed in 1.2.2. However, dysfunction in the microbiota has been associated with the development of numerous pathologies. Dysbiosis, defined as a change in the "balance" of microbes in the microbiota, can be induced through numerous stimuli including antibiotic use, diet, hygiene, and host immune function (Figure 1.7) (Sommer & Bäckhed, 2013; Tamboli *et al.*, 2004). This alteration in the intestinal microbiota has been associated with diseases such as inflammatory bowel disease (IBD), obesity, and antibiotic-associated diarrhoea (AAD), which I will briefly discuss here.

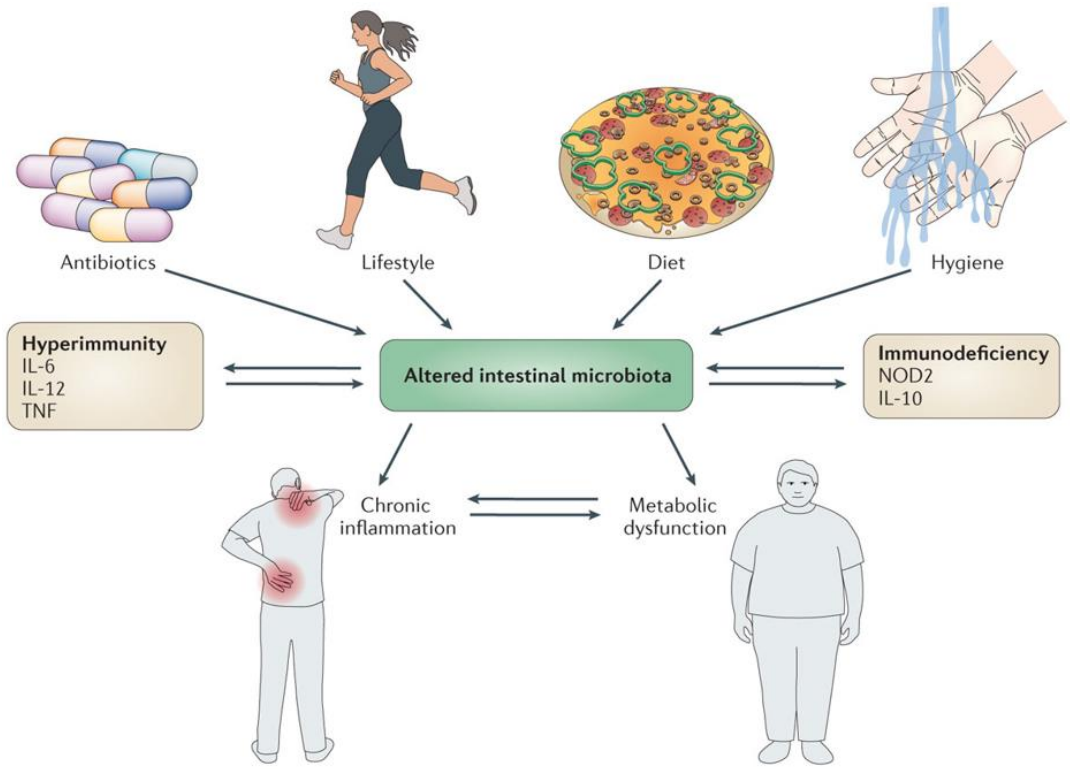

Figure 1.7: Factors associated with dysbiosis of the intestinal microbiota

Image taken from Sommer and Bäckhed (2013).

IBD is a term that primarily refers to ulcerative colitis (UC) and Crohn's disease (CD), two conditions which are characterised by severe abdominal pain, vomiting, bloody diarrhoea, and weight loss which drastically decrease the quality of life of affected patients (Pizzi *et al.*, 2006). The exact causes behind IBD are presently unknown, with the current consensus suggesting that both CD and UC have multiple triggers, including those shown in Figure 1.7 (for a more thorough analysis of these factors, see Bernstein and Shanahan (2008)). Of particular interest is the role of the microbiota, as diversity is decreased in patients with CD and UC relative to patients without IBD (Ott *et al.*, 2004; Walker *et al.*, 2011). The underlying reason for this decrease is unknown, although the use of antibiotics in early childhood has been correlated with an increased risk of developing IBD (Hviid *et al.*, 2011; Kronman *et al.*, 2012; Shaw *et al.*, 2010, 2011). Additionally, IBD has been linked to decreased levels of *Akkermansia muciniphila* and increased numbers of *Ruminococcus gnavus*

and *Ruminococcus torques* (Belzer & de Vos, 2012; Png *et al.*, 2010). Crucially, while changes in the levels of a particular microbial species has been associated with IBD, it is important to note that no single microbe has been definitively implicated in the development of these diseases. Thus, it remains to be determined whether the decrease in the diversity of the microbiota is a cause or symptom of IBD (Joossens *et al.*, 2011; Manichanh *et al.*, 2012; Sokol *et al.*, 2006; Walker *et al.*, 2011; Willing *et al.*, 2009).

Obesity is a global health crisis, as more than 1.9 billion adults were reported as overweight in 2014, with 600 million of those classified as obese, representing 39% and 13% of the world's adult population, respectively (WHO, 2015). Similar to IBD, obesity is a multifactorial disease based on lifestyle, environmental, and genetic influences (Figure 1.7). One component which is thought to contribute to the development of obesity is the gut microbiota, due to the role these bacteria play in the digestion of food and the acquisition of energy. The role of the microbiota in obesity was first demonstrated by Turnbaugh *et al.* (2006), who demonstrated that transfer of the microbiota from obese mice into germ-free mice significantly increased body fat relative to control mice with a "healthy" microbiota after 2 weeks. Interestingly, this change in body fat was not due to an increased calorific intake, but rather an increased energy harvest (Turnbaugh *et al.*, 2006). Similar findings have also been reported in germ-free mice which were inoculated with the faecal microbiota of twins who were discordant for obesity, as mice with the obese microflora showed higher adiposity than those with the lean microbiota (Ridaura *et al.*, 2013). A reported difference between the healthy and obese microbiota is the level of diversity, as diet-induced obese mice, as well as obese humans, have a distinct decrease in microbial diversity (Turnbaugh *et al.*, 2008; Turnbaugh *et al.*, 2009). This reduction in humans is associated with lifestyle factors, as diets have changed to more energy dense foods, the microbiota has adapted to utilise this increased energy resource. Obesity and associated metabolic disorders, such as type II diabetes, will likely be a key health challenge of the 21st century and as such, a thorough understanding of the role of the gut microbiota in obesity, and how we can modify it, may offer solutions to this epidemic.

While there are multiple factors contributing to the onset of obesity and IBD, the development of AAD has a more defined aetiology. AAD is an umbrella term for the development of diarrhoea in patients after the administration of antibiotics (Bartlett, 2002). However, I will focus on AAD associated with *C. difficile* infection, which occurs in 10-20% of cases of AAD (Bartlett, 2002). Severe infection by *C. difficile* is characterised by repeated episodes of diarrhoea, which can contain blood, as well as the formation of pseudomembranes on the colonic epithelium, which develop after the use of antibiotic therapy for an unrelated condition (Bartlett, 2002). The specific reason behind the onset of *C. difficile*-induced AAD is thought to be due to the unintentional removal of the microbiota by antibiotic treatment, which removes the natural colonisation resistance that the microbiota provides (Borriello & Barclay, 1986; Young & Schmidt, 2004). Disruption of the microbiota has been confirmed in patients with recurrent *C. difficile* diarrhoea, which demonstrated a reduction in microbial diversity compared to patients with either a single incidence of *C. difficile*-induced diarrhoea or those who were not infected (Chang *et al.*, 2008). The lack of bacterial competition allows *C. difficile*, which may naturally be present in the microflora, to establish a persistent infection and induce diarrhoea, despite further antibiotic therapy (Bartlett, 2002). Thus, restoration of the gut microbiota via faecal microbial transplant has been explored as a potential therapeutic avenue. Systematic reviews of studies reporting on the effectiveness of faecal microbial transplants on *C. difficile* infection have demonstrated that this therapy has considerable potential, with cure rates of approximately 90% in patients with recurrent infection (Gough *et al.*, 2011; Kassam *et al.*, 2013; Li *et al.*, 2016). Although both randomised controlled trials and long term safety studies are required to support the use of faecal microbial transplant, this treatment demonstrates the important role of the gut microbiota in reducing the impact of infection.

1.2.3. The *Lactobacillus* genus

The *Lactobacillus* genus consists of over 200 recorded species of lactobacilli (http://www.ncbi.nlm.nih.gov/taxonomy), isolated from a range of locations including faeces, breast milk, animals, and plants (Walter, 2008). Lactobacilli are Gram-positive, non-spore forming facultative anaerobes of the *Firmicute* phylum which produce lactic acid and have been traditionally used in the development of numerous products including yoghurt, cheese, and other fermented foods (Claesson *et al.*, 2007). In addition to their use in food fermentation, lactobacilli are also a normal resident of the human microbiota; with isolates identified in the oral cavity, the GIT, and the vagina of mammals, including humans (Walter, 2008). It is important to note that the overall numbers of lactobacilli in the distal GIT is low relative to other bacterial species, representing 0.01% of the total adult faecal microbiota (~10^6 CFU/ g) (Harmsen *et al.*, 2002). In the proximal GIT, the number of *Lactobacillus* species increases to approximately 1% of the total microbial population (Walter, 2008). In the adult stomach and small intestine however, the proportion of lactobacilli which are transient relative to autochthonous isolates remains to be determined (Walter, 2008). In contrast to findings in adults, lactobacilli were detected in 45% of infants at 10^8-10^9 CFU/ g of faeces over the first 6 months from birth (Ahrné *et al.*, 2005; Grönlund *et al.*, 2000). This difference in adult and infant microbial ecology is primarily due to differences in diet, as lactobacilli are acquired from the mother's breast milk and are amongst the pioneer colonisers of the infant GIT (Karlsson *et al.*, 2011; Martín *et al.*, 2003; Solís *et al.*, 2010). Additionally, *Lactobacillus* spp. from the mother's vagina have been shown to temporarily colonise approximately 1 in 4 infants after birth (Matsumiya *et al.*, 2002). These studies demonstrate that the mother is a reservoir of lactobacilli for infants and as such, the loss of exposure to these species, through both Caesarean section and formula-feeding, may have an impact on the development of the newborn microbiota (see section 1.1.2).

The role of *Lactobacillus* in the GIT is varied, due to the heterogeneous characteristics of this genus; however, certain strains have been identified with

probiotic features which can improve host intestinal function, such as aiding the resolution of diarrhoea.

1.2.4. Use of *Lactobacillus* in the treatment of diarrhoea

Numerous meta-analyses have investigated the impact of *Lactobacillus* spp. on diarrhoea, with positive outcomes reported in the prevention of AAD (21-64% reduction) (Kale-Pradhan *et al.*, 2010; McFarland, 2006, 2015; Pattani *et al.*, 2013; Szajewska *et al.*, 2006) as well as a reduction in the duration of acute infectious diarrhoea by between 16.8 – 26.4 hours (Allen *et al.*, 2010; Huang *et al.*, 2002; Szajewska *et al.*, 2013; Urbańska *et al.*, 2016; Van Niel *et al.*, 2002). It is important to note that while meta-analyses provide a powerful technique to evaluate the efficacy of a particular therapeutic regimen by pooling data from multiple studies, probiotic characteristics are strain-specific and thus, effects observed at the genus and species level may not be applicable to every strain of a particular species. Thus, while these meta-analyses are useful in the general assessment of probiotics on GI disorders, differences in the probiotic strains used as well as variation in study methodology (such as length of treatment, follow up period, and the selection of inpatients versus outpatients) should be appreciated when considering these findings. However, two systematic reviews of *L. rhamnosus* GG have shown that treatment with this strain reduces the incidence of AAD by 30% (McFarland, 2006; Szajewska *et al.*, 2006). Additionally, both *L. rhamnosus* GG and *L. reuteri* DSM 17938 decrease the duration of acute infectious diarrhoea by 26.4 hours and 24.82 hours, respectively (Szajewska *et al.*, 2013; Urbańska *et al.*, 2016). These studies demonstrate that specific *Lactobacillus* strains can improve diarrhoeal conditions. Potential mechanisms behind these probiotic characteristics will be discussed.

1.2.4.1. Mechanisms of probiotic action

In vitro studies investigating the probiotic effects of lactobacilli have aimed to identify the specific mechanisms behind probiotic action, which can be broadly classified as microbe-host and microbe-microbe interactions. Microbe-host mechanisms include modulation of the host immune response, epithelial barrier integrity, and mucin production (Mack *et al.*, 2003; Resta-Lenert & Barrett, 2003; Vizoso Pinto *et al.*, 2009). Microbe-microbe inhibitory actions include the production of antimicrobial compounds as well as competition for nutrients and binding sites (Lebeer *et al.*, 2008). These protective actions can be widespread amongst probiotic *Lactobacillus* or specific at either the species or strain level (Figure 1.8) (Hill *et al.*, 2014).

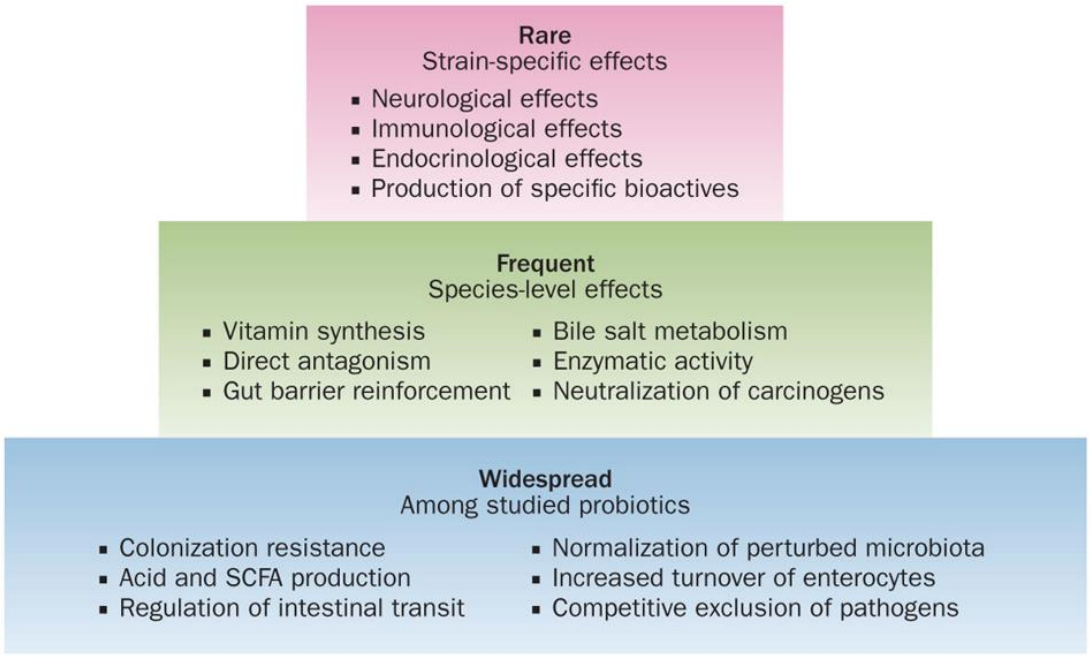

Figure 1.8: Distribution of potential protective characteristics amongst probiotics

Beneficial characteristics can be widespread amongst probiotics or specific to certain species or strains. Image taken from Hill *et al.* (2014).

Adhesion to the host is a frequently investigated probiotic characteristic, as intestinal colonisation, even if transient, is thought to be necessary to allow a probiotic to deliver its beneficial effects (Tuomola *et al.*, 2001). Thus, the identification of specific proteins which enhance this process has been a topic of much interest (Vélez *et al.*, 2007). Bacterial adhesins are generally classified according to their binding target (e.g. mucus-binding protein), the location on the bacterial cell (e.g. surface layer protein), or the mode of attachment to the bacterial surface (e.g. sortase-dependent proteins) (Vélez *et al.*, 2007). The adhesive potential of probiotics is normally assessed *in vitro* using mucus-deficient intestinal epithelial cell lines, such as Caco-2, HT-29, and T84, and thus binding efficiencies may not be applicable to the *in vivo* situation (further discussed in 1.4).

Interestingly, adhesion can also have anti-pathogenic effects through the competitive exclusion of pathogens from the intestinal epithelium. Pathogen exclusion is thought to be dependent on either competition for mutual binding sites/receptors or steric hindrance, where the probiotic cell physically blocks the access of a pathogen to the epithelial surface (Lebeer *et al.*, 2008). These characteristics *in vivo* would be similar to colonisation resistance, where the probiotic bacteria and the host microbiota outcompete the infectious agent by preventing the pathogen from gaining a foothold in the intestinal milieu.

In addition to inhibition of pathogens through “passive” mechanisms, *Lactobacillus* strains also produce a battery of antimicrobial compounds which can either kill or reduce the growth of competitor bacteria (Servin, 2004). The organic acids produced by *Lactobacillus* have potent antibacterial properties, as high lactic acid-producing strains inhibited *S*. Typhimurium, EHEC, and *C. difficile* infection *in vitro* (De Keersmaecker *et al.*, 2006; Makras *et al.*, 2006; Ogawa *et al.*, 2001; Tejero-Sariñena *et al.*, 2012). Similar effects have been reported *in vivo*, where production of lactic acid was associated with increased protective effects against the infection of chickens and mice with *Campylobacter jejuni* and *S*. Typhimurium, respectively (Annuk *et al.*, 2003; Neal-McKinney *et al.*, 2012). Likewise to lactic acid, the production of hydrogen peroxide by *Lactobacillus* has been associated with anti-pathogenic effects, although this is more common in vaginal isolates rather than intestinal

lactobacilli (Servin, 2004). However, hydrogen peroxide produced by intestinal *Lactobacillus johnsonii* strains NCC 533 and 933 can kill *S.* Typhimurium and so this method of inhibition may be more prevalent amongst other lactobacilli in the gut (Atassi & Servin, 2010; Pridmore *et al.*, 2008).

Lactobacillus species also produce bacteriocins, which are small heat-stable peptides targeted against related microbes inhabiting a similar niche (Eijsink *et al.*, 2002). The mechanism of action of these peptides is generally through the formation of pores in the membrane of target bacteria, causing the intracellular contents to leak out from the cell (Eijsink *et al.*, 2002; O'Connor *et al.*, 2015). The best described bacteriocin is nisin, a class I bacteriocin (lantibiotic) produced by the lactic acid bacterium *Lactococcus lactis*, which has been used in the food manufacturing industry for the last 50 years due to its broad-spectrum inhibitory activity against Gram-positive bacteria, particularly the food contaminant *Listeria monocytogenes* (Delves-Broughton *et al*., 1996). While the inhibitory effects of these peptides are generally assessed *in vitro,* the protection of mice by *Lactobacillus salivarius* UCC118 against *L. monocytogenes* was dependent on the production of the class II bacteriocin Abp118, demonstrating that these proteins are also effective *in vivo* (Corr *et al.*, 2007). Interestingly, a small number of bacteriocins produced by lactobacilli can also kill Gram-negative bacteria, such as the *L. salivarius* 1077 peptide L-1077 which inhibited both *C. jejuni* and *Salmonella enterica* serovar Enteritidis *in vitro*, as well as reducing the levels of these pathogens in the liver and spleen of infected chickens (Svetoch *et al.*, 2011). It is important to note that these antimicrobials can inhibit both pathogenic and commensal bacteria, as the administration of Abp118-producing *L. salivarius* UCC118 altered the microbiota in both murine and porcine models (Murphy *et al.*, 2012; Riboulet-Bisson *et al.*, 2012). While there were no reported changes in the health of animals which consumed the bacteriocin-producing lactobacilli, alterations to the microbiota may have unintended side effects and thus, the impact of these antimicrobials on the microbial community requires further investigation.

In addition to antimicrobial characteristics, probiotic lactobacilli can also interact with the host. These interactions can have beneficial effects, such as

strengthening the epithelial barrier, which can be weakened during GI conditions such as IBD and infection (Lebeer *et al.*, 2008). Beneficial effects of probiotics on epithelial barrier function have been demonstrated *in vitro*, as *L. plantarum* DSM 2648 and *L. rhamnosus* GG attenuated the decrease in TER associated with EPEC and EHEC infection of intestinal epithelial cells (Anderson *et al.*, 2010; Johnson-Henry *et al.*, 2008). The protection of epithelial barrier function has been associated with the prevention of TJ proteins ZO-1, claudin-1, and occludin dissociation from the lateral surfaces of the epithelial cells (Johnson-Henry *et al.*, 2008; Miyauchi *et al.*, 2009; Roselli *et al.*, 2007). Similar findings have been reported *in vivo*, as *L. rhamnosus* OLL2838 and the mixed species probiotic product VSL#3 (containing *Bifidobacterium longum*, *B. infantis*, *B. breve, L. acidophilus*, *L. casei*, *L. delbrueckii* subsp*. Bulgaricus, L. plantarium,* and *Streptococcus salivarius* subsp. *Thermophilus*) inhibited TJ protein redistribution in mice with induced colitis (Madsen *et al.*, 2001; Mennigen *et al.*, 2009; Miyauchi *et al.*, 2009). Similar effects have also been reported in humans, as *L. plantarum* WCFS1 increased occludin localisation to TJs, relative to mock-treated controls (Karczewski *et al.*, 2010). However, while these studies have demonstrated changes in epithelial barrier function induced by *Lactobacillus* species, the specific mechanisms for these effects, i.e. the molecules and associated receptors which initiate these changes, remain poorly defined (Lebeer *et al.*, 2008). One protein which has shown promising effects on maintaining TJ integrity is the *L. rhamnosus* GG-derived protein p40, which alleviates DSS-induced colitis in mice via interaction with the epidermal growth factor (EGF) receptor (Yan *et al.*, 2011). Whether similar proteins, which utilise the same mechanism, are present in other *Lactobacillus* strains requires further investigation.

1.2.5. *Lactobacillus reuteri*

The gut symbiont *Lactobacillus reuteri* is an autochthonous inhabitant of the mammalian gut, with the taxonomic classification shown in Figure 1.9 (Walter *et al.*, 2011). *L. reuteri* isolates have been identified in numerous mammals,

including humans, mice, pigs, hamsters, cattle, rats, and dogs (Casas & Dobrogosz, 2000; Korhonen *et al.*, 2007; Reuter, 2001; Salzman *et al.*, 2002). In the porcine gut, *L. reuteri* is one of the most numerous *Lactobacillus* species isolated from the ileum, where biofilms of lactobacilli and other commensals bind directly to the intestinal surface (Walter, 2008). In human adults, *L. reuteri* has been detected throughout the GIT and in faeces, and has been noted as a predominant species in the small intestine, although this is controversial as other studies have suggested that only 4% of the population has an indigenous *L. reuteri* population (Dal Bello *et al.*, 2003; Hayashi *et al.*, 2005; Reuter, 2001; Valeur *et al.*, 2004). In addition to the GIT, *L. reuteri* has also been isolated from both the vagina and breast milk, and strains sequestered from these environments have been widely associated with probiotic health benefits, including the synthesis of vitamins such as B_{12} and folate, as well as the prevention and treatment of infectious diarrhoea (Abrahamsson *et al.*, 2009; Saulnier *et al.*, 2011; Shornikova *et al.*, 1997a; Shornikova *et al.*, 1997b; Urbańska *et al.*, 2016).

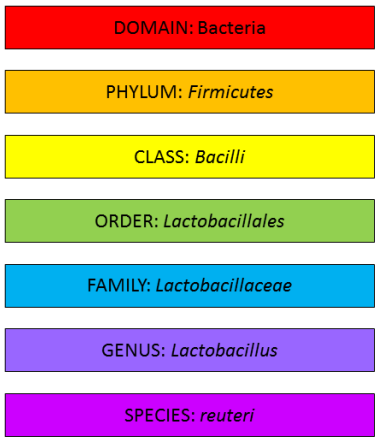

Figure 1.9: Taxonomic classification of *L. reuteri*

1.2.5.1. Interaction of *L. reuteri* with the host

Phylogenetic analysis of *L. reuteri* strains isolated from numerous backgrounds (human, mouse, rat, pig, chicken, and turkey) has determined that *L. reuteri* demonstrates host specificity (Oh *et al.*, 2010). These findings have been confirmed experimentally, as *L. reuteri* strains sequestered from rodents (mouse and rat) outcompeted isolates from others backgrounds in germ-free mice (Oh *et al.*, 2010). Additionally, murine *L. reuteri* strains lacking host-specific genes demonstrated reduced levels of colonisation of the murine foregut, further demonstrating that certain genes enhance ecological fitness in *L. reuteri* (Frese *et al.*, 2013). These genes have numerous roles in the host, such as survival of gastric acid and encoding adhesins, which enhance binding to the mucosa, such as Mucus binding protein (MUB) and Cell and mucus binding protein (CmbA), produced by *L. reuteri* ATCC 53608 and ATCC PTA 6475, respectively (Table 1.2). The evolution of these host-specific adhesins demonstrates the symbiotic relationship that mammals and birds have with *L. reuteri*, as the development of these evolutionary lineages is thought to span millions of years (Oh *et al.*, 2010). The identification of these adhesins offers a method for the evaluation of potential probiotics for both human and animal consumption, as the presence of adhesins may enhance *L. reuteri* persistence in the host.

Protein	Strain(s)	Origin	Function in the GIT	References
CnBP/ MapA	DSM 20016, RC-14, 104R	Human, hamster, pig	Adhesion to cells or mucus	Roos *et al.* (1996), Heinemann *et al.* (2000), Miyoshi *et al.* (2006)
Mub	ATCC 53608[1]	Pig	Adhesion to mucus and/or IgA, cell aggregation	Roos and Jonsson (2002), MacKenzie *et al.* (2010)
CmbA	ATCC PTA 6475[2], DSM 20016[3], ATCC PTA 4659[4], ATCC PTA 5289[5]	Human	Adhesion to cells and mucus	Jensen *et al.* (2014)
Lsp	100-23	Rat	Adhesion to forestomach epithelium	Walter *et al.* (2005)
Lr70902	100-23	Rat	Adhesion to forestomach epithelium	Frese *et al.* (2013)
GtfA/ Inu	TMW1.106	Sourdough fermentation	Cell aggregation/ biofilm formation	Walter *et al.* (2008)

Table 1.2: *L. reuteri* proteins associated with adhesion to the mucosa

Alternative strain names; [1]1063, [2]MM4-1a, [3]JCM112, [4]MM2-3, [5]FJ1.

1.2.5.2. Probiotic characteristics of *L. reuteri*

The application of *L. reuteri* as a probiotic has increased in recent years, as numerous studies have identified beneficial effects on the host, including the attenuation of diarrhoea and immunomodulation (summarised in Table 1.3). In addition to these host effects, *L. reuteri* can also inhibit other microbes, as certain strains produce 3-hydroxypropionaldehyde, a potent antimicrobial commonly referred to as reuterin (Talarico *et al.*, 1988). Reuterin is produced as an intermediate compound during the fermentation of glycerol into 1, 3-propanediol, which is used to reoxidise NAD^+ from NADH (pathway shown in Figure 1.10) (Lüthi-Peng *et al.*, 2002; Schaefer *et al.*, 2010). While *L. reuteri* has a high resistance to reuterin, this anti-microbial has broad inhibitory activity against Gram-positive and Gram-negative bacteria, as well as yeast, fungi, and protozoa (Axelsson *et al.*, 1989; Casas & Dobrogosz, 2000; Chung *et al.*, 1989). Interestingly, *L. reuteri* actively secretes reuterin into its local environment when other bacteria are present, indicating that this mechanism may be used to gain a competitive advantage in the GIT milieu (Schaefer *et al.*, 2010). As the production of reuterin has been demonstrated by *L. reuteri* JCM 1112 in the murine intestine *in vivo*, this further supports this mode of action (Morita *et al.*, 2008). Due to reuterin's broad inhibitory effects on microbial pathogens, this compound has numerous potential applications, such as a food preservative and as a pharmaceutical product (Vollenweider & Lacroix, 2004).

Strain	Effect	References
ATCC PTA 6475	Reduced diarrhoea, anti-inflammatory, reuterin producer, increased bone density, improved sociability	Emara *et al.* (2013), Francavilla *et al.* (2014), Preidis *et al.* (2012), Spinler *et al.* (2008), Jones and Versalovic (2009), Lin *et al.* (2008), Britton *et al.* (2014), Collins *et al.* (2016), McCabe *et al.* (2013), Gao *et al.* (2015), Thomas *et al.* (2012), Buffington *et al.* (2016).
ATCC 55730/ DSM 17938	Immunomodulation, reduced diarrhoea, reduced NEC, reduced colic	Valeur *et al.* (2004), Francavilla *et al.* (2012), Dinleyici *et al.* (2014), Gutierrez-Castrellon *et al.* (2014), Shornikova *et al.* (1997a), (Urbańska *et al.*, 2016), Shornikova *et al.* (1997b), Tubelius *et al.* (2005), Savino *et al.* (2015), Oliva *et al.* (2012), Twetman *et al.* (2009), Liu *et al.* (2014), Hunter *et al.* (2012), Bird *et al.* (2016)
ATCC PTA 4659	Anti-inflammatory, reuterin producer, increased mucus thickness	Spinler *et al.* (2008), Liu *et al.* (2010), Ahl *et al.* (2016).

Table 1.3: Summarised probiotic characteristics of three *L. reuteri* strains

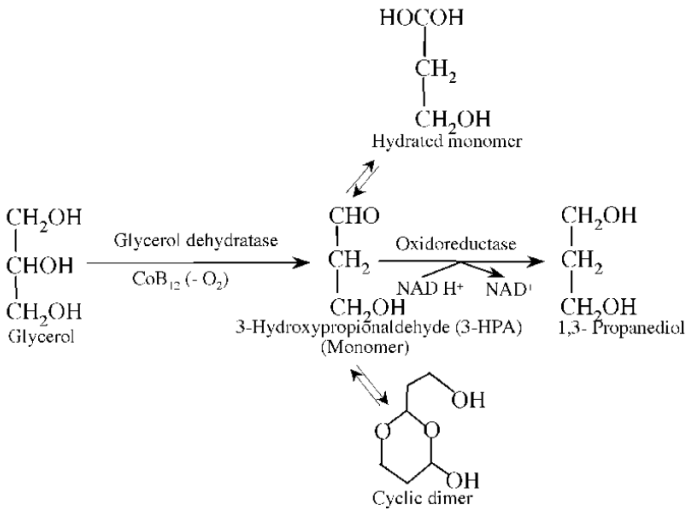

Figure 1.10: Reuterin synthesis pathway

Reuterin is produced by *L. reuteri* as an intermediate compound during the production of 1, 3- Propanediol from glycerol. Reuterin is composed of an equilibrium mixture of hydrated monomeric, monomeric, and cyclic dimeric forms of 3-hydroxypropionaldehyde. Image taken from Casas and Dobrogosz (2000).

In addition to reuterin, some *L. reuteri* strains (such as LTH2584) produce reutericyclin, a broad-spectrum antibiotic that alters the electrochemical gradient of the Gram-positive bacterial cell membrane (Gänzle *et al.*, 2000). Reutericyclin has demonstrated inhibitory effects against numerous important pathogens, including *Staphylococcus aureus*, *Bacillus subtilis*, and *C. difficile* (Gänzle *et al.*, 2000; Gänzle & Vogel, 2003; Hurdle *et al.*, 2011). While this antibiotic shows promising effects, it still remains to be determined whether it is produced *in vivo*, as only *L. reuteri* isolates from sourdough have currently been shown to produce this compound (Gänzle & Vogel, 2003).

While numerous *L. reuteri* strains synthesise the anti-microbial reuterin, other effects are strain-specific. Thus, we will discuss the demonstrated probiotic characteristics of two of the most commonly used human *L. reuteri* isolates, ATCC PTA 6475 and ATCC 55730.

1.2.5.2.1. *L. reuteri* ATCC PTA 6475

L. reuteri ATCC PTA 6475, also known as MM4-1a, were originally isolated from the breast milk of a healthy Finnish woman, and is currently used in formulations by the probiotic company Biogaia (Stockholm, Sweden) (Liu *et al.*, 2010). Investigations into the probiotic characteristics of ATCC PTA 6475 have revealed that this strain can alleviate the effects of gastritis *in vivo* when used in combination with *L. reuteri* DSM 17938, as two randomised controlled trials in humans have identified a reduction in *Helicobacter pylori* symptoms, including diarrhoea (Emara *et al.*, 2013; Francavilla *et al.*, 2014). ATCC PTA 6475 monoculture has also demonstrated anti-diarrhoeal effects, as probiotic supplementation reduced the production of diarrhoea by 1 day in rota-virus-infected mice (Preidis *et al.*, 2012). Additionally, germ-free mice pre-treated with ATCC PTA 6475 prior to EHEC infection shed decreased numbers of EHEC as well as maintained body weight, relative to non-treated littermates (Eaton *et al.*, 2011). The mechanism behind the reduction of diarrhoea is unknown however, ATCC PTA 6475 is a reuterin producer and this strain has been linked to the inhibition of numerous enteric pathogens *in vitro*, including EHEC, ETEC, and *S. enterica*, which may partially explain these probiotic effects (Spinler *et al.*, 2008). An alternative mechanism may be associated with the modulation of the host inflammatory state, as ATCC PTA 6475 has demonstrated potent anti-inflammatory effects, both *in vitro* and *in vivo* (Jones & Versalovic, 2009; Lin *et al.*, 2008; Preidis *et al.*, 2012). The anti-inflammatory characteristics of ATCC PTA 6475 have also been associated with an increased bone density in both male and oestrogen-deficient female mice (Britton *et al.*, 2014; Collins *et al.*, 2016; McCabe *et al.*, 2013). A potential mechanism behind the immunomodulatory effects of ATCC PTA 6475 is the production of histamine from dietary L-histidine, which suppresses protein kinase A signalling, subsequently reducing the production of inflammatory cytokines (Gao *et al.*, 2015; Thomas *et al.*, 2012). Inhibition of the inflammatory response through the production of histamine by *L. reuteri* has been demonstrated both *in vitro* and *in vivo*, further supporting this mechanism (Gao *et al.*, 2015; Thomas *et al.*, 2012).

Interestingly, a recent study by Buffington *et al.* (2016) has identified that supplementation of ATCC PTA 6475 into mice with social disorders, induced via maternal high fat diet, improved sociability. While the authors linked this effect to the neuropeptide oxytocin, which can be induced by ATCC PTA 6475 (Poutahidis *et al.*, 2013), the immunomodulatory characteristics of this strain may also have an effect, as inflammation has also been associated with altered brain development (Bolton & Bilbo, 2014). The implications of these findings, both ethically and scientifically, remain to be determined as these data suggest that specific probiotics may impact the host in ways which have not previously been appreciated. As such, further study is required to fully understand the effects of ATCC PTA 6475 on the host.

1.2.5.2.2. *L. reuteri* ATCC 55730

L. reuteri ATCC 55730 were first isolated from the breast milk of a Peruvian mother who lived in the Andes, and have previously been used in probiotic products by Biogaia (Liu *et al.*, 2010). As ATCC 55730 contained a plasmid with antibiotic resistance, this strain has now been superseded by the plasmid-cured daughter strain DSM 17938, which has maintained the probiotic characteristics of ATCC 55730 (Liu *et al.*, 2010; Rosander *et al.*, 2008).

Similarly to ATCC PTA 6475, the impact of ATCC 55730/ DSM 17938 on diarrhoea has been an area of investigation in numerous studies. These findings have implied that the administration of either ATCC 55730 or DSM 17938 can reduce both the occurrence and symptoms of acute diarrhoea in children (Dinleyici *et al.*, 2014; Francavilla *et al.*, 2012; Gutierrez-Castrellon *et al.*, 2014; Shornikova *et al.*, 1997a; Urbańska *et al.*, 2016). However, one study did not identify any beneficial effects of DSM 17938 against nosocomial diarrhoea in children, although the reason behind this contrast with the other studies is unknown (Wanke & Szajewska, 2012). Interestingly, DSM 17938 specifically reduced the incidence of both EPEC- and rotavirus-associated diarrhoea in infants (Savino *et al.*, 2015; Shornikova *et al.*, 1997b). Similar findings have also been reported in adults, as the incidence of GI and

respiratory illness decreased by 60% in volunteers consuming ATCC 55730, relative to those in the placebo group (Tubelius *et al.*, 2005). These probiotic strains were well tolerated with no side effects in either healthy or immunocompromised individuals and thus, represent a potential option to treat diarrhoea (Mangalat *et al.*, 2012; Wolf *et al.*, 1998).

In contrast to ATCC PTA 6475, ATCC 55730 has often been described as an immunostimulatory probiotic (Liu *et al.*, 2010). In healthy human volunteers, ATCC 55730 increased the number of B lymphocytes in the duodenum and $CD4^+$ T-lymphocytes in the ileum (Valeur *et al.*, 2004). Immune stimulation has also been observed *in vitro*, as ATCC 55730 secreted products stimulated the production of TNF-α by the monocyte cell line THP-1 (Lin *et al.*, 2008). However, in children with active rectal UC, the direct administration of ATCC 55730 by enema to the inflamed area reduced mucosal inflammation, as well as increasing the levels of the anti-inflammatory cytokine interleukin-10 (IL-10) at the site of inflammation (Oliva *et al.*, 2012). Similar findings have also been reported in patients with active gingivitis, as volunteers using chewing gums containing *L. reuteri* ATCC 55730 and ATCC PTA 5289 demonstrated decreased levels of pro-inflammatory IL-8 and tumour necrosis factor-α (TNF-α) (Twetman *et al.*, 2009). Thus, these findings suggest that ATCC 55730 and DSM 17938 have immunomodulatory features, rather than only stimulating the immune response. This is supported by recent findings in a mouse model of necrotising enterocolitis (NEC), as DSM 17938 decreased numbers of T-effector cells and increased T-regulatory cells in the inflamed tissue, as well as decreasing mortality and disease severity (Liu *et al.*, 2014). Similar findings have been reported in high-risk human neonates, as prophylactic treatment with DSM 17938 significantly reduced the risk of NEC (Hunter *et al.*, 2012).

Infantile colic is defined as excessive crying with no apparent cause and is a common condition in infants up to 6 months (Sung *et al.*, 2012). A recent meta-analysis of 444 infants identified that treatment with ATCC 55730 and DSM 17938 decreased crying 2.3-fold, relative to infants given either placebo or standard therapy (simethicone) (Bird *et al.*, 2016). Importantly, there were no apparent side effects associated with *L. reuteri* supplementation, indicating that this therapy would be safe for future usage (Bird *et al.*, 2016).

1.3. Innate mechanisms of host defence

The intestinal epithelium, which provides the first line of defence against foreign antigens and bacteria, is composed of a single layer of enterocytes, Paneth cells, enteroendocrine cells, and goblet cells, which work in unison to regulate the movement of nutrients and water out of the lumen, while preventing microbial access across the epithelium. Homeostasis in the GIT is maintained by the epithelial cells through multiple mechanisms, such as the production of the mucus layer and the innate immune response (Johansson & Hansson, 2011c; McGuckin *et al.*, 2011). The failure of these barriers is a basis for dysbiosis in the gut, as well as the development of IBD (Neuman & Nanau, 2012). Whilst the mucus layer and the innate immune response inhibit bacterial access, these defences can be modified through microbial interaction, including both EPEC and *Lactobacillus*, and thus the role of bacteria on these systems remains an important area of research.

1.3.1. Mucus

1.3.1.1. Intestinal mucus production

The lining of the GIT encompasses the largest surface area of any part of the human body, totalling approximately 400 m^2 in adults, and is covered by a thick layer of mucus (Mowat & Viney, 1997). The mucus layer is a biochemical coating which protects the intestinal epithelium by lubricating the mucosal surface, preventing mechanical damage from food passing through the intestines. The mucus layer also acts as a barrier and a habitat for microbes, preventing bacterial access to the epithelium while also providing a glycan-rich environment for commensal bacteria to inhabit.

The thickness and structure of the mucus layer is dependent on the location in the GIT, which corresponds with the role of each section in digestion as well as the bacterial burden (Johansson *et al.*, 2011b). The current understanding of mucus thickness is based on findings in rodents, although a more recent study with human colonic biopsies was in agreement with these observations

(Figure 1.11) (Atuma *et al.*, 2001; Gustafsson *et al.*, 2012). In the stomach, a thick mucus layer is required to protect the epithelium from the harsh acidic conditions which could damage the epithelial surface (Ermund *et al.*, 2013). The mucus structure changes in the small intestine, as the mucus thickness decreases and becomes loose and penetrable (Ermund *et al.*, 2013). This change in the mucus facilitates the absorption of nutrients by villi while also protecting the epithelial surface, as the rapid transit time of digested food, the efficient capture of bacteria by the mucus as well as the production of antimicrobial compounds, inhibits microbial access to the epithelium (Ermund *et al.*, 2013). In the distal small intestine and the large bowel (ileum and colon, respectively), the mucus thickness increases drastically, as the bacterial load reaches its peak in the colon (Ermund *et al.*, 2013). As the transit time of food in the colon is reduced, the thick outer mucus layer provides a habitat for the large microbial population, which extract inaccessible nutrients from digested material in the lumen (Bäckhed *et al.*, 2005). These changes in mucus thickness represent a dynamic response to the specific challenges that the regions of the GIT encounter.

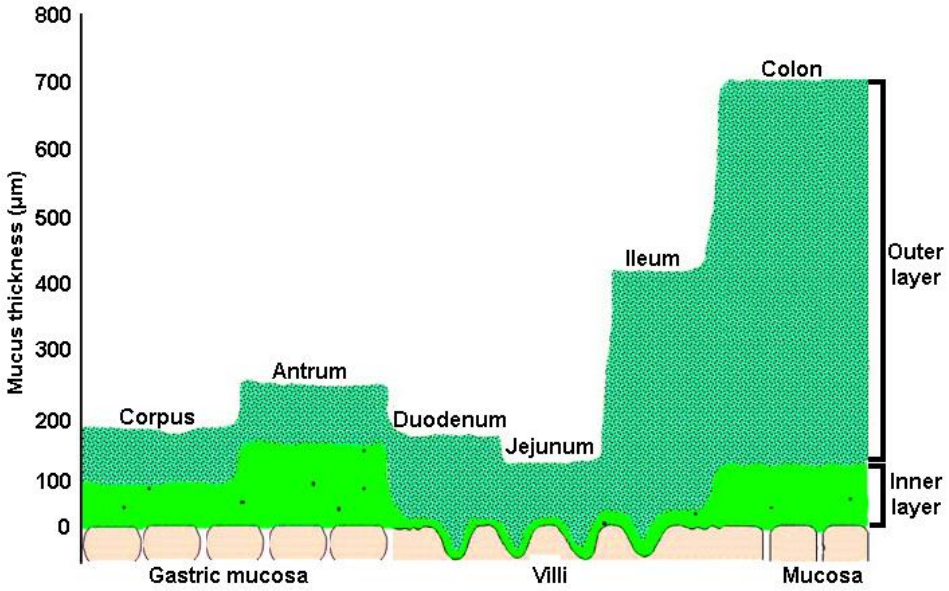

Figure 1.11: Mucus thickness throughout the rat GIT

The thickness of the inner and outer mucus layers change dependent on the location in the GIT. Image adapted from Juge (2012).

In addition to variable mucus thickness, the structure of the mucus layer also differs between GI locations (Atuma *et al.*, 2001). In the rat colon, the mucus forms an inner (~150 µm) and an outer (~550 µm) layer, the former excluding bacterial access to the epithelium whereas the latter facilitates bacterial colonisation (Figure 1.12A) (Johansson *et al.*, 2011a). These layers demonstrate different physical properties, as the outer mucus layer can be easily removed via aspiration, while the inner layer remains adherent (Atuma *et al.*, 2001; Gustafsson *et al.*, 2012). The outer mucus layer is formed from the inner layer via the cleavage of cysteine residues in the mucin proteins, which initiates a four-fold expansion of the mucin glycoprotein while maintaining the structural integrity of the mucus layer (Figure 1.12B) (Johansson *et al.*, 2011a; Johansson *et al.*, 2008).

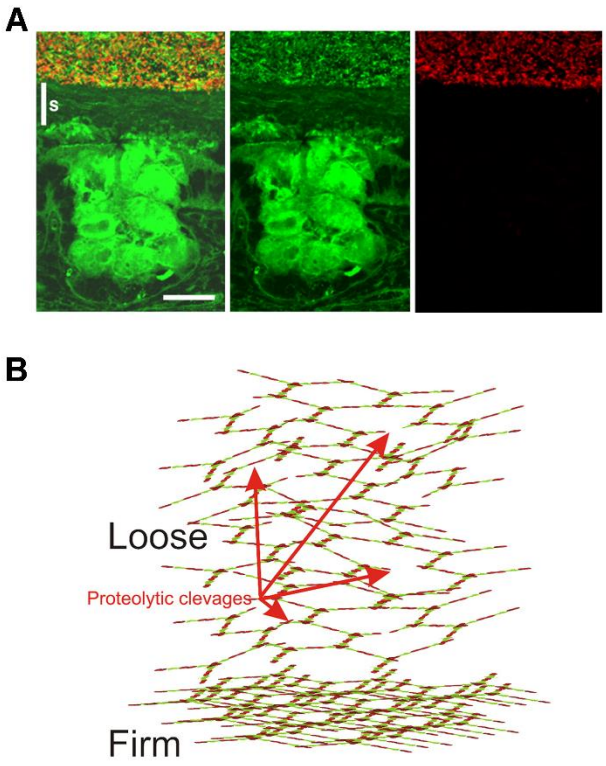

Figure 1.12: The inner and outer mucus layers of the colon

(A) The inner stratified (S) and outer mucus layer (green) where the former is devoid of bacteria (red) whereas the latter demonstrates distinct bacterial colonisation. Scale bar = 20 µm. Image taken from Johansson *et al.* (2008). (B) Cleavage of cysteine residues in the MUC2 mucin initiates a four-fold expansion of the glycoprotein. Images taken from Johansson *et al.* (2011a).

In contrast to the colon, the mucus layer of the small intestine is comprised of a single layer of loose mucus, which can be aspirated to reveal the villous surface (Atuma *et al.*, 2001; Ermund *et al.*, 2013). However, microbial access to the small intestinal epithelium is rarely observed, as bacteria are inhibited by antibacterial compounds such as defensins and RegIII lectins, which are released by Paneth cells and enterocytes (Figure 1.13) (Bevins, 2006; Vaishnava *et al.*, 2011).

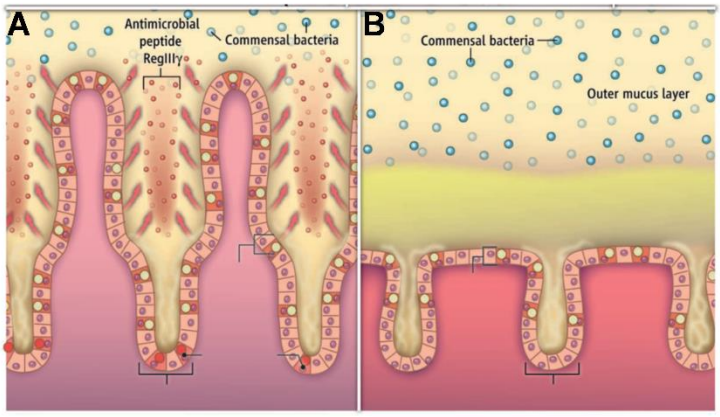

Figure 1.13: Mucus structure in the small and large intestine

Bacterial access to the small intestinal epithelium is inhibited through the production and secretion of anti-microbial compounds into the loose mucus layer (A). In the large intestine, the dense inner mucus layer inhibits bacterial movement to the epithelium (B). Image adapted from Johansson and Hansson (2011c).

Mucus has an essential role in inhibiting the access of biological and chemical insults to the epithelial surface. This has been demonstrated in mice deficient in Muc2, the primary secreted mucin in the murine small and large intestine, which spontaneously develop a severe colitis, characterised by the production of bloody stools as well as reduced weight gain (Van der Sluis *et al.*, 2006). The induction of colitis is partly due to the microbiota contacting the gut epithelium. Mice deficient in *O*-glycan formation, and hence demonstrated altered mucus properties, showed reduced colitis when treated with broad-spectrum antibiotics, which depleted aerobic and anaerobic bacteria (Fu *et al.*, 2011). Furthermore, when Muc2-deficient mice were exposed to A/E pathogen *C. rodentium*, the symptoms of infection were amplified, as these mice rapidly lost weight and demonstrated a 90% mortality rate, whereas wildtype mice recovered from infection after one week (Bergstrom *et al.*, 2010). These studies demonstrate the importance of mucus in maintaining a healthy GIT.

1.3.1.2. Mucin structure and production

The mucus layer is produced by secretion of mucin glycoproteins from epithelial goblet cells. Up to date, 17 mucins have been identified in humans, with 10 of these produced in the GIT (Arike & Hansson, 2016). Mucins can be divided into two groups, secreted gel-forming mucins (MUC2, -5AC, -5B, and -6) and membrane-bound mucins (MUC1, -3, -4, -12, -13, and -17) (Arike & Hansson, 2016). All mucin glycoproteins share a common core structure, a protein domain which is rich in proline, threonine, and serine residues (called the PTS domain), and can be over 2000 amino acids long (Arike & Hansson, 2016). The serine and threonine residues of the PTS domain are heavily glycosylated, forming a distinct "bottle brush" structure, contributing up to 70% of the mucin mass (Figure 1.14) (Arike & Hansson, 2016; Johansson *et al.*, 2011b; Juge, 2012; Lindén *et al.*, 2008a).

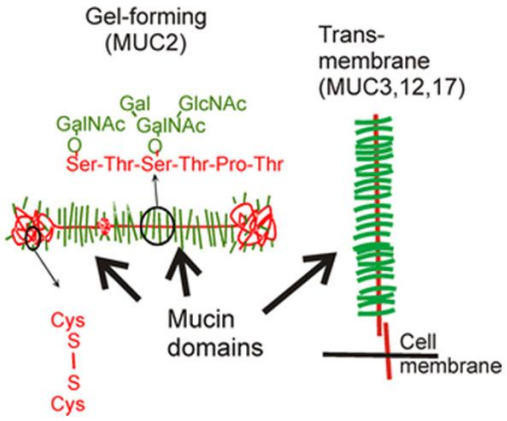

Figure 1.14: Structure of gel-forming and transmembrane mucins

Mucin glycoprotein structure with the protein core (red) and oligosaccharides decorations (green). Image taken from (Johansson *et al.*, 2011b)

Bacterial access to the epithelium throughout the GIT is inhibited by the adhesion of bacteria to mucins which mimic cell surface receptors, trapping microbes away from the epithelium (Lindén *et al.*, 2008a). Trapped bacteria

are subsequently removed during the turnover of mucus through sheer mechanical force, which is generated from the movement of digested food through the intestines (Kerss *et al.*, 1982). In the small and large intestine, MUC2 is the predominant secreted mucin, which forms a net-like structure upon release from the goblet cell, acting as a filter to prevent microbial passage to the epithelium (Johansson *et al.*, 2011a). The formation of the MUC2 mucin in the goblet cell has been relatively well defined. Briefly, the MUC2 protein dimerises at the C-terminus in the endoplasmic reticulum before transportation to the Golgi body (Figure 1.15A) (Ambort *et al.*, 2012a; Johansson *et al.*, 2011b). In the Golgi body, the PTS domains of the apomucin are glycosylated and N-terminal oligomerisation occurs, before the mucin molecules are packed into vesicles (Ambort *et al.*, 2012a; Johansson *et al.*, 2011b). Upon release from the goblet cell, the mucin molecules expand and combine to form the net-like structure, linked by covalent and non-covalent bonds (Figure 1.15B) (Ambort *et al.*, 2012b; Johansson *et al.*, 2011b).

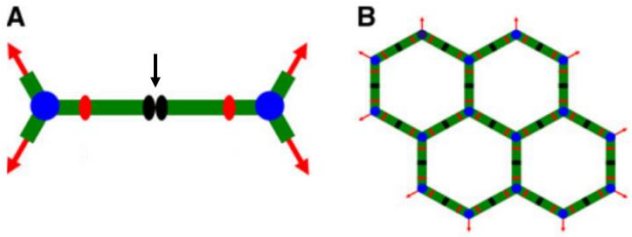

Figure 1.15: Formation of the MUC2 mucin structure

(A) C-terminal dimerisation of MUC2 apomucin (indicated by the arrow), (B) MUC2 polymeric net-like structure. Image adapted from Johansson *et al.* (2011b).

One of the key roles of glycan decorations of MUC2 are to protect the mucin from degradation by host digestive enzymes (Arike & Hansson, 2016). The glycans of the MUC2 mucin are themselves resistant to proteases and further prevent the access of these enzymes to the vulnerable mucin protein core

(Lidell *et al.*, 2006). Additionally, glycans also play an important role in the selection of the host microbiota, as *O*-glycans act as adhesion sites and nutrient sources for commensal bacteria (Juge, 2012; Koropatkin *et al.*, 2012; Tailford *et al.*, 2015b).

1.3.1.3. Mucus as a habitat for the microbiota

The mucus layer facilitates the colonisation of a diverse range of microbes, as mucin *O*-glycans have a diverse structure which offer numerous binding sites for different bacteria (Derrien *et al.*, 2010; Juge, 2012). Bacterial localisation in the GIT is influenced by mucin glycan structures, as these carbohydrates differ across the GIT, which may be a host mechanism to encourage the growth of certain bacteria in specific areas of the gut (Donaldson *et al.*, 2016). In mice, differences in glycan structures have been identified across the GIT, as the levels of sulfation, fucosylation, and sialyation of mucins was dependent on the location in the small and large intestine (Larsson *et al.*, 2013). The importance of glycosylation in bacterial binding to mucins has been demonstrated in animal models, as mice deficient in the production of *O*-glycans with a core-1 structure demonstrated an altered gut microbiota, with increased abundance of *Bacteroidetes* relative to *Firmicutes*, whereas wildtype mice were the inverse (Sommer *et al.*, 2014). Bacterial adhesion to mucins is mediated by a variety of surface receptors, such as the MUB protein of *L. reuteri* ATCC 53608, which binds to terminally sialyated glycans (Etzold *et al.*, 2014a; MacKenzie *et al.*, 2010). The MUB protein contains multiple repeats constituted of Ig- and mucus binding domains with homology to the MucBP domain of *L. monocytogenes* (Boekhorst *et al.*, 2006; Etzold *et al.*, 2014a; Juge, 2012; MacKenzie *et al.*, 2009). While MUB is unique to ATCC 53608, surface proteins containing the MucBP domain are located throughout all sequenced *L. reuteri* genomes such as the CmbA adhesin from human *L. reuteri* isolates (Etzold *et al.*, 2014b; Jensen *et al.*, 2014; MacKenzie *et al.*, 2010). Other *Lactobacillus* species also produce surface proteins with mucus-binding domains, including *L. rhamnosus* GG (mucus-binding factor protein) and *L. plantarum* strains WCFS1 and 299v (mannose-specific adhesin)

(Pretzer *et al.*, 2005; von Ossowski *et al.*, 2011). The prevalence of adhesins containing mucus-binding characteristics indicates the importance of these proteins in *Lactobacillus* colonisation/survival in the host. Besides specific mucus binding proteins, bacterial extracellular appendages also interact with intestinal mucins, such as the *L. rhamnosus* GG SpaCBA pilin and the probiotic *E.coli* Nissle 1917 flagella (Juge, 2012; Kankainen *et al.*, 2009; Lebeer *et al.*, 2012; Nishiyama *et al.*, 2015; Troge *et al.*, 2012).

In addition to providing binding sites for the microbiota, the mucus layer also acts as a food source, as glycans from mucins can be utilised by bacterial species that produce mucin-degrading enzymes (Koropatkin *et al.*, 2012; Tailford *et al.*, 2015a). *Ruminococcus, Bifidobacterium*, *Bacteroides*, and *Akkermansia* species have all been identified as mucin degraders however, these effects, as well as the specific glycans targeted, are strain specific (Crost *et al.*, 2013; Crost *et al.*, 2016; Derrien *et al.*, 2004; Ruas-Madiedo *et al.*, 2008; Sonnenburg *et al.*, 2005). For example, *A. muciniphilia* demonstrates a narrow glycan range whereas *Bacteroides thetaiotaomicron* targets both dietary- and host-derived glycans (Derrien *et al.*, 2004; Sonnenburg *et al.*, 2005).

As a consequence of mucin degradation, mucinolytic bacteria produce a range of metabolites, including short chain fatty acids (SCFA), particularly butyrate, which aid in the maintenance of epithelial homeostasis and provide an energy source for colonocytes (Hamer *et al.*, 2008). Butyrate can also increase the production of mucus by IEC's (Barcelo *et al.*, 2000; Finnie *et al.*, 1995; Hatayama *et al.*, 2007; Tazoe *et al.*, 2009). The exact mechanism remains unknown but the ERK signalling cascade has been implicated (Tazoe *et al.*, 2009). It is important to note that the majority of studies have investigated the impact of butyrate on the colon rather than the small intestine. However, butyrate, as well as the SCFAs acetate and propionate, are detected in ileostomy effluent, which indicates that SCFAs are present in the small intestine and thus may have similar beneficial effects (Zoetendal *et al.*, 2012). In addition to effects on the host, butyrate also influences bacterial virulence and reduces the expression of the key *S. enterica* pathogenicity island SPI1, which is necessary for epithelial colonisation (Gantois *et al.*, 2006). The health

benefits of SCFAs on maintaining host homeostasis remains an area of active research.

1.3.1.4. Pathogen subversion of the mucus layer

In order to initiate infection in the healthy GIT, pathogens must either infiltrate or degrade the mucus layer (Figure 1.16) (reviewed in McGuckin *et al.* (2011)). The flagellum is a key bacterial appendage which facilitates movement through mucus as well as enhancing microbial persistence by binding to mucins, as demonstrated for EPEC, EHEC, and *C. difficile* (Erdem *et al.*, 2007; Tasteyre *et al.*, 2001). Interestingly, the EPEC flagellum is not essential for the colonisation of human duodenal biopsies with an intact mucus layer, as a *fliC*-deficient mutant demonstrated enhanced binding relative to the wildtype (Schüller *et al.*, 2009). This implies that the interaction between flagella and the mucus may inhibit epithelial infection (Schüller *et al.*, 2009). However, components of the *C. difficile* flagella specifically bind to mucus from the mouse caecum but not the porcine stomach, which could indicate either host or GIT location selection by *C. difficile* (Tasteyre *et al.*, 2001).

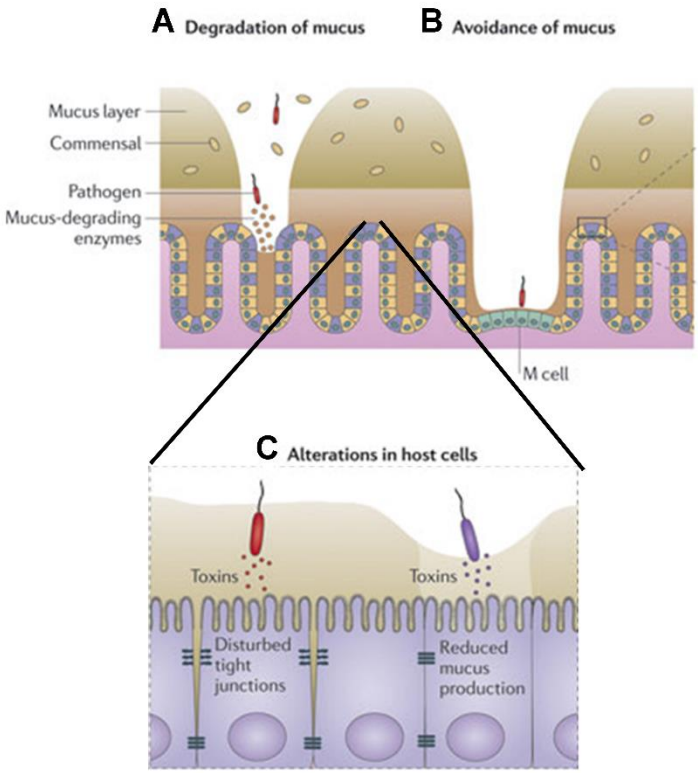

Figure 1.16: Pathogenic mechanisms to subvert the mucus layer

Pathogens can either (A) degrade the mucus layer with extracellular mucinases, (B) target microfold (M) cells which sample the gut microbiota and are not covered by a thick mucus layer, or (C) secrete toxins which diffuse through the mucus and disrupt TJs and inhibit mucus production. Image adapted from McGuckin *et al.* (2011).

Some GI pathogens produce enzymes which degrade mucus (Figure 1.16A) (McGuckin *et al.*, 2011). Mucinase production has been identified in numerous pathogens including EAEC (Pic), EHEC (StcE), and *Vibrio cholerae* (TagA) (Dutta *et al.*, 2002; Grys *et al.*, 2005; Szabady *et al.*, 2011). The EHEC metalloprotease StcE cleaves the protein core of the mucin glycoprotein, degrading the mucus coating and revealing the epithelial surface for colonisation (Angel *et al.*, 2012; Grys *et al.*, 2005). Additionally, the degradation of the mucus layer creates a nutrient-poor environment for commensal bacteria, which reduces colonisation resistance by the host

microbiota against EHEC infection (Bäumler & Sperandio, 2016). Alternatively, *H. pylori* modifies the local environment to reduce mucus viscoelasticity, without degrading the mucus layer (Celli *et al*., 2009). The stomach mucus forms an effective barrier at a low pH however, *H. pylori* releases a urease which hydrolyses urea and elevates the pH of the mucus from 4 to 6 (Celli *et al.*, 2009). By altering the rheological properties of the mucus, *H. pylori* can move freely toward the epithelium, to escape from the harsh acidic environment of the stomach lumen (Celli *et al.*, 2009; Montecucco & Rappuoli, 2001).

In the small intestine, microfold (M) cells, present in the dome epithelium of Peyer's patches, sample antigens from the lumen and transport them to underlying immune cells (Wang *et al.*, 2014b). The sampling of the microbiota is an essential component of the localised immune system in the gut, which allows the host to prepare an antigen-specific IgA response against potential insults, without inducing an inflammatory response (Neutra *et al.*, 2001). For effective sampling in the lumen, M cells have minimal mucus coverage, due to a lack of goblet cells in the Peyer's patch, and thus offer a preferential site for infection in the small intestine (Figure 1.16B) (McGuckin *et al.*, 2011; Wang *et al.*, 2014b). Infection of M cells has been demonstrated by numerous invasive GI pathogens, including *S.* Typhimurium, *S. flexineri,* and *L. monocytogenes* (Jensen *et al.*, 1998; Jepson & Clark, 2001; Jones *et al.*, 1994; Sansonetti *et al.*, 1996).

Some GI pathogens produce toxins which travel through the functional mucus layer and access the epithelium (Figure 1.16C) (McGuckin *et al.*, 2011). These toxins can alter the mucus in various ways, such as *H. pylori*-secreted cytotoxins CagA and VacA, which directly inhibit mucin synthesis and secretion (Beil *et al.*, 2000; Byrd *et al.*, 2000). Alternatively, toxins can modify the mucus layer indirectly, such as those produced by *V. cholerae*, which impact on the normal functioning of the cell and further induce apoptosis or weaken TJs in the epithelial barrier (Arce *et al.*, 2005; Fasano *et al.*, 1997; Lucas, 2010). Through interaction with the epithelium, cholera toxin induces the secretion of water into the lumen which disrupts the mucus layer and induces diarrhoea, providing an opportunity for *V. cholerae* to both establish

infection in the host and disseminate into the external environment (Lucas, 2010).

The current understanding of the interaction between EPEC and the mucus is limited however, the infection of mice with the related A/E pathogen *C. rodentium* induced the depletion of Muc2 from goblet cells, mediated by the host immune response, indicating that the detection of A/E bacteria induced changes in the mucus layer (Bergstrom *et al.*, 2008). Additionally, EPEC produces SslE (secreted and surface-associated lipoprotein from *E. coli*; also produced by ETEC and ExPEC), a secreted enzyme which has mucinolytic properties *in vitro* and *in vivo* (Luo *et al.*, 2014; Nesta *et al.*, 2014; Valeri *et al.*, 2015). While this enzyme offers a mechanism behind EPEC access to the epithelium, the impact of this mucinase during human infection still remains to be determined.

1.3.2. Innate immune response

1.3.2.1. Innate immune response to pathogens

If a pathogen subverts the mucus layer and gains access to the epithelium, the host then initiates innate immune defence mechanisms against the invading microbe (Figure 1.17). This response detects ubiquitous microbial molecules, referred to as microbe-associated molecular patterns (MAMPs), which are detected by pattern recognition receptors (PRRs). PRRs are comprised of Toll-like receptors (TLRs), receptors that detect MAMPs at the host cell surface or in endosomes, and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), which detect intracellular MAMPs (Figure 1.18) (Chen *et al.*, 2009). Due to the constant evolution of pathogens which can quickly adapt to avoid host defences, PRRs have evolved to identify a limited range of highly conserved motifs which are not produced by higher eukaryotes, such as peptidoglycan, lipopolysaccharide (LPS) , and flagellin, detected by TLR2, TLR4, and TLR5, respectively (Aderem & Ulevitch, 2000). The detection of a MAMP initiates a cascade via the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein

kinase (MAPK) signalling pathways, which activate the pro-inflammatory transcription factors NF-κB and activator protein-1 (AP-1), respectively (Aderem & Ulevitch, 2000; Chen *et al.*, 2009; de Grado *et al.*, 2001). The target genes of NF-κB include the cytokines interleukin (IL)-1β, IL-6, IL-8, TNF-α, and interferon-γ (IFN-γ), which are secreted from the host cell and induce the chemotactic migration of macrophages, phagocytes, neutrophils, and dendritic cells to the site of infection (Tak & Firestein, 2001). These immune cells attack pathogens with DNA-damaging free-radicals (such as H_2O_2 and NO) as well as engulfing and phagocytosing microbes (Knight, 2000).

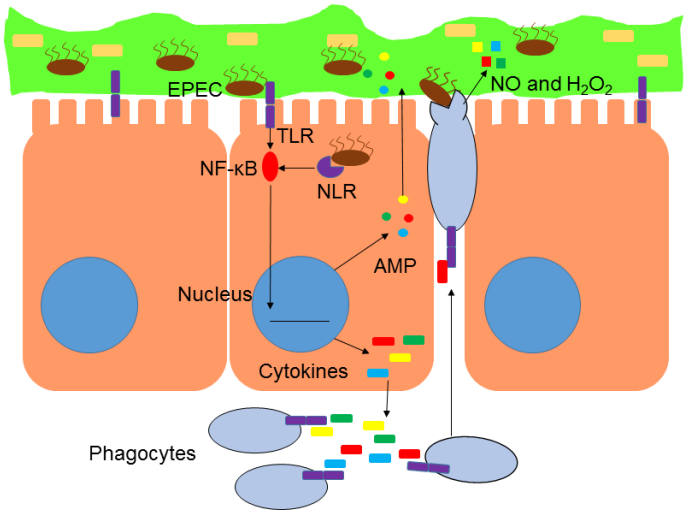

Figure 1.17: Innate immune response in the GIT

The innate immune response is initiated upon detection of MAMPs by PRRs (either TLRs or NLRs). The PRRs initiate the NF-κB signalling cascade, upregulating the expression of pro-inflammatory genes, which encode secreted antimicrobial proteins (AMP) and inflammatory cytokines. The secretion of cytokines recruits phagocytes to the site of infection, inducing transepithelial migration to the apical surface. These immune cells release free-radicals (such as NO and H_2O_2) and phagocytose invading microbes.

Furthermore, the detection of pathogens by the intestinal epithelium (through TLRs and NLRs) can induce the production and secretion of antimicrobial

peptides, such as defensins, REGIII lectins, and cathelicidins, which integrate into the bacterial cell wall and/or membrane and form pores, resulting in osmotic leakage and cell death (Mukherjee & Hooper, 2015). These small peptides are produced by Paneth cells, enterocytes and goblet cells and are specifically targeted against bacteria, as these antimicrobials have net positive charges, whereas the bacterial cell wall has a net negative charge, allowing electrostatic interactions between the peptide and the bacterial cell (Dürr *et al.*, 2006; Fujii *et al.*, 1993; Mukherjee *et al.*, 2014). In contrast, only the cytoplasmic surface of the eukaryotic cell membrane is negatively charged, thus the controlled secretion of these cationic peptides outside the membrane, prevents interaction with the host cell (Mukherjee & Hooper, 2015).

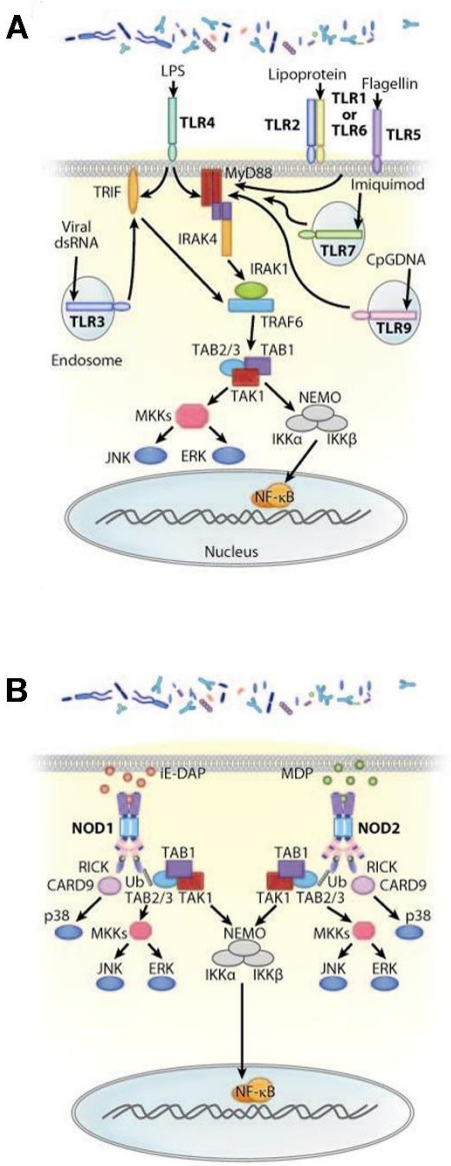

Figure 1.18: Inflammatory signalling pathway activation by PRRs upon detection of MAMPs

(A) TLR-mediated and (B) NLR-mediated signalling pathways. Image adapted from Chen *et al.* (2009).

1.3.2.2. Innate immune response against EPEC infection

The innate immune response raised against EPEC infection has been well investigated and has been associated with neutrophil infiltration at the site of infection (Moon *et al.*, 1983). The migration of neutrophils across the epithelial surface is mediated by the chemoattractant IL-8, a cytokine which is significantly increased during EPEC infection (Khan *et al.*, 2008; Nadler *et al.*, 2010; Pearson *et al.*, 2011; Ruchaud-Sparagano *et al.*, 2007; Savkovic *et al.*, 1997; Schüller *et al.*, 2009; Sharma *et al.*, 2006; Zhou *et al.*, 2003). Flagellin, the principle component of the EPEC flagellum, has been implicated as the primary immunostimulatory factor, as flagellin induced the production of IL-8 in both *in vitro* (HT-29 and T84) and *ex vivo* (duodenal biopsies) models (Khan *et al.*, 2008; Schüller *et al.*, 2009; Sharma *et al.*, 2006; Zhou *et al.*, 2003). Whilst flagellin has been identified as a key inducer of the IL-8 response, it is important to note that the loss of *fliC* does not completely abrogate the production of IL-8, implying that other immunostimulatory factors are involved (Khan *et al.*, 2008; Schüller *et al.*, 2009; Sharma *et al.*, 2006). While EPEC LPS, DNA, and EspC do not induce an IL-8 response *in vitro*, the additional immunogenic factor(s) remain unknown (Sharma *et al.*, 2006).

Interestingly, the innate immune response against EPEC infection is thought to contribute to the onset of diarrhoea, as migrating neutrophils initiate the release of chloride ions by the epithelium, which induces fluid secretion from epithelial cells into the lumen (Crane *et al.*, 2002; Crane *et al.*, 2007; Viswanathan *et al.*, 2009). However, EPEC counteracts the host immune response via the secretion of effector proteins into the host cell.

1.3.3. The effect of EPEC on innate defences

The innate immune response raised against EPEC perturbs the normal intestinal milieu, creating a hostile environment, which aids in the clearance of the pathogen from the host. However, the EPEC genome encodes a vast repertoire of effector proteins that dampen the host response to infection (Kaper *et al.*, 2004). These effectors target the NF-κB and the MAPK signalling

pathways and effectively inhibit the immune response through the mechanisms described below.

The NF-κB transcription factor is a key mediator in the propagation of the innate immune response against EPEC infection. NF-κB is comprised of two subunits, p50 and p65, which are dimerised at the Rel homology domain on the N-terminus of each subunit (Pearson *et al.*, 2011). In the non-inflamed cell, NF-κB is located in the cytoplasm in an inactive form bound to IκB however, upon initiation of the pro-inflammatory cascade, IκB is phosphorylated by IκB kinase (IKK), which induces IκB ubiquitination (Tak & Firestein, 2001). The removal of IκB subsequently activates NF-κB, which translocates to the nucleus and initiates the transcription of genes containing a κB enhancer element, such as IL-8 (Figure 1.17A) (Kunsch & Rosen, 1993; Tak & Firestein, 2001). The EPEC effector protein NleE prevents NF-κB activation by inhibiting phosphorylation of IKK and subsequent degradation of IκB (Figure 1.19) (Nadler *et al.*, 2010; Newton *et al.*, 2010; Vossenkämper *et al.*, 2010; Yen *et al.*, 2010). NleB has a similar, though less pronounced, effect on IKK (Gao *et al.*, 2013; Li *et al.*, 2013; Nadler *et al.*, 2010; Pearson *et al.*, 2011). In addition, NleB reduces the GAPDH-mediated degradation of TNF receptor associated factor-2 (TRAF2), which is activated during the TNF-α-induced inflammatory response, which further inhibits NF-κB activation (Gao *et al.*, 2013; Li *et al.*, 2013). In contrast to NleE and NleB, NleC directly targets the NF-κB heterodimer by cleaving the p65 subunit at the N-terminus, degrading NF-κB (Figure 1.19) (Baruch *et al.*, 2011; Mühlen *et al.*, 2011; Pearson *et al.*, 2011; Yen *et al.*, 2010). The effector proteins NleH1 and NleH2 also target the NF-κB complex, specifically the non-Rel component human ribosomal protein S3 (RPS3), which inhibits the localisation of NF-κB to κB enhancer sites (Gao *et al.*, 2009; Pham *et al.*, 2012; Wan *et al.*, 2007). However, despite substantial homology between these proteins, these effectors induce different effects on RPS3, as NleH1 and NleH2 inhibit and activate RPS3, respectively (Gao *et al.*, 2009; Pham *et al.*, 2012). Interestingly, despite NleH1 reducing RPS3 translocation to the nucleus, an EHEC Δ*nleH1* mutant strain induced a more severe disease in infected pigs, which was associated with an enhanced inflammatory response, even though little diarrhoea was produced (Gao *et al.*,

2009). As both NleH1 and NleH2 can interact and bind to each other, this suggests that these proteins modulate the innate immune response in unison however, the mechanism behind this effect remains unknown (Pham *et al.*, 2012). Finally, the EPEC Tir protein also inhibits the NF-κB pathway through the recruitment of Src homology region 2 domain-containing phosphatase-1 (SH-1), which subsequently binds to and inhibits TRAF6 phosphorylation, preventing the activation of TGF-β activated kinase-1 and further downstream processes (Figure 1.19) (Ruchaud-Sparagano *et al.*, 2011; Yan *et al.*, 2012).

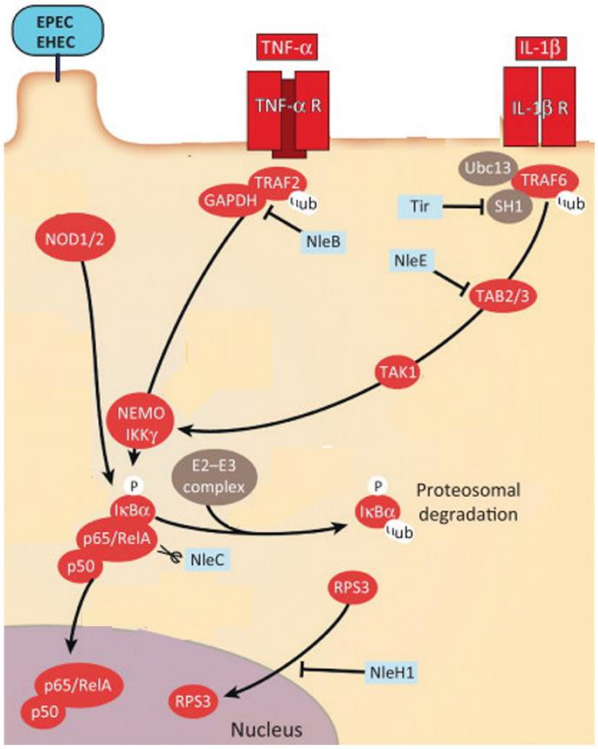

Figure 1.19: Inhibition of the NF-κB inflammatory pathway by EPEC effector proteins

EPEC effector proteins (blue) target both inflammatory cascade proteins (red) and helper proteins (brown) which initiate the innate immune response. Image adapted from Raymond *et al.* (2013).

While the NF-κB pathway is the predominant mechanism responsible for the innate immune response against EPEC infection, the MAPK pathway also contributes to inflammation through the induction of AP-1 (Figure 1.20). However, activation of the MAPK pathway is inhibited by EPEC effector proteins such as NleD, which cleaves JNK and p38, subsequently preventing the nuclear translocation of AP-1 (Marchés *et al.*, 2005). Interestingly, the loss of *nleD* does not reduce IL-8 protein production (Baruch *et al.*, 2011; Marchés *et al.*, 2005). Similarly to NleD, NleC also targets and cleaves p38, demonstrating the multifunctional activity of this protein, which also targets the p65 subunit of NF-κB (discussed above) (Sham *et al.*, 2011). In contrast to other EPEC effector proteins, NleH2 stimulates an increase in AP-1 activity however, the mechanism behind this effect remains to be determined (Gao *et al.*, 2009).

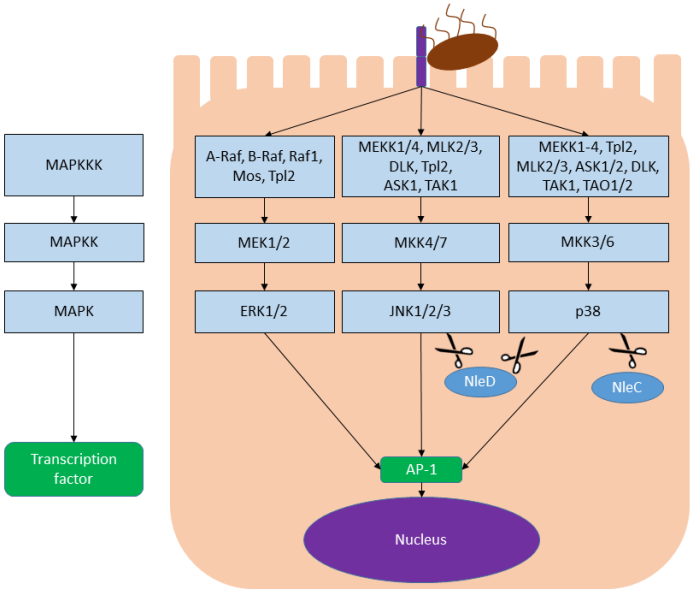

Figure 1.20: Inhibition of the MAPK signalling pathway by EPEC effector proteins

EPEC effector proteins NleD and NleC cleave signalling molecules in the MAPK signalling pathway. Image based on Morrison (2012).

1.3.4. The effect of *Lactobacillus* on innate defences

As the mucus layer is the primary colonisation site for lactobacilli in the GIT, as well as the initial barrier to infection, microbial modulation of this surface is an area of ongoing investigation (Juge, 2012; Walter, 2008). Upregulation of MUC3 gene and protein expression has been demonstrated by the probiotic strain *L. plantarum* 299v when incubated on HT-29 cells (Mack *et al.*, 2003; Mack *et al.*, 1999). Furthermore, changes in MUC3 expression in HT-29 cells were associated with a decrease in EPEC binding (Mack *et al.*, 2003; Mack *et al.*, 1999). *Lactobacillus* also modulate the production of secreted mucins, as MUC2 protein levels in LS174T cells and mice were increased by the *L. rhamnosus* GG secreted protein p40, which interacted with the EGF receptor (Wang *et al.*, 2014a). Furthermore, *L. reuteri* ATCC PTA 4659 and R2LC increased the mucus thickness in mice with DSS-induced colitis and in healthy controls (Ahl *et al.*, 2016).

In addition to enhancing the mucus layer, some *Lactobacillus* strains also have potent immunomodulatory characteristics, which can aid in the maintenance/ restoration of gut homeostasis during inflammatory events. Moreover, the influence of probiotics on ailments with persistent GI inflammation, such as IBD, has shown some promising outcomes, although further randomised controlled trials are required to ensure clinical efficacy (Ghouri *et al.*, 2014). The effects of probiotics on the innate immune response have generally been determined by measuring changes in pro-inflammatory cytokine production, using both *in vitro* and *in vivo* model systems. *Lactobacillus* strains are usually classified as either anti-inflammatory or immunostimulatory, such as *L. reuteri* ATCC PTA 6475 and *L. reuteri* ATCC 55730, respectively (previously discussed in 1.2.4.1.1 and 1.2.4.1.2). Importantly, immunomodulatory effects can be dependent on either the model system used or the immune stimulus, as *L. rhamnosus* GG decreased the production of IL-8 in Caco-2 cells treated with flagellin, yet induced IL-8 production in HT-29 cells infected with *S.* Typhimurium (Lopez *et al.*, 2008; Pinto *et al.*, 2009). Furthermore, mice with DSS-induced colitis presented with an increased disease severity when treated with *L. rhamnosus* GG relative to mice which did not receive the

probiotic, demonstrating that probiotic choice can influence clinical outcome *in vivo* (Mileti *et al.*, 2009).

The exact mechanisms responsible for the immunomodulatory characteristics of *Lactobacillus* strains are often poorly defined however, inhibition of IκB degradation has been suggested in multiple studies (Ko *et al.*, 2007; Lopez *et al.*, 2008; Ma *et al.*, 2004; Petrof *et al.*, 2009; Tien *et al.*, 2006). Alternatively, some *Lactobacillus* strains increase the production of anti-inflammatory cytokines by dendritic cells, such as IL-10 (Christensen *et al.*, 2002; Drakes *et al.*, 2004; Fernandez *et al.*, 2011; O'Hara *et al.*, 2006). Additionally, secreted products produced by lactobacilli have been associated with decreased expression of inflammatory cytokines (Coconnier *et al.*, 2000; Nemeth *et al.*, 2006). In a small number of studies, the decrease in inflammation has been directly associated with specific compounds, such as the anti-oxidant glutathione and histamine, which are produced by *L. fermentum* 5716 and *L. reuteri* ATCC PTA 6475, respectively (Peran *et al.*, 2006; Thomas *et al.*, 2012). Whether the production of these compounds is widespread amongst anti-inflammatory lactobacilli remains to be determined and is an area of ongoing research.

1.4. Model systems for studying bacterial interaction with the intestinal mucosa

1.4.1. Cultured epithelial cell lines

The most frequently used model for the assessment of both *Lactobacillus* and EPEC interactions with the intestinal epithelium are cultured epithelial cell lines. Generally, either HeLa cells, derived from a cervical carcinoma, or colon carcinoma cells have been used to determine the effects of lactobacilli and EPEC on microbial binding, the innate immune response, and mucin regulation (Law *et al.*, 2013). As the intestinal epithelium exhibits distinct polarity, cell-based models have been adapted to resemble this structure, with polarised Caco-2 and T84 cells frequently being used to determine the impact of bacterial interaction with the epithelia. However, the interactions between

microbes and cancer cell lines is known to differ from those which occur at the intestinal epithelium, which is due to a number of reasons (Law *et al.*, 2013). Firstly, the intestinal epithelium consists of four main cell types (enterocytes, goblet cell, enteroendocrine cells, and Paneth cells), whereas cultured epithelial cells represent a single cell type. Thus, the interaction and cross-communication between the various cell types in the epithelium is not observed in cultured cell line based models. Furthermore, the most frequently used IECs (HT-29, Caco-2, and T84) are enterocyte-like cells, which do not secrete mucins and are thus not protected by a mucus layer. As the mucus protects the epithelium *in vivo*, the absence of this layer removes an essential component of intestinal defence against microbes in the healthy intestinal environment (Johansson *et al.*, 2011b; Johansson *et al.*, 2011a; Johansson *et al.*, 2008). Thus, the use of non-mucus producing IECs to evaluate probiotic binding is more representative of ailments with a perturbed mucus barrier, such as IBD, rather than the healthy GIT. Finally, immortal cancer cell lines have undergone substantial genomic mutations which enable these cells to continuously propagate, whereas non-malignant cells enter senescence. This presents a key problem, as these mutations can alter the basic cell physiology and the function of the cell.

However, cancer cell lines enable the researcher to dissect the gut epithelium from the complex environment of the GIT, which can assist in the identification of novel bacterial features. Nonetheless, it is crucial to complement results from cell-based assays with more physiologically relevant models, to ensure that these findings are representative of the *in vivo* situation (Law *et al.*, 2013).

1.4.2. *In vivo* model systems

The identification of relevant *in vivo* models for EPEC infection remains an active area of investigation, as experiments on infants would be highly unethical (Goosney *et al.*, 2000; Law *et al.*, 2013). A small number of studies have been performed on adult volunteers to assess EPEC virulence (Donnenberg *et al.*, 1993b; Levine & Edelman, 1984; Levine *et al.*, 1985;

Tacket *et al.*, 2000; Vallance & Finlay, 2000). It is important to note that stomach acid neutralisation and a large EPEC dose were required to induce diarrhoea in adults, which does not represent the conditions required for natural infection (Nataro & Kaper, 1998).

Investigators have used gnotobiotic piglets as an *in vivo* infection model for human EPEC infection and identified EPEC colonisation in the porcine midileum (Moon *et al.*, 1983; Tzipori *et al.*, 1985). However, the required maintenance costs for larger animals and maintaining germ-free status, as well as few genetically modified lines, encourages the use of alternative model systems. Mice have also been utilised to investigate EPEC infection of the GIT, with Savkovic *et al.* (2005) identifying mucosal colonisation and neutrophil migration to the epithelium. However, these data are conflicting with other reports, which show minimal colonisation of the mouse GIT by EPEC (Klapproth *et al.*, 2005; Mundy *et al.*, 2006). In contrast, streptomycin-treated and neonatal mice supported EPEC adhesion when treated with a high bacterial dose, suggesting colonisation resistance by the intestinal microbiota as a cause for absent EPEC binding in conventional mouse models (Dupont *et al.*, 2016; Royan *et al.*, 2010). Although EPEC-infected germfree and neonatal mice demonstrated A/E lesion formation at the epithelial surface, these mice do not develop watery diarrhoea after infection (Dupont *et al.*, 2016; Meador *et al.*, 2014; Vallance & Finlay, 2000). Thus, no murine model to date presents with the complete repertoire of clinical symptoms associated with EPEC infection of human infants (Law *et al.*, 2013; Vallance *et al.*, 2002).

The natural murine pathogen *C. rodentium* has often been used as a proxy for EPEC to examine intestinal infection by A/E pathogens, as the *Citrobacter* genome contains a LEE PAI which shares 41 open reading frames with the EPEC LEE PAI (Deng *et al.*, 2001). However, 32% of the *C. rodentium* genome is not shared with EPEC, which includes at least seven putative T3S effector proteins (Petty *et al.*, 2010). Further differences from EPEC infection include location of intestinal colonisation, as *C. rodentium* adhere to the murine cecum and colon, and different pathologies, as *C. rodentium* infection does not induce profuse, watery diarrhoea in the host (Law *et al.*, 2013). Additionally, *C. rodentium* infection is not limited to infant mice (Law *et al.*,

2013). In contrast, infection of weaned rabbits with rabbit EPEC demonstrated a similar phenotype to human EPEC colonisation, as the infection was age restricted and induced a similar tissue pathology, alongside potentially lethal diarrhoea (Law *et al.*, 2013; Moon *et al.*, 1983; Robins-Browne *et al.*, 1994). However, the severity of diarrhoea in infant rabbits inhibits the investigation into moderate changes in disease phenotype (Law *et al.*, 2013). Furthermore, there are few genetically modified rabbit lines, which limits the evaluation of the host response to EPEC infection (Law *et al.*, 2013).

In contrast to EPEC infection studies, clinical studies investigating probiotic characteristics are more prevalent, as *Lactobacillus* species are generally regarded as safe (GRAS) and can thus be consumed without risk of harm to the participant, which reduces ethical and safety concerns (Salminen *et al.*, 1998). Furthermore, *L. reuteri* strain ATCC 55730 was not detrimental in immunocompromised patients, further supporting the GRAS status of these microbes (Wolf *et al.*, 1998). In addition to human volunteer studies, animals have been used to investigate probiotic characteristics including mice, rats, chickens, and pigs. However, as *L. reuteri* strains demonstrate ecological adaption to their original host species, this limits the use of animals when investigating the probiotic characteristics of human-derived strains, as findings in one species may not be applicable to another (Walter, 2008).

1.4.3. *Ex vivo* model systems

To overcome the lack of relevant *in vivo* models for EPEC infection studies, Knutton and colleagues developed an *in vitro* organ culture (IVOC) system, which used intestinal mucosal biopsy samples to investigate the interaction of A/E *E. coli* with the intestinal epithelium (Knutton *et al.*, 1987). IVOC has numerous advantages over traditional cell culture, including the presence of all major epithelial cell types and the protective mucus layer (Fang *et al.*, 2013). A limitation of IVOC is that EPEC infection is not restricted to the mucosal surface. However, a polarised system (pIVOC) has been developed, which confines bacterial access to the epithelial biopsy surface, enabling the

investigation of the innate immune response against EPEC infection (Schüller *et al.*, 2009).

IVOC is a valuable model for the study of microbe-host interactions at the intestinal mucosa however, there are a number of limitations which prevent the routine use of this system. Firstly, IVOC studies require ethical approval, as samples are acquired from patients in addition to any biopsies which are taken for diagnostic use. Secondly, access to human tissue can be unpredictable due to the need for patient consent. Thirdly, different host backgrounds can result in considerable variation between experiments. Finally, IVOC experiments are limited to approximately eight hours, as tissue survival is reduced in the absence of blood supply. While there are limitations to the IVOC method, this system is currently regarded as the gold standard for the investigation of pathogenic *E. coli* interaction with the intestinal mucosa (Girard *et al.*, 2007; Phillips & Frankel, 2000; Schüller *et al.*, 2009).

Human intestinal tissue has also been used in the investigation of probiotic *Lactobacillus* characteristics, such as samples from patients with IBD to determine the impact of probiotic strains on inflammation (Tsilingiri *et al.*, 2012). The interaction between lactobacilli and GI pathogens has also been explored with intestinal tissue from the porcine gut (Bogovič Matijašić *et al.*, 2006; Collins *et al.*, 2010). Nonetheless, the use of intestinal tissue for probiotic research remains a niche area.

1.5. Aims and objectives

The overarching aim of this PhD project was to determine the influence of probiotic *L. reuteri* on EPEC infection of the human intestinal epithelium, with particular focus on 1) EPEC binding, 2) mucus production, and 3) pro-inflammatory host response. Both *in vitro* and *ex vivo* human intestinal epithelial cell models were used in this study.

CHAPTER TWO

Methods and Materials

2.1. Bacterial strains and growth conditions

2.1.1. Bacterial strains

The bacterial strains used in this study are listed in Table 2.1. All experiments using EPEC were performed in a containment level 2 facility.

Strain	Description	Reference
L. reuteri		
ATCC PTA 6475	Human isolate	Oh *et al.* (2010)
ATCC PTA 6475 CmbA-	Human isolate	Etzold *et al.* (2014b)
DSM 20016	Human isolate	Oh *et al.* (2010)
LMS11-3	Human isolate	Oh *et al.* (2010)
ATCC 55730	Human isolate	Oh *et al.* (2010)
ATCC 53608	Pig isolate	Oh *et al.* (2010)
ATCC 53608 MUB-	Pig isolate	MacKenzie *et al.* (2010)
100-23C	Rat isolate	Oh *et al.* (2010)
LB54	Chicken isolate	Oh *et al.* (2010)
EPEC		
E2348/69	EPEC wildtype (O127:H6)	Levine *et al.* (1978)
CVD452	EPEC *escN* mutant	Jarvis *et al.* (1995)
AGT01	EPEC *fliC* mutant	Girón *et al.* (2002)
UMD864	EPEC *espB* mutant	Donnenberg *et al.* (1993a)

Table 2.1: Bacterial strains used in this study

2.1.2. Culture methods

L. *reuteri* were routinely cultured in de Man, Rogosa and Sharpe (MRS) culture medium (Oxoid), which is selective for *Lactobacillus* by low pH (6.2 ± 0.2). *L. reuteri* broth cultures were inoculated from frozen glycerol stocks and grown standing in an anaerobic cabinet (5% CO_2, 10% H_2 and 85% N_2, Don Whitley Scientific) at 37 °C overnight. *L. reuteri* colonies were cultured at 37 °C on MRS agar (1.5% w/v agar, Formedium) plates overnight under anaerobic conditions.

EPEC were routinely cultured in Lennox Lysogeny Broth (LB) (Formedium) and grown standing in aerobic conditions at 37 °C overnight. EPEC colonies

were cultured at 37 °C on LB agar (1.5% w/v agar) plates overnight under aerobic conditions.

EPEC cultures were stored as streaks on LB plates for up to one month at 4 °C before re-streaking. EPEC deletion mutants were selected with the appropriate antibiotic at the following concentrations: kanamycin (50 µg/ mL) and chloramphenicol (25 µg/ mL).

2.1.3. Cryopreservation and thawing of strains

Stock cultures of *L. reuteri* and EPEC were preserved in 15% (v/v) glycerol solution. A 50% glycerol solution was prepared in deionised distilled H_2O (ddH_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.

2.2. Cell culture

All cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4500 mg/ L glucose and 3700 mg/ L sodium bicarbonate (Sigma). DMEM medium was supplemented with 4 mM L-glutamine (Sigma) and 10% foetal bovine serum (FBS, Sigma). Cells were maintained at 37 °C in a 5% CO_2 atmosphere. Media used during routine cell culture were warmed to 37 °C prior to use. Cells were grown in 25 cm^2 culture flasks (Sarstedt and Greiner Bio-One) and passaged at full confluency.

HT-29 (ECACC 91072201) and LS174T cells (ECACC 87060401), originally isolated from human colon adenocarcinoma, were used between passages 5 to 20 and 7 to 27, respectively.

2.2.1. Trypsinisation and passaging of cell lines

At confluency, the cell culture media were removed and cells were washed twice with 4 mL PBS (Sigma) to remove residual FBS, which can deactivate trypsin. Cells were washed once with 0.5 mL 0.25% trypsin- 0.02% ethylenediaminetetraacetic acid (EDTA) solution (T/E, Sigma) before 0.5 mL T/E were applied. Cells were incubated at 37 °C until cell detachment from the flask (approximately 5 min). Trypsin was deactivated by resuspending the cells in 4.5 mL supplemented DMEM, and cells were split at dilution ratios of 1:10 into a new 25 cm^2 culture flask. Cells were split approximately every 7 days and the cell culture media were replaced every 2 days, to prevent acidification.

2.2.2. Determination of cell concentration and cell seeding

After trypsinisation, 10 µL of cell suspension was mixed with 10 µL trypan blue (Sigma) to identify nonviable cells. Trypan blue is unable to permeate the cell membrane of live cells, and only dead cells are stained blue. Viable cells were counted using an improved Neubauer haemocytometer (Hawksley; depth 0.1 mm) and an inverted light microscope (Zeiss Invertoskop ID03). Two fields (1 x 10^{-4} mL/ field) (Figure 2.1) were counted and multiplied by 10^4 to determine the cell concentration. The volume of cell suspension required for seeding (x) was determined by the following formula;

$$x = (y * w) / z$$

y is the desired number of cells/ well, w is the number of wells to be seeded, and z is the cell concentration.

Cells were seeded into 24 well plates at 1 x 10^5 cells/ well (HT-29) or 1.5 x 10^5 cells/ well (LS174T). Cells were seeded onto sterile coverslips (CS), for microscopy analysis, or directly onto wells for all other experiments. Cells were grown to confluence (7 days) and, prior to infection, cell culture media were replaced with plain DMEM.

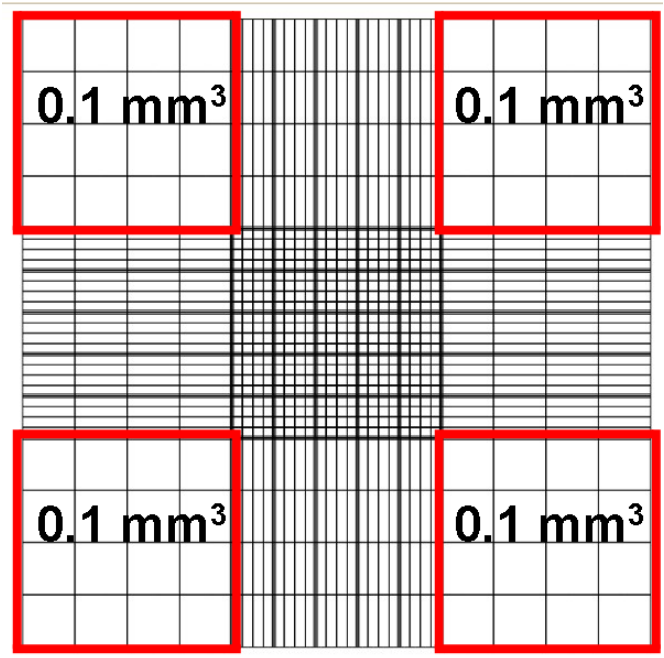

Figure 2.1: Neubauer cell counting chamber

Red boxes indicate the cell counting area with a defined volume of 0.1mm^3, equivalent to 10^{-4} mL.

2.2.3. Cryopreservation and resurrection of cell lines

2.2.3.1. Cryopreservation of cell lines

Cells were grown in 75 cm^2 culture flasks until confluence. Cells were trypsinised and resuspended in 5 mL supplemented DMEM, and the cell count was determined as described in 2.2.2. Cell concentration was adjusted to 2-4 x 10^6 cells/ mL, and 950 µL cell suspension was added to a freezing vial. 50 µL dimethyl sulfoxide (DMSO) (5% final concentration, Sigma) was added, and vials were placed in a freezing container (Mr Frosty, Nalgene), containing isopropanol (Sigma), 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.

2.2.3.2. Resurrection of cell lines

Frozen vials were removed from liquid nitrogen storage and warmed quickly to room temperature (RT). The cell suspension was transferred into 5 mL supplemented DMEM, resuspended and centrifuged for 7 min at 188 x *g* to remove DMSO. The supernatant was discarded, and the cell pellet was resuspended in 7 mL supplemented DMEM. The cell suspension was transferred into a 25 cm^2 culture flask and cultured as previously described.

2.3. Infection assay

2.3.1. Adhesion assay

Prior to inoculation, *L. reuteri* and EPEC were spun down at 18,000 x *g* for 5 min and resuspended in DMEM. Approximately 5 x 10^7 bacteria (Multiplicity of infection (MOI) of 50 bacteria/ cell) were incubated with the cell monolayers at 37 °C in a 5% CO_2 atmosphere for 1 h. The cell monolayers were washed with PBS (3x) to remove non-adherent bacteria, and processed according to further applications.

2.3.2. Protection assay

2.3.2.1. Short-term protection assay

For short-term protection assays, 5 x 10^8 *L. reuteri* (MOI 500) were pre-incubated with cell monolayers for 1 h. Subsequently, 5 x 10^6 EPEC (MOI 5) were added for 1 h. At the end of the experiment, cell monolayers were washed with PBS (3x), and processed according to further applications.

2.3.2.2. Long-term protection assay

Cells were pre-incubated with 5 x 10^8 *L. reuteri* for 4 h, and the cell culture media were tested with pH indicator strips (ThermoFisher, pH range 4.5-10.0 at 0.5 intervals) after the incubation to determine potential acidification.

Non-adherent lactobacilli were removed by washing with DMEM for 5 min on a rocking platform (30 rotation/ min), and cells were incubated with 5 x 10^6 EPEC for 1 or 3 h. At the end of the experiment, cells were washed with PBS (3x) and processed for further applications.

2.3.3. Contact killing assay

To assess the impact of *L. reuteri* on EPEC viability, 5 x 10^8 *L. reuteri* were pre-incubated for 1 h in 1 mL DMEM medium at 37 °C in 5% CO_2 atmosphere, and 5 x 10^6 EPEC were added and further incubated for 1 h. As 1% Triton X-100 (TX-100, Sigma) was used in protection assay experiments to release adherent bacteria (see 2.3.5), 1% TX-100 was added to the co-culture for 10 min after the EPEC incubation. EPEC survival was assessed by plating out serial dilutions of the co-culture on LB agar plates and viable EPEC were determined relative to the initial inoculum by plate counting.

2.3.4. Co-culture assay

To determine the effect of *L. reuteri* and EPEC on mucus production and the inflammatory response, 5 x 10^7 *L. reuteri* were incubated with 5 x 10^7 EPEC on cell monolayers at 37 °C in 5% CO_2 atmosphere for 6 h. Cell culture media were spun down at 18,000 *x g* for 5 min and the supernatant frozen at – 80 °C until processing. Cells were washed with PBS (3x) and processed for further applications.

2.3.5. Quantification of bacterial colonisation

Cells were lysed with 1% TX-100 in PBS for 10 min at RT to release adherent bacteria. The lysate was serially diluted in PBS, plated out and incubated overnight at 37 °C. Bacterial colonies were counted, and adherence was determined according to the following formula;

% adherence = (adherent bacteria/ mL/ inoculated bacteria/ mL) * 100

2.4. *In vitro* organ culture (IVOC) of human intestinal biopsy tissue

2.4.1. Ethical approval and collection of biopsy samples

This study was performed with ethical approval from the University of East Anglia Faculty of Medicine and Health ethics committee (ref 2010/11-030). All biopsies were collected through the Norwich Biorepository, which has National Research Ethics Service approval (ref 08/h0304/85+5). Samples were provided by the Gastroenterology Department at the Norwich and Norfolk University Hospital.

Up to six biopsies from the second part of the duodenum were obtained with informed consent from 87 adults (24-87 years; average age 64.7; 42 male and 45 female) undergoing routine upper endoscopy for investigation of GI symptoms (see Appendix 1 for a copy of the Norwich Biorepository information sheet and consent form for adult patients). Patients were not included in the study if they met any of the following criteria;

Previous infection of Human Immunodeficiency Virus or Hepatitis B.

The patient suffered from any conditions of the small bowel.

For studies investigating the immune response, patients were also excluded if they had used immunosuppressive medication within the past month.

Biopsies were taken from macroscopically normal areas, transported to the laboratory in IVOC medium and processed for experimentation within the next hour.

2.4.2. Culture of biopsy samples

Biopsies were maintained in IVOC medium (0.94 g NCTC 135 medium (Sigma), 0.22 g sodium bicarbonate (Sigma), and 1 g D-(+)-mannose (Sigma), to prevent bacterial adhesion by type I fimbriae, dissolved in 90 mL ddH_2O). The solution was filter sterilized with a 0.45 µm syringe filter (Sartorius Stedim), and combined with 90 mL DMEM and 20 mL newborn calf serum (Sigma). The medium was stored at 4 °C.

IVOC was performed as described previously (Knutton *et al.*, 1987). Briefly, biopsies were cut with a disposable scalpel to a diameter of 2-3 mm and orientated with the mucosal side upwards on a foam support (Simport) in a 12 well plate in 1 mL IVOC medium. IVOC medium in the well was adjusted to allow a thin film of medium to cover the biopsy. Biopsies were infected with 25 µL standing overnight culture (2.5×10^7 bacteria) and incubated on a seesaw rocker with 12 revolutions/ min in air with 5% CO_2 at 37 °C for up to 8 h. Medium was changed at 4 h and 6 h to maintain pH and prevent bacterial overgrowth. At the end of the experiment, biopsies were transferred to glass screw cap tubes and washed twice with PBS by vigorous shaking to remove mucus and non-adhering bacteria. Alternatively, biopsies were processed without washing to allow assessment of the mucus layer.

2.4.2.1. Polarised IVOC

The polarised IVOC technique was pioneered by Schüller *et al.* (2009), and is an adaption of IVOC which restricts bacterial access to the mucosal surface. For this, the biopsy was sandwiched between two Perspex disks (12 mm diameter, Faculty of Science Mechanical Workshop, University of East Anglia) with a central aperture (2 mm diameter), as demonstrated in Figure 2.2.

For mounting of the biopsy, a cellulose nitrate membrane filter (3 µm pore size, Whatman) was soaked in IVOC medium and placed onto the basal disk. The biopsy was placed centrally on the membrane and orientated with the mucosal side facing upwards under a dissection microscope. To prevent bacterial leakage, the apical disk was sealed to the mucosal biopsy surface with Histoacryl tissue glue (Braun Medical). The biopsy-containing unit was mounted into a Snapwell support (polycarbonate membrane, Corning) and placed into a 6 well plate with 3 mL IVOC medium in the basolateral chamber. For adherence assays, biopsies were submerged in 180 µL IVOC medium and infected with 20 µL standing overnight bacterial culture (2 x 10^7 CFU). The biopsies were incubated on a seesaw rocker at 37 °C in a 5% CO2 atmosphere for up to 6 h. Apical medium was removed after 2 h, and biopsies were washed with 200 µL IVOC medium. Biopsies were further incubated under slightly submerged conditions (~50 µl of medium) to reduce epithelial shedding. For protection assays, biopsies were pre-incubated with 10^9 *L. reuteri* in 300 µL IVOC medium for 2 h. Biopsies were washed with IVOC medium to remove non-adherent *L. reuteri* and biopsies were incubated with 2 x 10^7 EPEC for 4 h. At the end of the experiment, specimens were removed from the support, washed, and processed according to further applications.

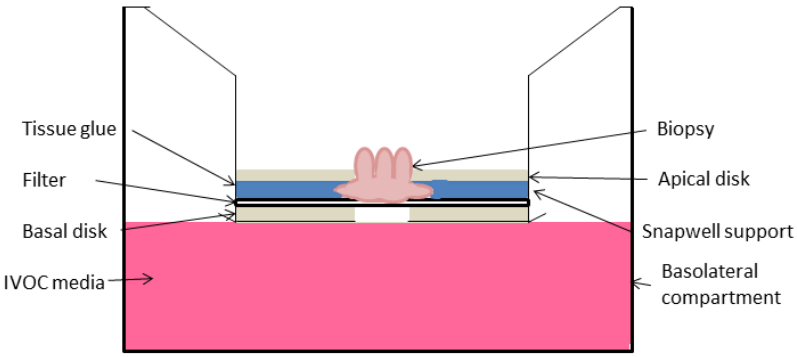

Figure 2.2: pIVOC system

Biopsies were mounted between two Perspex disks with a central aperture to limit bacterial access to the mucosal biopsy surface. Adapted from Schüller *et al.* (2009).

2.4.3. Scanning electron microscopy

Biopsies were fixed in 2.5% glutaraldehyde (Agar Scientific) in PBS overnight and stored at 4 °C until processing. All subsequent incubation steps were performed on a rocker. Samples were washed twice with PBS and once with ddH_2O for 10 min. The biopsies were dehydrated by subsequent incubation in 30%, 50%, 70%, and 90% acetone in ddH_2O for 15 min followed by two 15 min incubations in 100% acetone. The specimens were dried by incubation in tetramethylsilane (Sigma) for 10 min and air-dried for at least 10 min. Samples were orientated under a dissecting microscope and mounted with the mucosal surface facing upwards on aluminium stubs (TAAB Laboratory Equipment Ltd.) using silver paint (Agar Scientific). The biopsies were sputter-coated with gold (Polaron SC7640 sputter coater, Quorum Technologies), and viewed with a JEOL JSM 4900 LV scanning electron microscope (School of Environmental Sciences, UEA).

2.4.4. Biopsy cryosections

Biopsies with preserved mucus layers were embedded in optimal cutting temperature compound (Sakura) in a cryotube, snap-frozen in an ethanol/dry ice bath and stored at -80 °C. Serial sections of 7 µm were cut with a Microm HM550 cryostat (Thermo Scientific), picked up on poly L-lysine-coated slides (Agar Scientific) and air-dried for at least 30 min. Slides were stored at -80 °C until processing.

2.4.5. Quantification of bacterial colonisation of biopsies

Biopsies were transferred to microcentrifuge tubes and lysed in 1 mL 1% TX-100 for 15 min at RT. The specimens were homogenised with a sterile pestle (Sigma), and vortexed. Serial dilutions of the lysate were plated on LB agar, and adherent bacteria were quantified by plate counting.

2.4.6. Preparation of biopsy lysates for cytokine analysis

Biopsies were transferred to microcentrifuge tubes containing 250 µL ice cold lysis buffer (1% TX-100 and 0.5% protease inhibitor cocktail (Sigma) in PBS). Samples were incubated on ice for 5 min and homogenised with a sterile pestle. The lysate was centrifuged at 18,000 x *g* for 15 min at 4 °C to pellet insoluble proteins. The supernatant was removed and stored at -80 °C until further processing.

2.5. Fluorescence staining

2.5.1. Antibodies and dilutions

The antibodies used in this study are listed in Table 2.2.

Antigen	Host species	Dilution	Source
CmbA	Rabbit	1:250	D. MacKenzie
MUB	Rabbit	1:250	D. MacKenzie
Srr	Rabbit	1:250	D. MacKenzie
E. coli	Goat	1:500	abcam
NF-κB	Rabbit	1:200	Santa Cruz
MUC2	Mouse	1:250	Santa Cruz
MUC5AC	Rabbit	1:250	Santa Cruz

Table 2.2: Primary antibodies used in this study

All incubations with antibodies were performed at RT.

2.5.2. Staining of adherent cell lines and tissue cryosections

Slides containing cryosections were equilibrated to RT for 10 min, and sections were encircled with a PAP pen (Sigma) to prevent antibody crossover between sections. For mucus preservation, coverslips and cryosections were fixed with ice-cold methanol/ acetone (1:1; MeOH/ acetone) for 4 min on ice, and washed

once with PBS. For all other applications, fixation was performed in 3.7% formaldehyde in PBS (formalin) for 15 min at RT. All samples were stored at 4 °C in PBS until further processing.

Samples were incubated in 0.5% bovine serum albumin (BSA; Sigma), and 0.1% TX-100 (formalin-fixed samples only) in PBS for 20 min to block non-specific binding sites and permeabilise the cell membrane. All reagents were diluted in 0.5% BSA in PBS, as detailed in Table 2.2. Samples were washed once with PBS and incubated with the first primary antibody for 60 min at RT. Unbound antibody was removed by washing with PBS on a rocking platform for 10 min, and specimens were incubated with the secondary antibody for 30 min in the dark. Secondary antibodies were either Alexa Fluor-488 or Alexa Fluor-568 (IgG; Life Technologies) and were used at a 1:400 dilution. Unbound antibody was removed by washing, and incubations in primary/ secondary antibodies were repeated with the remaining antibodies. If required, samples were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; 1:5000; Roche) to label cell nuclei and/or fluorescein isothiocyanate (FITC)-conjugated phalloidin (1:200; Sigma) to visualise filamentous actin and actin pedestals. Samples were washed for 40 min with a single PBS change, air-dried for 5 min, and mounted in Vectashield (Vector Laboratories). Samples were stored at 4 °C in the dark until evaluation with a fluorescence microscope (Axiovert 200M or Axioimager M2, Zeiss).

2.5.3. Analysis of fluorescence microscopy images

To quantify EPEC actin pedestal and microcolony formation, images were taken from ten random fields of view at a set exposure time. EPEC were counted and each bacterium was classified on whether they were associated with an actin pedestal and whether they were contained in a microcolony (five or more bacteria grouped together).

To quantify mucin protein in LS174T cells, images were taken at set exposure times and analysed using ImageJ, a public domain imaging software (Wayne Rasband, NIH). Protein levels were determined by measuring fluorescence

intensity by analysis of integrated density. To reduce background, fluorescence intensity was limited to a minimum threshold for inclusion in the analysis which was kept constant for all images within an experiment.

2.6. Gene expression analysis using quantitative real time PCR (qPCR)

2.6.1. RNA extraction

Eukaryotic RNA from LS174T cells was extracted using the RNeasy Mini kit (Qiagen) with RNase-free tubes, tips, and reagents. Confluent LS174T cells were lysed with 350 µL RLT buffer, supplemented with 3.5 µL β-mercaptoethanol (Sigma) to inhibit intrinsic RNases. Cells were detached by scraping with a pipette tip. The lysate was homogenised by vortexing for 10 sec and stored at -80 °C until further processing. RNA extraction was performed according to the manufacturer's protocol for "RNA extraction from animal cells using spin technology". Genomic DNA was removed by "on-column" digestion with DNase I as described in the manufacturer's protocol. RNA was eluted in 30 µL RNase-free water and stored at -20 °C until further processing.

2.6.2. Assessment of RNA quality by agarose gel electrophoresis and spectrophotometry

RNA integrity was determined by agarose gel electrophoresis in Tris/Borate/EDTA (TBE) buffer (10x TBE:108 g Tris (Sigma), 55 g boric acid, and 40 mL 0.5 M EDTA, pH 8.0 (Sigma) in 1 L ddH_2O). RNA samples were labelled with DNA nontox dye (PanReac AppliChem) separated in a 1% (w/v) agarose (Sigma) gel at 250 mA and 90 V for 30 min (Consort E863), and visualised using a U:Genius ultra violet gel imager (Syngene). RNA integrity was confirmed by identification of the 28S and the 18S ribosomal RNA subunits (Figure 2.3).

RNA quantity and purity was assessed with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Protein contamination was evaluated by the ratio of absorbance at 260 nm and 280 nm with a value between 1.8 and 2.0 considered acceptable. The ratio of absorbance at 230 nm and 260 nm was used as a secondary measure to assess salt contamination, with a value between 1.5 and 2 considered acceptable. RNA quantity was determined by absorbance at 260 nm, and RNA concentrations were presented as ng/ µL.

Figure 2.3: Representative image of intact RNA

RNA degradation was assessed by agarose gel electrophoresis. Each lane represents an individual sample.

2.6.3. cDNA synthesis

Complementary DNA (cDNA) was synthesised from 1 µg RNA using the qScript cDNA supermix (Quanta Biosciences), according to the manufacturer's instructions, and a thermal cycler (Biometra Professional Trio). A reverse transcriptase-negative (RT-) control, with no added cDNA supermix, was run alongside the cDNA synthesis to assess DNA contamination during qPCR analysis. cDNA was diluted at a ratio of 1:3 with sterile nanopure H_2O and stored at 4 °C for up to 1 week or at -20 °C for long-term storage.

2.6.4. Primers used in this study

Target gene	Primer sequence	Product size (bp)	Reference
MUC1	F 5'CGACGTGGAGACACAGTTCA 3'	165	This Study
	R 5'AGAACACAGACCAGCACCAG 3'		
MUC2	F 5'ACTGCACATTCTTCAGCTGC 3'	233	This Study
	R 5'ATTCATGAGGACGGTCTTGG 3'		
MUC3A	F 5'ACCACCCTTACATCACGCAG 3'	121	This Study
	R 5'AAGCACACTGTCCCTGTTCC 3'		
MUC5AC	F 5'CTGGGGTCCTCATTCAGCAG 3'	212	This Study
	R 5'CCCGAATTCCATGGGTGTCA 3'		
MUC6	F 5'GCTTCGTATTCGACGGCAAC 3'	256	This Study
	R 5'ATGTCCACGACAAGGCTCAG 3'		
MUC12	F 5'TGAAGGGCGACAATCTTCCTC 3'	104	(F) Moal *et al.* (2005) (R) This Study
	R 5'AGTGTAGTCTGCCTCCAGGAT 3'		
MUC13	F 5'TGTAAACACAGCCACCAACCA 3'	158	This Study
	R 5'AAGTAGCTGTTGGGAAAGGTGT 3'		
MUC17	F 5'GTTTCAACACCACTGGCACC 3'	122	This Study
	R 5'CTGGTCCCGGTACTCCACTA 3'		
IL-8	F 5'TTGAGAGTGGACCACACTGC 3'	98	Ou *et al.* (2009)
	R 5'TGCACCCAGTTTTCCTTGG 3'		
YWHAZ	F 5'ACTTTTGGTACATTGTGGCTTCAA 3'	94	Jacob *et al.* (2013)
	R 5'CCGCCAGGACAAACCAGTAT 3'		
POLR2A	F 5'GATGGGCAAAAGAGTGGACTT 3'	180	Schüller *et al.* (2009)
	R 5' GGGTACTGACTGTTCCCCCT 3'		

Table 2.3: Primers used in this study

2.6.5. Primer design

All primers selected for this study were based on publically available human gene sequences (http://www.ensembl.org/index.html) and supplied by Sigma Genosys. Primers were designed using the PrimerBLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) with the following parameters:

- Primer length: 15-25 base pairs (bp)
- Melting temperature (Tm): 58 – 63 °C

- GC content: 20-80%
- Self-complementarity: <8

In addition, forward and reverse primers for a specific gene were designed to have a melting temperature within 2 °C of each other. When possible, the following additional parameters were employed:

- Amplicon length: 80 – 250 bp
- Amplicon GC content: <60%
- Spanning of exon-intron boundaries

Primers were evaluated for the formation of secondary structures and primer dimers using the Sigma design tool. Lyophilised oligonucleotides were resuspended in RNase-free water to a final concentration of 100 µM and stored at -20 °C.

2.6.6. qPCR

qPCR experiments were performed using SYBR® Green JumpStart™ *Taq* ReadyMix™ (SYBR) (Sigma). Each experiment was performed in a 96 well PCR plate (Sarstedt), with each well containing 5 µL SYBR Readymix, 1 µL primer solution (containing 10 µM forward and reverse primer), 0.1 µL internal reference dye, 1 µL cDNA, and 3 µL nanopure H_2O. The plate was kept on ice during pipetting to prevent amplification before the start of the cycling protocol. Samples were run in duplicate for each gene tested. Corresponding RT- and non-template controls (without cDNA) controls were included for each primer pair to assess DNA contamination of RNA preparations and reagents, respectively. The plate was sealed with transparent sealing film (Sarstedt) and spun down using a swing-rotor centrifuge at 2000 x *g* for 1 min, to gather reagents at the base of the wells. The qPCR reaction was performed using an ABI7500 Taqman real-time PCR system (Applied Biosystems) with the following cycling parameters:

Stage 1 – 1 cycle

- 95 °C for 2 min – Initial denaturation

Stage 2 – 40 cycles

- 95 °C for 30 sec – Denaturation
- 60 °C for 30 sec – Annealing
- 72 °C for 35 sec – Elongation

Stage 3 – 1 cycle

- 72 °C for 5 min – Final elongation (for gel electrophoresis)

Stage 4 – 1 cycle – Dissociation curve analysis

- 95 °C for 15 sec
- 65 °C for 60 sec
- 95 °C for 15 sec
- 60 °C for 15 sec

Amplification and dissociation curves were analysed using Taqman SDS software.

2.6.7. Primer validation

Primer specificity was assessed by dissociation curve analysis (single peak) and agarose gel electrophoresis to determine product size. Primer efficiency was determined by amplifying two-fold serial dilutions (1:2 to 1:32) of template cDNA. Cycle threshold (C_T) values were log transformed and plotted against dilutions, and the slope of the regression line was determined. Slopes of -3.0 to -3.6 were considered acceptable, indicating 85 to 115% amplification efficiency according to the formula;

Amplification efficiency = (10^(-1/slope-1)) *100.

2.6.8. Relative quantification of gene expression ($\Delta\Delta C_T$)

The $\Delta\Delta C_T$ method determines relative changes in gene expression between treated and non-treated samples (Livak & Schmittgen, 2001). C_T values for the gene of interest (GOI) were normalised by subtraction of the geometric mean of the reference genes (*YWHAZ* and *POLR2A*), the normalised value referred to as ΔC_T. To determine the relative change in gene expression ($\Delta\Delta C_T$) of the GOI, the non-treated ΔC_T was subtracted from the ΔC_T of the treated sample. Fold expression values were determined as follows:

$$\text{Fold-expression} = 2^{-\Delta\Delta CT}$$

2.7. Quantification of IL-8 expression by ELISA

IL-8 production was quantified using the human IL-8 enzyme-linked immunosorbent assay (ELISA) development kit (Peprotech) according to the manufacturer's instructions. Reactions were performed in Nunc Maxisorb immunoassay 96 well plates (Fisher Scientific), and colour development was quantified using a Benchmark Plus Microplate Spectrophotometer plate reader and Microplate Manager 5.2.1 software (Bio-Rad). IL-8 production in biopsy lysates was normalised against total protein content, determined using the DC protein colorimetric assay (Bio-Rad) according to the manufacturer's protocol for microtitre plates. The standard curve was prepared with 0.1 – 0.5 µg/ mL BSA in lysis buffer and 10 µL of sample/standard were used for analysis.

2.8. Statistical analysis

All data are shown as means ± standard errors of the mean (SE). Statistical analysis was performed using GraphPad Prism software (version 5). Student's T-test was used to determine differences between two groups. One-way ANOVA with Tukey's multiple comparisons test was used to determine differences between multiple groups. Non-parametric Kruskal-Wallis with Dunn's post hoc test was used for quantification of IL-8 production in biopsies.

All gene expression data were $\log_{10}$ transformed before statistical analysis. A *P* value of <0.05 was considered significant with degrees of statistical significance presented as follows: *=P<0.05, **=P<0.01, ***=P<0.001.

CHAPTER THREE

L. reuteri inhibition of EPEC adherence to the human intestinal epithelium

The work contained in this chapter was the basis of the following publication:

Walsham, A. D., MacKenzie, D. A., Cook, V., Wemyss-Holden, S., Hews, C. L., Juge, N., & Schüller, S. (2016). *Lactobacillus reuteri* Inhibition of Enteropathogenic *Escherichia coli* Adherence to Human Intestinal Epithelium. *Frontiers in microbiology*, *7*.

(See Appendix 2)

3.1. Introduction and objectives of study

Adhesion to the host is an important step in EPEC pathogenesis (Knutton *et al.*, 1987). EPEC adhere to the epithelium by forming A/E lesions, which have been observed in both human *in vitro* and *ex vivo* models (Knutton *et al.*, 1989; Knutton *et al.*, 1987). A/E lesions are characterised by intimate attachment of EPEC to the host cell, effacement of underlying microvilli and the polymerisation of actin beneath the bacterium (Knutton *et al.*, 1989; Moon *et al.*, 1983). Formation of the A/E lesion reduces the absorptive surface and, alongside the effects of other T3S effector proteins, contributes to the onset of diarrhoea (Moon *et al.*, 1983; Viswanathan *et al.*, 2009). Therefore, inhibition of EPEC binding to the host provides an attractive target for prevention and treatment strategies.

Probiotics offer a solution to inhibit pathogen binding, with those which persevere within the host being particularly valuable (Juge, 2012; Kleerebezem *et al.*, 2010). Probiotic persistence in the GIT is seen as beneficial as this increases the interaction time between the host and the probiotic to deliver beneficial effects, such as competing with pathogens to reduce infection (Lebeer *et al.*, 2008). To investigate probiotic-pathogen interaction, *in vitro* models have been developed which typically use three intervention protocols: pre-incubation of the probiotic before infection (protection), inoculation of pathogen and probiotic together (competition), and probiotic administration after infection (displacement). Protection assays simulate the use of probiotics as a preventative measure before infection, whereas displacement assays investigate the effect of probiotics after infection, resembling therapeutic intervention. Previous studies investigating the impact of lactobacilli on EPEC adherence to epithelial cell lines have demonstrated that pre- or co-incubation of live *Lactobacillus* spp. reduced EPEC binding (Bernet *et al.*, 1994; Coconnier *et al.*, 1993a; Forestier *et al.*, 2001; Sherman *et al.*, 2005). In addition, non-viable lactobacilli have also shown antagonistic effects against diarrhoeagenic *E. coli* adhesion to intestinal cell lines (Chauvière *et al.*, 1992b; Coconnier *et al.*, 1993a; Coconnier *et al.*, 1993b). Competitive exclusion has been suggested as one

mechanism responsible for the reduction in pathogen binding by probiotics, through both physical inhibition (steric hindrance) and competition for mutual binding sites (Chan *et al.*, 1985). While the specific host receptors are generally undefined, carbohydrate-containing receptors have previously been linked to adhesion of lactobacilli and enteric pathogens to host cells (Adlerberth *et al.*, 1996; Lee & Puong, 2002; Neeser *et al.*, 2000). Interestingly, cell-surface adhesins isolated from *L. crispatus* and *L. helveticus* reduced *S.* Typhimurium, EIEC, EHEC, and EPEC binding to epithelial cells and extracellular matrix proteins, suggesting mutual receptors (Chen *et al.*, 2007; Horie *et al.*, 2002; Johnson-Henry *et al.*, 2007).

However, the accessibility of cell-surface receptors to probiotic bacteria in a healthy individual is uncertain, as the epithelium is covered by a thick layer of mucus (Atuma *et al.*, 2001; Juge, 2012). The mucus layer of the GIT is the primary colonisation site for commensal bacteria, facilitating host-microbe and microbe-microbe interactions (Johansson *et al.*, 2011a). Yet, most studies investigating adherence of probiotic bacteria and their interaction with pathogens have generally used non-mucus producing IECs, such as Caco-2, HT-29, and T84 cells (Navabi *et al.*, 2013a; van Klinken *et al.*, 1996). As these cell lines lack a functional mucus layer, the observed interactions may better represent a diseased state such as IBD, in which the mucus layer has been compromised, rather than the healthy mucosa prior to infection (Sheng *et al.*, 2012).

In this study, we sought to determine the effects of *L. reuteri* on EPEC binding to the intestinal epithelium in the absence and presence of a mucus layer. For this, we selected the colon adenocarcinoma cell lines HT-29 and LS174T, as these cell lines represent the two interfaces for *L. reuteri* to exert an effect against EPEC binding; at the epithelial surface (HT-29) and in the mucus layer (LS174T). HT-29 cells resemble the enterocytes of the small intestine and produce MUC3, a membrane-bound mucin which is present in the small and large intestine (Mack *et al.*, 2003), and have been used in a number of studies investigating inhibition of pathogen colonisation by lactobacilli (Candela *et al.*, 2008; Mack *et al.*, 1999; Rousset, 1986; Zhang *et al.*, 2010b). In contrast, LS174T cells are goblet cell-like and produce MUC2 and MUC5AC, the major

secreted mucins of the intestines and stomach, respectively (Kuan *et al.*, 1987; van Klinken *et al.*, 1996). In addition to cancer cell lines, the IVOC model was utilised with human duodenal biopsies. IVOC demonstrates greater physiological relevance to the GIT, as biopsies demonstrated the native gut structure and were covered by a representative mucus layer. While IVOC is considered the gold standard for the investigation into EPEC interaction with the intestinal epithelium, few studies have utilised this model for probiotic research (Collins *et al.*, 2010).

3.2. Results

3.2.1. *L. reuteri* adhesion to HT-29 cells is strain specific

To select adhering *L. reuteri* strains for further protection assays, several *L. reuteri* isolates, from human, pig, chicken, and rat (Table 2.1), were incubated with HT-29 cells for 1 h. Bacterial adhesion was initially evaluated by fluorescence microscopy using antibodies specific to *L. reuteri* adhesins. As shown in Figure 3.1A, adhesion varied between *L. reuteri* strains with the rat isolate 100-23C demonstrating minimal adhesion, with few bacteria attached to HT-29 cells, while the human strain ATCC PTA 6475 and the pig isolate ATCC 53608 displayed greater adhesion, with bacteria localised across the monolayer. We also observed that ATCC 53608 demonstrated aggregative properties.

Bacterial binding to HT-29 cells was further quantified by plating out serial dilutions of cell lysates and counting colony forming units (CFU). Whereas ATCC 53608 demonstrated the highest adherence to HT-29 cells, the human strains ATCC PTA 6475 and DSM 20016 exhibited similar adhesion as EPEC and isolates ATCC 55730 and LB54, from human and chicken respectively, exhibited the lowest binding potential (Figure 3.1B). As the strains with the greatest binding, we selected ATCC PTA 6475 and ATCC 53608 for further analysis. Additionally, these *L. reuteri* isolates showed distinct binding patterns, as aggregates of ATCC 53608 were bound to individual HT-29 cells.

In contrast, fewer ATCC PTA 6475 were bound to individual cells, but these bacteria were more dispersed across the epithelial surface.

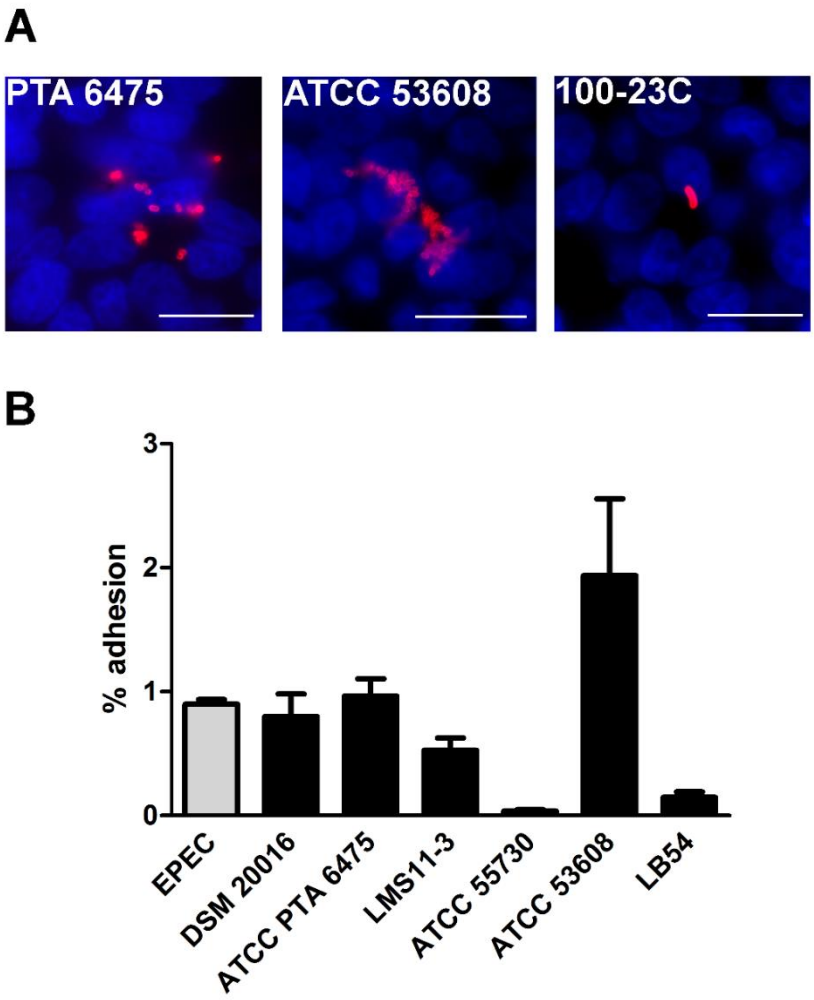

Figure 3.1: *L. reuteri* adhesion to HT-29 cells is strain specific

HT-29 cells were incubated with various *L. reuteri* strains and EPEC for 1 h. Adherent bacteria were observed by immunofluorescence staining (A) and quantified by plate counting (B). (A) ATCC PTA 6475, ATCC 53608 and 100-23C were stained with antisera specific to the adhesins CmbA, MUB, and SRR respectively (red) while cell nuclei were labelled with DAPI (blue). Images are representative of three independent experiments performed in duplicate. Scale bars = 10 µm. (B) Bacterial adhesion is expressed as a percentage relative to the inoculated dose. Data are shown as means ± standard error of the mean (SE) of three independent experiments performed in duplicate.

3.2.2. Mucus binding proteins increase *L. reuteri* adhesion to HT-29 and LS174T cells

The role of *L. reuteri* adhesins, such as CmbA (ATCC PTA 6475) and MUB (ATCC 53608), has previously been investigated, and binding to mucus (CmbA and MUB) and Caco-2 cells (CmbA) has been demonstrated (Jensen *et al.*, 2014; MacKenzie *et al.*, 2010). We aimed to identify the importance of the adhesins CmbA and MUB in binding to mucus-producing LS174T and mucus-deficient HT-29 cells. The presence of MUC2 was investigated in both cell lines by immunofluorescence staining, which demonstrated that MUC2 was only expressed in LS174T cells (Figure 3.2A).

To investigate the role of CmbA and MUB in *L. reuteri* adhesion to mucus and the epithelium, LS174T and HT-29 cells were incubated with ATCC PTA 6475, ATCC 53608, and isogenic adhesin deletion mutants (ATCC PTA 6475 CmbA- and ATCC 53608 MUB-) for 1 h. Quantification of adhered bacteria revealed that the absence of MUB significantly reduced adherence to HT-29 and LS174T cells (Figure 3.2B). We also observed a reduction in the binding of the CmbA- mutant relative to the wildtype strain, but this did not reach significance. Furthermore, the adhesion of all strains to LS174T cells was significantly higher than HT-29 cells.

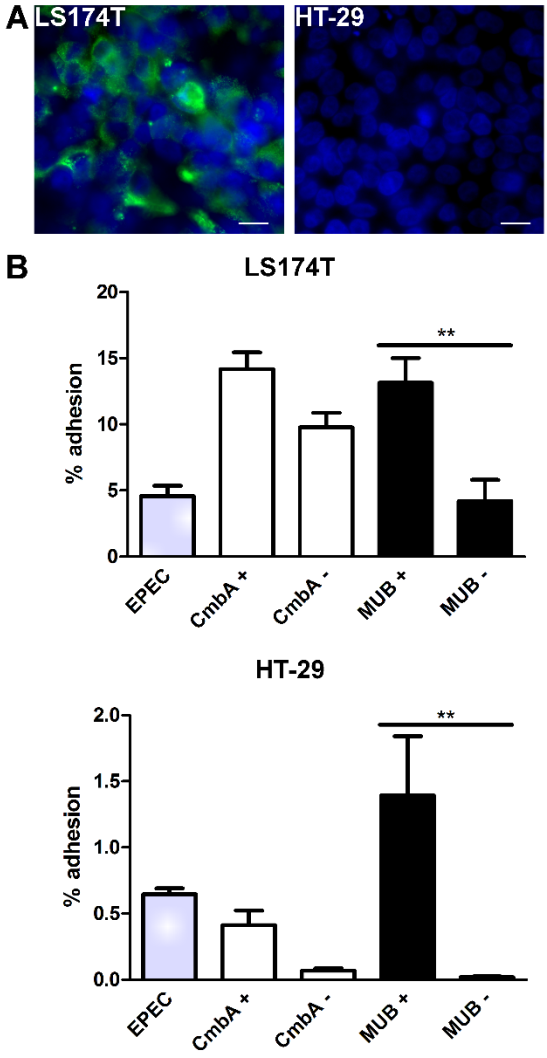

Figure 3.2: Mucus binding proteins enhance *L. reuteri* adhesion to HT-29 and LS174T cells

(A) Immunofluorescence staining confirmed production of MUC2 (green) in LS174T but not HT-29 cells. Cell nuclei were stained with DAPI (blue). Images are representative of three independent experiments performed in duplicate. Scale bar = 10 µm.

(B) EPEC, *L. reuteri* ATCC PTA 6475, ATCC 53608, or isogenic adhesin mutants (ATCC PTA 6475 CmbA- and ATCC 53608 MUB-) were incubated with HT-29 and LS174T cells for 1 h. Adhesion of bacteria is expressed as a percentage relative to the inoculated dose. Data are shown as means ± SE of three independent experiments performed in duplicate. ** = P <0.01.

3.2.3. *L. reuteri* inhibits EPEC adhesion to HT-29, but not LS174T cells

To investigate the effect of *L. reuteri* on EPEC adhesion, we pre-incubated HT-29 and LS174T cells with *L. reuteri* before EPEC infection. To determine the pre-treatment period for further experiments, HT-29 cells were pre-incubated with a 100x excess of *L. reuteri* for 1 or 3 h before addition of EPEC for 1 h. EPEC adhesion was assessed by plate counting. As shown in Figure 3.3A, pre-treatment of HT-29 cells with *L. reuteri* ATCC PTA 6475 and ATCC 53608 for 1 and 3 h significantly inhibited EPEC adhesion. As the protective effect did not significantly change between the 1 and 3 h pre-treatment, we used a 1 h pre-incubation for further experiments. We then determined the minimal dose required to inhibit EPEC binding by pre-incubating HT-29 cells with a 100x, 10x or 1x (equivalent numbers) excess of *L. reuteri,* relative to the EPEC inoculum, before infecting with EPEC for 1 h. While 100x excess of ATCC PTA 6475 or ATCC 53608 significantly inhibited EPEC adhesion, no effect was observed with a 10x or 1x excess of *L. reuteri* (Figure 3.3B). In contrast to HT-29 cells, pre-treatment with a 100x *L. reuteri* excess did not significantly reduce EPEC binding to LS174T cells (Figure 3.3C).

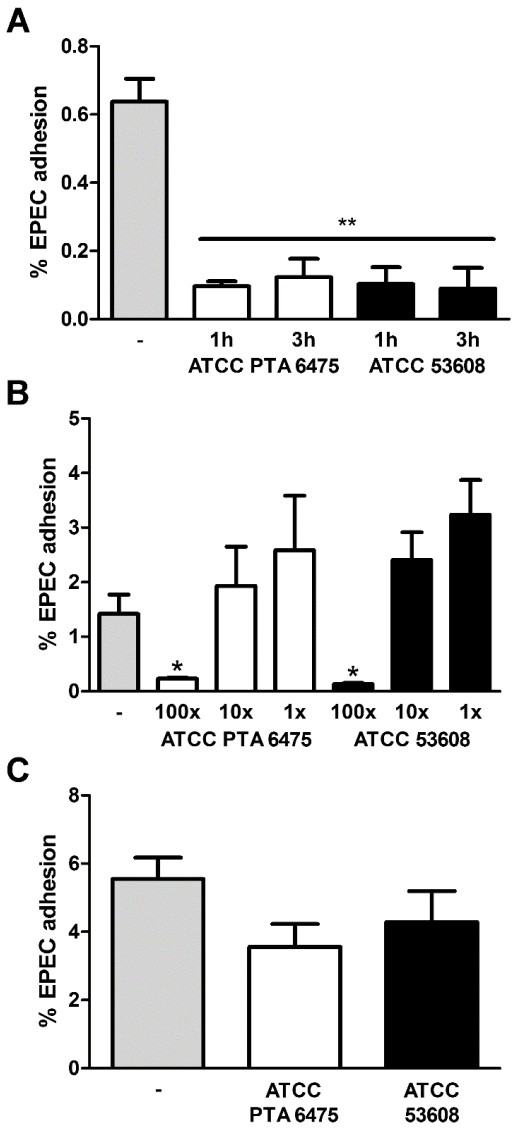

Figure 3.3: *L. reuteri* pre-incubation inhibits EPEC adhesion to HT-29, but not LS174T cells

(A) HT-29 cells were pre-incubated with a 100x excess of *L. reuteri* ATCC PTA 6475, ATCC 53608, or left untreated (-) for 1 h or 3 h before EPEC was added for 1 h. Cell-associated EPEC were quantified by plate counting and adhesion was expressed as a percentage relative to the EPEC inoculum. Data are shown as means ± SE of three independent experiments performed in duplicate. * = P <0.05; ** = P <0.01.

(B) HT-29 cells were pre-incubated with a 100x, 10x or 1x (equivalent numbers) excess of *L. reuteri* ATCC PTA 6475, ATCC 53608 or left untreated (-) for 1 h before the addition of EPEC for 1 h. Cell-associated EPEC were

quantified by plate counting, and adhesion was expressed relative to inoculated EPEC. Data are shown as means ± SE of three independent experiments performed in duplicate. * = P <0.05.

(C) LS174T cells were pre-incubated with a 100x excess of *L. reuteri* ATCC PTA 6475, ATCC 53608, or left untreated (-) for 1 h before the addition of EPEC for 1 h. Cell-associated EPEC were quantified by plate counting, and adhesion was expressed relative to inoculated EPEC. Data are shown as means ± SE of three independent experiments performed in duplicate.

To determine whether inhibition of EPEC adhesion to HT-29 cells by *L. reuteri* was dependent on *L. reuteri* adhesion to the epithelium, HT-29 cells were incubated with a 100x excess of *L. reuteri* for 2 h (total incubation time of *L. reuteri* and HT-29 cells in previous experiments) and washed to remove unbound bacteria before the addition of EPEC for 1 h. In addition, the effect of *L. reuteri* secreted products on EPEC adherence was investigated by incubating HT-29 cells with *L. reuteri*-conditioned cell culture medium (DMEM pre-incubated with *L. reuteri* for 2 h) before infection with EPEC for 1 h. As shown in Figure 3.4, EPEC binding was not significantly reduced by adherent *L. reuteri* or conditioned medium.

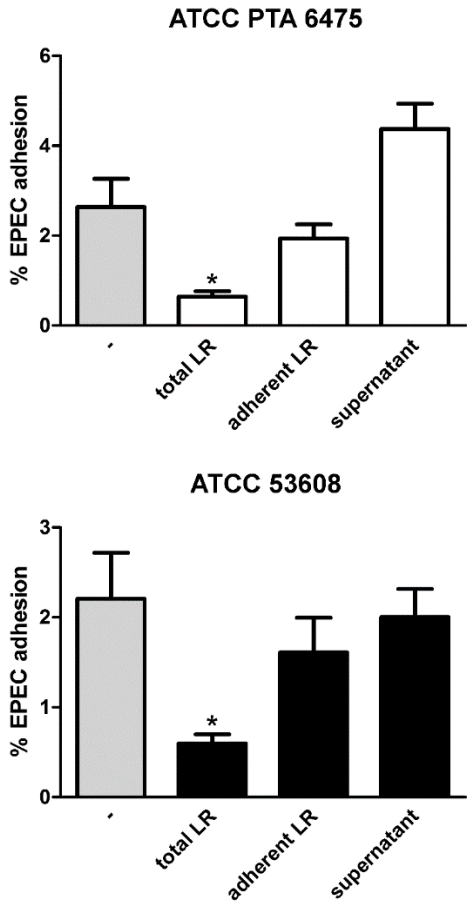

Figure 3.4: Inhibition of EPEC adhesion is independent of adherent *L. reuteri* and secreted anti-microbial compounds

HT-29 cells were exposed to the following treatments before incubation with EPEC for 1 h: *L. reuteri* for 1 h (total LR), *L. reuteri* for 2 h and subsequent removal of non-adherent bacteria (adherent LR), *L. reuteri*-conditioned medium for 2 h (supernatant), or non-treated (-). Cell-associated EPEC were quantified by CFU and adhesion was expressed as a percentage relative to inoculated EPEC. Data are shown as means ± SE of three independent experiments performed in duplicate. * = P <0.05.

3.2.4. *L. reuteri* ATCC 53608 decreases EPEC viability in co-culture

While secreted products from *L. reuteri* did not affect EPEC adherence, we further investigated whether *L. reuteri* or its secreted products reduced EPEC viability. A 100x excess of *L. reuteri* or *L. reuteri*-conditioned cell culture medium was pre-incubated for 1 h in cell-free wells before the addition of EPEC for 1 h. While incubation with ATCC PTA 6475 or conditioned media did not affect the number of EPEC recovered, incubation with ATCC 53608 demonstrated a reduction in recovered EPEC, although this did not reach significance (Figure 3.5A).

As described in previous studies (MacKenzie *et al.*, 2010) and demonstrated in Figure 3.1A, ATCC 53608 forms autoaggregates mediated by the adhesin MUB. This property may have caused the reduced EPEC CFU counts observed in the previous assay by “trapping” EPEC bacteria within bacterial clusters. To investigate this hypothesis, co-culture experiments were repeated with ATCC 53608 MUB-, which does not aggregate (Figure 3.5B). In addition, some co-cultures were treated with TX-100, the detergent we used to lyse the HT-29 and LS174T cell monolayers, at the end of the experiment to determine whether TX-100 affected aggregate formation or EPEC recovery. As shown in Figure 3.5C, co-incubation with ATCC 53608 or the MUB-deficient mutant significantly decreased the recovery of EPEC. In addition, TX-100 treatment did not impact on EPEC growth or ATCC 53608-mediated reduction of EPEC retrieval.

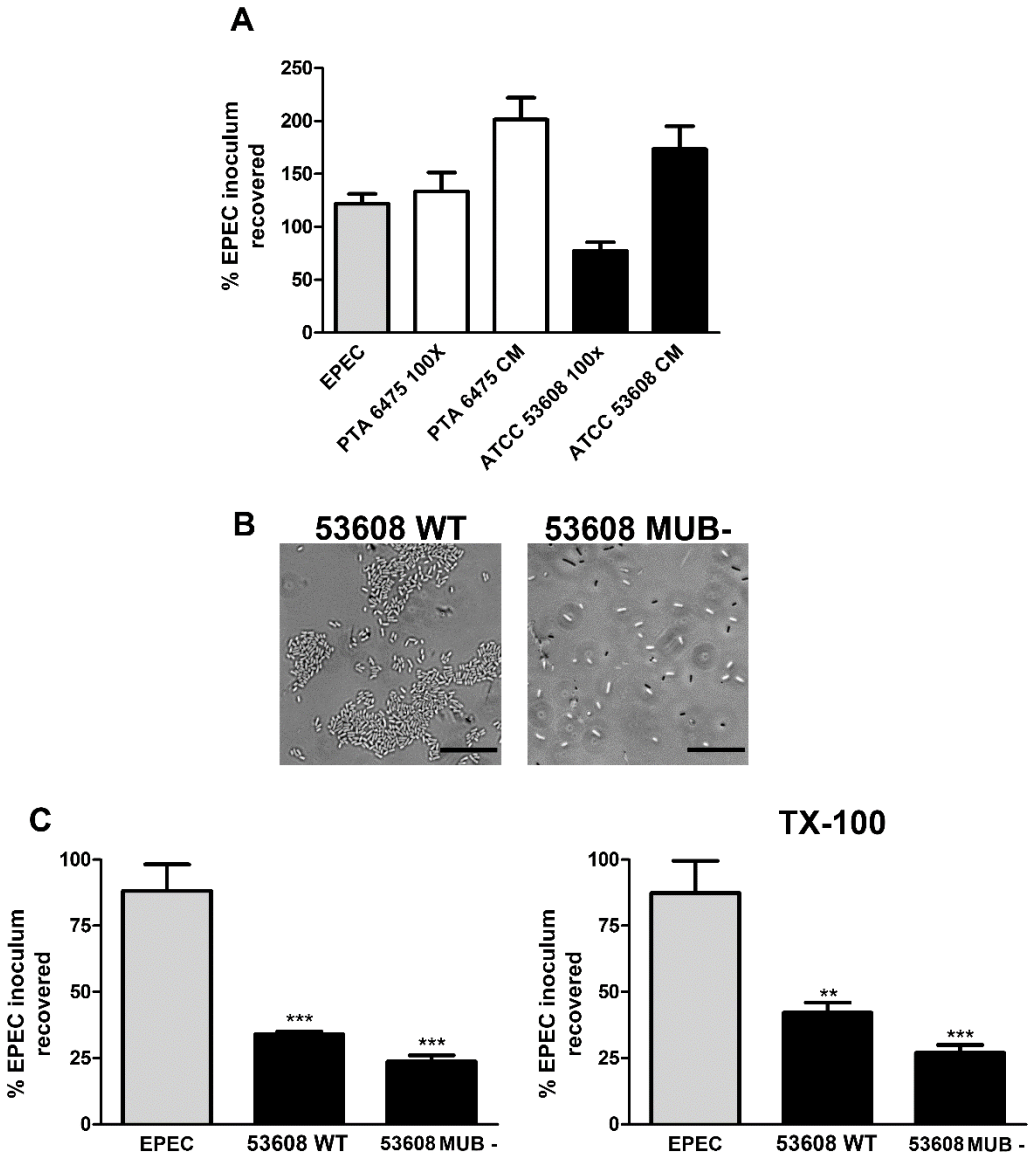

Figure 3.5: Co-incubation with *L. reuteri* ATCC 53608 reduces EPEC recovery independent of aggregate formation

(A) *L. reuteri* ATCC PTA 6475, ATCC 53608 (100x excess), conditioned medium, or untreated medium was pre-incubated for 1 h before the addition of EPEC for 1 h. Recovered EPEC were quantified by plate counting and expressed as a percentage relative to the number of inoculated bacteria. Data are shown as means ± SE of three independent experiments performed in duplicate.

(B) Phase contrast images of *L. reuteri* ATCC 53608 wildtype and ATCC 53608 MUB-. Images are representative of three independent experiments performed in duplicate. Scale bars = 10 µm.

(C) *L. reuteri* ATCC 53608 wildtype or a MUB-deficient mutant (53608 MUB-) were pre-incubated for 1 h before the addition of EPEC for 1 h. At the end of the experiment, some co-cultures were treated with TX-100, and EPEC were quantified by plate counting. Recovered EPEC are expressed as a percentage relative to the number of inoculated EPEC. Data are shown as means ± SE of three independent experiments performed in duplicate. Asterisks denote a significant difference from EPEC only control. ** = P <0.01; *** = P <0.001

3.2.5. Adherent *L. reuteri* ATCC PTA 6475 inhibit EPEC adhesion to HT-29 but not LS174T cells

In our previous experiments, we did not observe inhibition of EPEC binding to HT-29 cells by adherent *L. reuteri* after 2 h incubation (Figure 3.4). However, after a 1 h incubation, few *L. reuteri* were bound to HT-29 cells (Figure 3.1A). To increase *L. reuteri* adhesion, incubations were extended to 4 and 6 h, and adhesion was assessed by immunofluorescence. Whereas binding of *L. reuteri* was similar after 4 or 6 h incubation (data not shown), detachment of HT-29 and LS174T cell monolayers increased with longer incubations. Therefore, a 4 h pre-incubation period was selected for further studies.

As shown in Figure 3.6A, *L. reuteri* ATCC PTA 6475 and ATCC 53608 extensively adhered to LS174T cells, while binding to HT-29 cells was less pronounced. In addition, aggregate formation of ATCC 53608 was evident on HT-29 cells, as previously observed (Figures 3.6A). Having established efficient *L. reuteri* adherence, the protection assay was modified. Cells were pre-incubated with *L. reuteri* (100x excess) for 4 h, non-adherent *L. reuteri* were removed, and EPEC were added for 1 h. Results demonstrated in Figure 3.6B showed that adherent ATCC PTA 6475, but not ATCC 53608, significantly inhibited EPEC adherence to HT-29 cells. In contrast, no effect was observed in LS174T cells (Figure 3.6B).

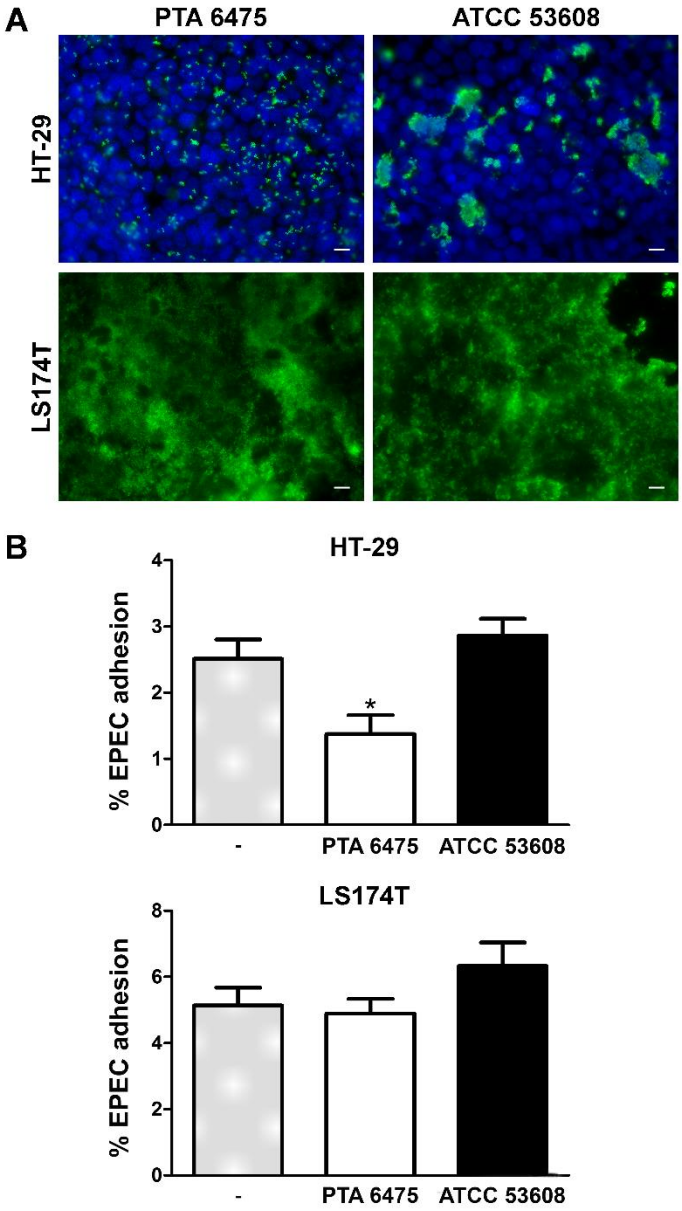

Figure 3.6: Adherent *L. reuteri* ATCC PTA 6475 inhibit EPEC binding to HT-29 but not LS174T cells

(A) Confluent HT-29 and LS174T cells were incubated with ATCC PTA 6475 or ATCC 53608 for 4 h. Adherent *L. reuteri* (green) were visualised by immunofluorescence microscopy. Cell nuclei were counterstained with DAPI (blue). Colour channels are merged for HT-29 whereas only the green channel is shown on LS174T cells, to improve clarity of *L. reuteri*. Images are representative of three independent experiments performed in duplicate. Scale bars = 10 µm.

(B) HT-29 and LS174T cells were pre-incubated with ATCC PTA 6475, ATCC 53608 (100x excess), or left untreated (-) for 4 h. Non-adherent bacteria were removed and EPEC were added for 1 h. Cell-bound EPEC were quantified by plate counting, and adhesion was expressed as a percentage relative to inoculated EPEC. Data are shown as means ± SE of three independent experiments performed in duplicate. * = P <0.05.

3.2.6. Pre-incubation with *L. reuteri* ATCC PTA 6475 increases the formation of EPEC microcolonies on LS174T cells

As LS174T cells produce mucus, CFU counts in cell lysates may not provide an accurate measure of EPEC adherence to the epithelial surface. To differentiate between mucus- and cell surface-bound bacteria, we utilised the fluorescent actin staining test to identify actin pedestal formation, a characteristic of A/E lesion formation (Figure 3.7A). EPEC were incubated with LS174T cells for 3 h to enable pedestal formation, and total EPEC and EPEC associated with pedestals were quantified by immunofluorescence microscopy and counting. While pre-treatment with *L. reuteri* did not influence EPEC A/E lesion formation (Figure 3.7B), more prominent clusters of EPEC were observed in LS174T cells pre-incubated with ATCC PTA 6475 (Figure 3.7C). Quantification of EPEC contained within these microcolonies confirmed that significantly more EPEC were located in microcolonies after pre-incubation with ATCC PTA 6475 compared to ATCC 53608- and non-treated cells (Figure 3.7D).

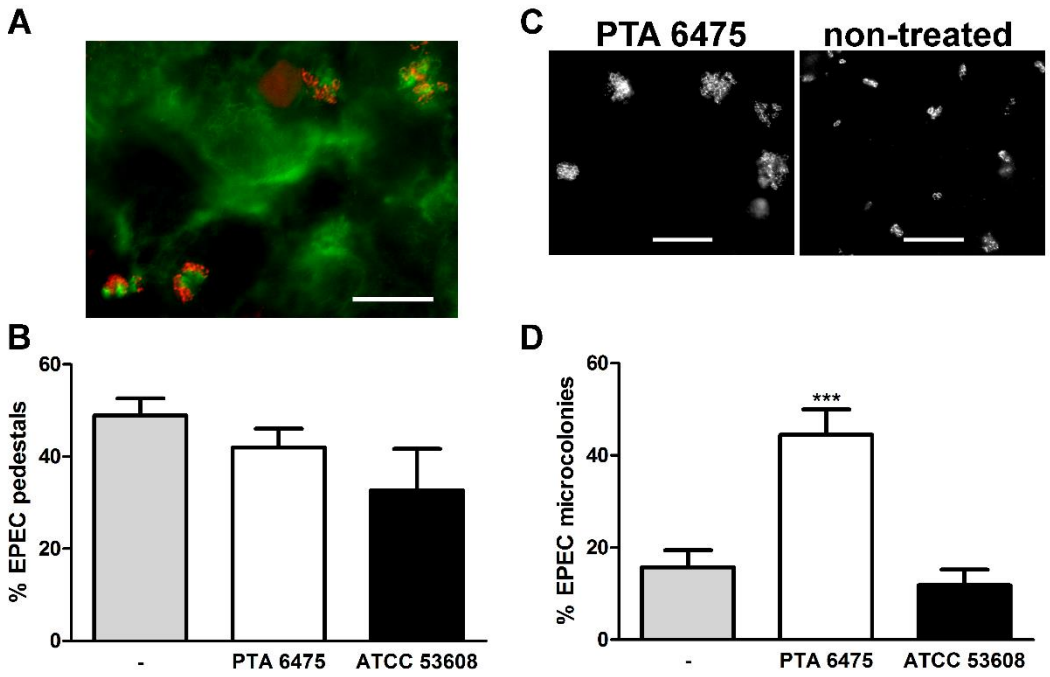

Figure 3.7: Adherent ATCC PTA 6475 enhance EPEC microcolony formation in LS174T cells

(A) LS174T cells were incubated with EPEC (red) for 3 h. Actin pedestal formation was visualised by immunofluorescence staining for filamentous actin (green) and *E. coli* (red). Image representative of four independent experiments performed in duplicate. Scale bar = 10 µm.

(B) Quantification of EPEC associated with actin pedestals. LS174T cells were pre-incubated with ATCC PTA 6475, ATCC 53608 or left untreated (-) for 4 h, and EPEC were added for 3 h. Pedestal formation was expressed as a percentage relative to the total number of adherent EPEC. Data are shown as means ± SE of four independent experiments performed in duplicate.

(C) Pre-incubation of LS174T cells with ATCC PTA 6475 increased EPEC microcolony formation compared to untreated cells (-). EPEC were visualised by immunofluorescence staining and are shown in black and white to increase clarity. Image is representative of four independent experiments performed in duplicate. Scale bars = 10 µm.

(D) Quantification of EPEC microcolony formation (five or more bacteria). EPEC contained within microcolonies were expressed as a percentage

relative to the total number of adherent EPEC observed. Data are shown as means ± SE of four independent experiments performed in duplicate. *** = P <0.001

3.2.7. *L. reuteri* is localised in the mucus layer of human duodenal biopsies

While cultured cells are a useful tool in the assessment of host-microbe interactions, these cell lines lack the complex epithelial structure formed from multiple cell types (Law *et al.*, 2013). Thus, findings using cultured cells require further validation in more physiologically relevant model systems, such as IVOC. Therefore, to further investigate bacterial binding to the human intestinal mucosa, duodenal biopsies were inoculated with *L. reuteri* ATCC PTA 6475, ATCC 53608, or EPEC for 8 hours, and colonisation was assessed by scanning electron microscopy (SEM). To distinguish between bacterial localisation in the mucus layer and at the epithelial surface, biopsies were fixed with either the mucus layer present or removed to reveal the epithelium. We identified that EPEC were localised in the mucus layer and at the epithelial surface of the biopsy, with prominent A/E lesion formation and microvillous elongation across the villous surface (Figure 3.8A). In contrast, both *L. reuteri* strains were located exclusively in the mucus layer, with no apparent binding to the epithelium (Figure 3.8A, images shown for ATCC PTA 6475 only). This was confirmed by immunofluorescence staining of cryosections, which demonstrated that *L. reuteri* were restricted to the mucus layer (Figure 3.8B).

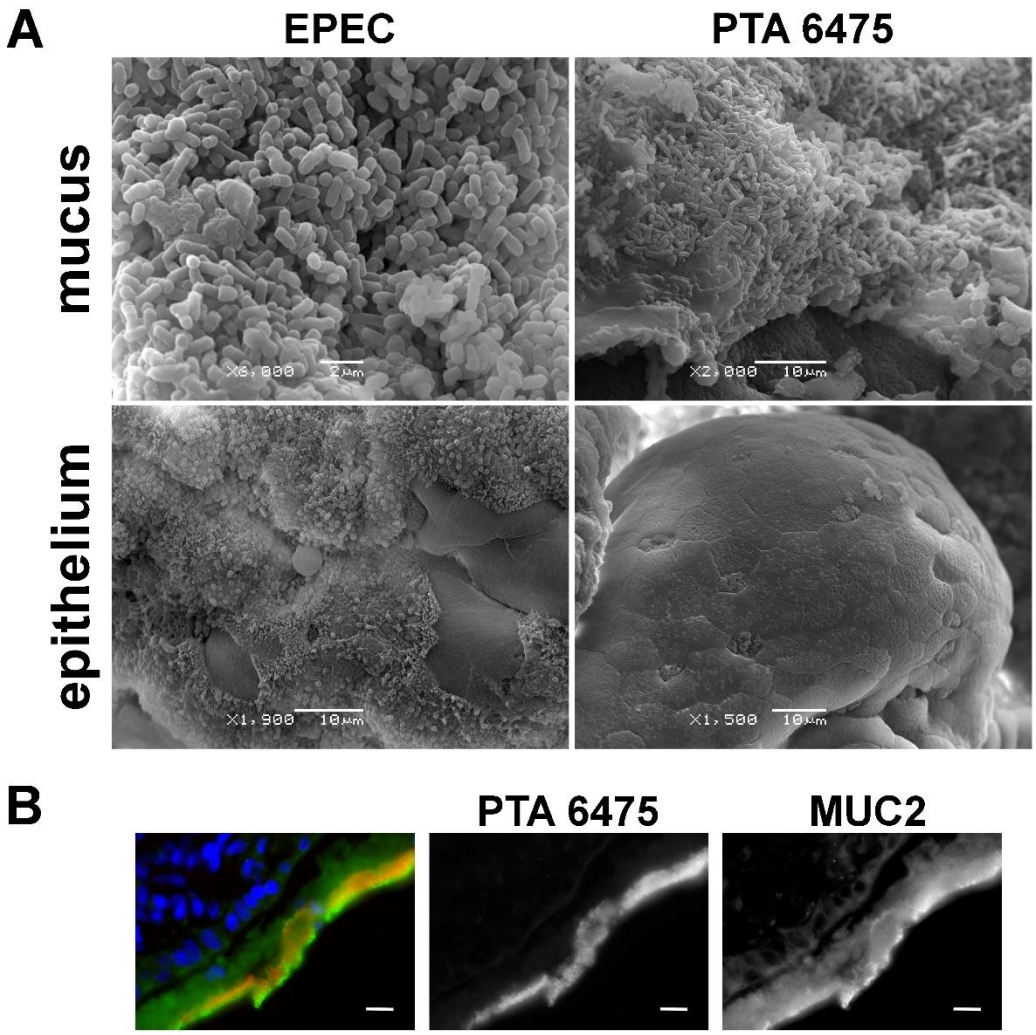

Figure 3.8: *L. reuteri* localise to the mucus layer of human duodenal biopsies

(A) SEM of duodenal biopsies incubated with *L. reuteri* ATCC PTA 6475 or EPEC for 8 h. Biopsies were fixed with the mucus layer present (mucus) or washed to allow visualisation of the epithelium (epithelium). Images are representative of three independent experiments performed in duplicate.

(B) Fluorescence microscopy of duodenal biopsies incubated with *L. reuteri* ATCC PTA 6475 for 8 h. Cryosections were stained for MUC2 (green), ATCC PTA 6475 (red) and cell nuclei (blue). Images are representative of three independent experiments performed in duplicate. Scale bars = 10 µm.

3.2.8. Pre-incubation with *L. reuteri* inhibits EPEC colonisation of the intestinal epithelium by polarised IVOC

To quantify EPEC adherence to the duodenal biopsy epithelium, a polarised IVOC (pIVOC) model restricting bacterial access to a defined mucosal surface area was used (Schüller *et al.*, 2009). The time period of EPEC infection was optimised to enable sufficient colonisation of the epithelium without epithelial shedding. Biopsies were infected for 6 to 8 h, and EPEC colonisation and epithelial integrity were assessed by SEM. While no epithelial shedding was observed on non-infected controls (8 h incubation), substantial shedding of the epithelium occurred after 7 and 8 hours of EPEC infection (Figure 3.9). In contrast, epithelial integrity was maintained after 6 h of infection (Figure 3.9), and this period was chosen for further pIVOC experiments.

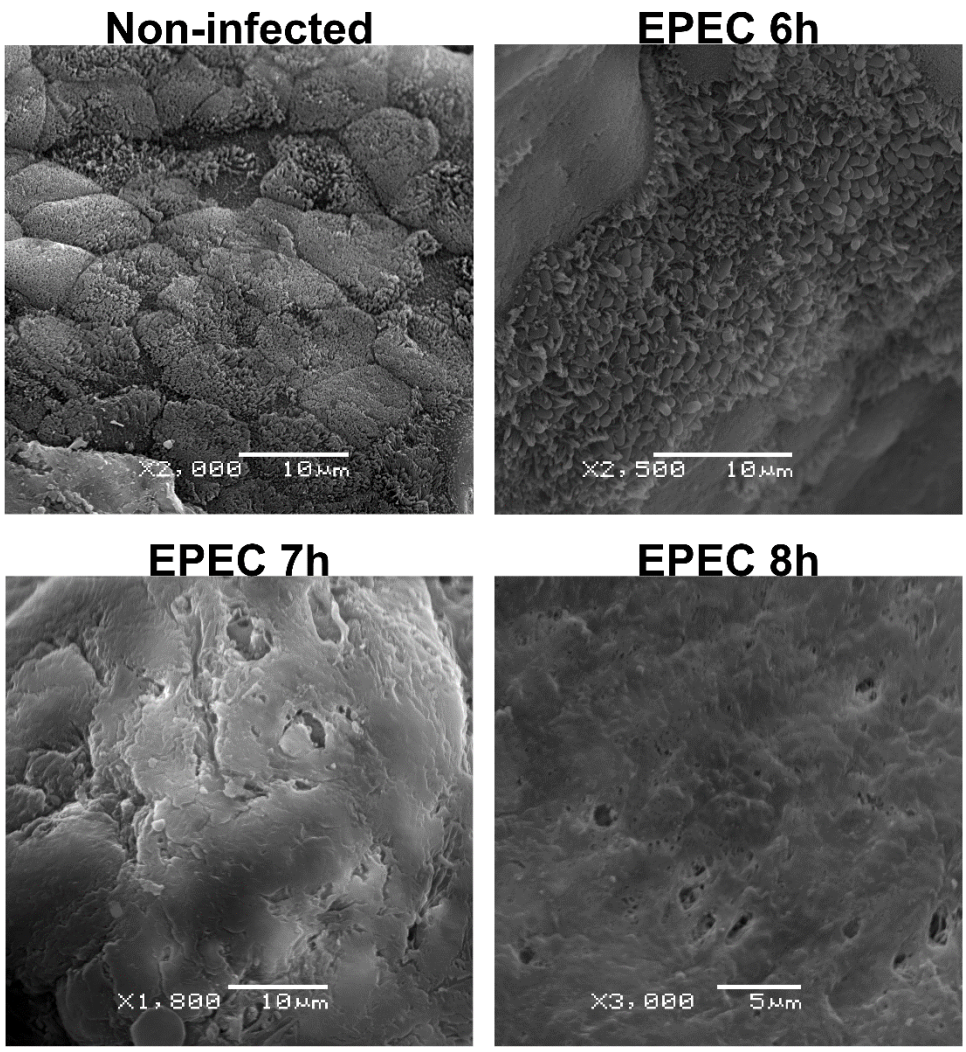

Figure 3.9: EPEC colonisation of human duodenal biopsy epithelium in pIVOC

Biopsies were infected with EPEC or left non-infected for 6 to 8 h and examined by SEM. The epithelium was preserved on both uninfected (8 h) and biopsies incubated with EPEC for 6 h, demonstrated by the discernible brush border. However, shedding of the biopsy epithelium and exposure of the basement membrane was observed after 7 and 8 h of EPEC infection. Images are representative of eight independent experiments performed in duplicate.

3.2.9. *L. reuteri* reduces EPEC colonisation of human duodenal biopsy epithelium

To investigate the influence of *L. reuteri* on EPEC colonisation *ex vivo,* duodenal biopsies were pre-incubated with 50x excess of ATCC PTA 6475, ATCC 53608, or left untreated (-) for 2 h. After removal of non-adherent *L. reuteri,* biopsies were infected with EPEC for 4 h. At the end of the experiment, the mucus layer was removed by washing and EPEC colonisation of the epithelium was quantified by plating out biopsy lysate and determining CFUs. Our data revealed a significant reduction in EPEC colonisation by *L. reuteri* compared to non-treated biopsies (Figure 3.10).

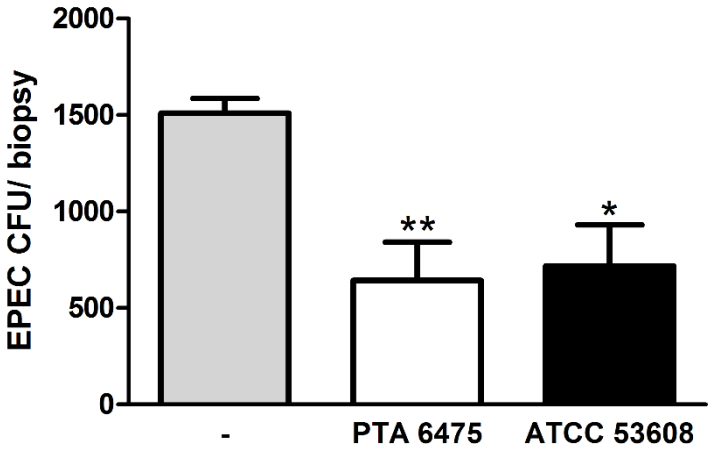

Figure 3.10: *L. reuteri* ATCC PTA 6475 and ATCC 53608 significantly inhibit EPEC colonisation of duodenal biopsy epithelium

Duodenal biopsies were pre-incubated with ATCC PTA 6475, ATCC 53608, or left untreated (-) for 2 h, non-adherent bacteria were removed, and EPEC were added for 4 h. Cell-bound EPEC were quantified by plate counting and adhesion is presented as EPEC CFU/ biopsy. Data are shown as means ± SE of four independent experiments performed in duplicate. * = P <0.05; ** = P <0.01.

3.3. Discussion

The colonisation efficacy of probiotic bacteria is often assessed using epithelial cancer cell lines (Joint FAO/WHO Working Report, 2002; Vélez *et al.*, 2007). However, most of these cell lines lack a functional mucus layer which is likely to impact on bacterial binding, as the mucus layer is the primary habitat for commensal bacteria (Johansson *et al.*, 2008; Navabi *et al.*, 2013a). In this study, we examined the effects of *L. reuteri* on EPEC colonisation of the human intestinal epithelium using enterocyte- and goblet cell-like cell lines as well as human duodenal biopsies as models of human infection. Through this investigation, we aimed to develop our understanding of probiotic-pathogen interaction and evaluate the probiotic potential of *L. reuteri*.

3.3.1. Influence of *L. reuteri* strain selection on epithelial adhesion

In this study, we demonstrated that *L. reuteri* binding to HT-29 cells is strain-dependent. This is consistent with previous findings, which have emphasised the importance of strain specificity when investigating *Lactobacillus* adhesion to IECs (Chauviere *et al.*, 1992a; Tuomola & Salminen, 1998). One study identified variable adhesion efficacy between a selection of *Lactobacillus* species to HT-29 cells, with strain-specific cell surface molecules aiding *L. reuteri* adhesion (Wang *et al.*, 2008). These cell surface molecules may have evolved due to adaptation of lactobacilli to their local environment (Frese *et al.*, 2011). Niche adaptation has previously been documented in *L. reuteri,* as genomic clades which contain adhesins that enhance adhesion to the host organism have been identified in strains isolated from humans, rodents, and pigs (Oh *et al.*, 2010; Wegmann *et al.*, 2015). Supporting previous studies, deletion of host-specific adhesin genes in the rodent strain *L. reuteri* 100-23 diminished colonisation of *Lactobacillus*-free mice (Frese *et al.*, 2011). Therefore, the presence of specific adhesins in *L. reuteri* provides a rationale for the selection of candidate probiotics. However, adhesion studies are required to confirm binding efficacy.

Interestingly, ATCC 53608, a porcine isolate, demonstrated the highest adherence of the *L. reuteri* strains examined. Similarities between the porcine and human GIT (Heinritz *et al.*, 2013) may account for this characteristic, and thus the porcine gut may provide an additional avenue for the isolation of novel probiotic strains. It is important to note the ecological variation within the porcine and human GIT however, with numerous *Lactobacillus* species forming biofilm layers directly contacting the squamous epithelium of the pig stomach (Fuller *et al.*, 1978). Within humans, no such niche has been identified and relatively few *Lactobacillus* species are thought to be persistent in the GIT (Walter, 2008). Efficient adhesion of ATCC 53608 to human IECs may be linked to the formation of aggregates mediated by the MUB protein, as ATCC 53608 MUB- did not aggregate and showed diminished adherence to IEC's (MacKenzie *et al.*, 2010; Wadstroum *et al.*, 1987).

L. reuteri strains ATCC PTA 6475, DSM 20016, and LMS11-3, originally isolated from human hosts, demonstrated comparable binding to HT-29 cells, whereas human strain ATCC 55730 showed reduced adhesion. These findings are supported by previous studies, which demonstrated high adherence of ATCC PTA 6475 (referred to as MM4-1a) and DSM 20016 to HT-29, Caco-2, and LS174T cells, whereas the ATCC 55730-derived strain DSM 17938 showed reduced binding (Jensen *et al.*, 2012). Interestingly, ATCC 55730 is currently marketed as a dietary supplement, and previous studies have demonstrated colonisation of the stomach and duodenum of human volunteers, suppression of *Helicobacter pylori* infection, and reduction of diarrhoeal episodes in children (Francavilla *et al.*, 2008; Valeur *et al.*, 2004; Weizman *et al.*, 2005). Therefore, the lack of correlation between *in vitro* and *in vivo* data suggests that probiotic screening with cell line models requires further validation with more physiologically relevant model systems.

Mucus binding proteins (MBPs) facilitate adhesion of lactobacilli to the host (reviewed in Juge (2012); Lebeer *et al.* (2008); Van Tassell and Miller (2011); Vélez *et al.* (2007)) and have been identified within a range of *Lactobacillus* species, such as the *L. rhamnosus* GG SpaCBA pillin (Kankainen *et al.*, 2009) and the *L. reuteri* 104R surface protein MapA (Miyoshi *et al.*, 2006; Rojas *et al.*, 2002). MBPs typically consist of a sorting peptide, an LPxTG motif in the

C-terminus (anchoring the MBP to the bacterial cell wall), and multiple repeats of the mucus binding domain mub, which shares homology with the bacterial mucin binding protein domain Pfam-MucBP, a sequence located in a number of *L. monocytogenes* proteins (Boekhorst *et al.*, 2006; Juge, 2012). The MUB protein of ATCC 53608 and the CmbA protein of ATCC PTA 6475 have been characterised as MBPs which mediate adhesion to mucus and the epithelium *in vitro* (Etzold *et al.*, 2014a; Jensen *et al.*, 2014; Roos & Jonsson, 2002). In our study, we identified that MUB and CmbA enhanced *L. reuteri* binding to both mucus-secreting LS174T cells and mucus-deficient HT-29 cells. As HT-29 cells have been shown to express cell-bound MUC3 when stimulated by *L. rhamnosus* GG and *L. plantarum* 299v, this may suggest a potential receptor for *L. reuteri* binding (Mack *et al.*, 2003).

While CmbA and MUB enhance *L. reuteri* adhesion, ATCC PTA 6475 CmbA- and ATCC 53608 MUB- still partially bound to LS174T cells. This finding suggests that additional cell surface proteins may contribute to the adhesion of these *L. reuteri* strains to mucus. One study identified three *L. acidophilus* NCFM cell surface proteins, FbpA, Mub, and SlpA, which contributed to adhesion to Caco-2 cells (Buck *et al.*, 2005). Furthermore, these binding proteins may be multifactorial in purpose, with additional roles to cellular adhesion. The elongation factor Tu protein of *L. johnsonii* La1, a guanosine nucleotide protein which assists protein synthesis in the cytoplasm, has been identified as an adhesin-like molecule on the bacterial cell surface (Granato *et al.*, 2004). While our findings are supported by these studies, the presence of additional adhesins on *L. reuteri* ATCC PTA 6475 requires further investigation.

We further observed increased adhesion of *L. reuteri* and EPEC to LS174T compared to HT-29 cells, suggesting that mucus enhanced microbial binding. This is in agreement with our data on duodenal biopsies, which demonstrated *L. reuteri* localisation to the mucus. Previous studies have demonstrated increased binding of *L. rhamnosus* GG, *L. johnsonii* La1, and *L. bulgaricus* ATCC 11842 human intestinal mucus compared to resected human colonic tissue (Ouwehand *et al.*, 2002). Put together with our data, these findings suggest preferential lactobacilli binding to mucus, potentially due to MBPs

such as MUB and CmbA. While further investigation is required to confirm that enhanced *L. reuteri* binding to LS174T cells is mediated by the adhesion of MBPs to mucins, these findings support the use of mucus-producing models when investigating probiotic adhesion

3.3.2. Influence of *L. reuteri* on EPEC adherence *in vitro*

In this study, we report that *L. reuteri* ATCC PTA 6475 and ATCC 53608 significantly inhibit EPEC adhesion to HT-29 cells. One mechanism of pathogen inhibition is competitive exclusion, where probiotics compete with a pathogen to access binding sites on the epithelial surface. Previous reports investigating competitive exclusion have suggested that either pre-incubation of the probiotic or co-incubation with the pathogen is required, as pathogen displacement is uncommon (Lebeer *et al.*, 2008). One study investigating pre-incubation, co-incubation, and displacement of DAEC and ETEC binding by *L. acidophilus* La1 demonstrated that inhibition of *E. coli* binding to Caco-2 cells was only achieved during protection and co-incubation studies (Bernet *et al.*, 1994). A similar study investigating the effect of *L. salivarius* and *L. plantarum* on *S. aureus* adhesion to Caco-2 cells identified that only pre-incubation and competition assays inhibit *S. aureus* binding (Ren *et al.*, 2012). As previous reports have demonstrated successful inhibition of pathogenic *E. coli* adhesion by pre-incubation with *Lactobacillus* species (Bernet *et al.*, 1994; Coconnier *et al.*, 1993a), we selected this intervention protocol for our study.

We initially investigated the influence of *L. reuteri* pre-incubation time on EPEC binding. We determined that 1 and 3 hour pre-incubation of *L. reuteri* inhibited EPEC adhesion to HT-29 cells. A previous study identified that 1 hour pre-incubation of *L. acidophilus* ATCC 4356 and *S. thermophilus* ATCC 19258 significantly inhibited EIEC adhesion to Caco-2 cells (Resta-Lenert & Barrett, 2003). Longer pre-incubation periods have also been reported, with 6 hour pre-incubation of either *L. acidophilus* R0052 or *L. rhamnosus* R0011 inhibiting both EPEC and EHEC adhesion to polarised T84 cells (Sherman *et al.*, 2005).

Previous studies investigating the protective effects of lactobacilli on EPEC adhesion to non-mucus producing IECs have found different dosage requirements with equivalent numbers of *L. acidophilus* La1 inhibiting EPEC adherence to Caco-2 cells (Bernet *et al.*, 1994; Coconnier *et al.*, 1993a). Interestingly, the study by Coconnier *et al.* (1993a) also found that pre-incubation with heat-killed *L. acidophilus* decreased EPEC binding too Caco-2 cells, although a 10-fold excess was required for this effect. Similarly, EPEC binding to polarised T84 cells was reduced by *L. acidophilus* and *L. rhamnosus* when pre-incubated at 10x and 1,000x excess, respectively (Sherman *et al.*, 2005). Furthermore, *L. rhamnosus* and a selection of *L. acidophilus* strains inhibited EHEC adherence to HT-29 cells when incubated at 1,000x excess (Kim *et al.*, 2008). Thus, our findings that a 100-fold excess of *L. reuteri* reduced EPEC binding agree with other studies investigating the protective characteristics of *Lactobacillus*.

It has been demonstrated that lactobacilli produce anti-microbial compounds (Servin, 2004). Some *L. reuteri* strains, including ATCC PTA 6475 and ATCC 53608, produce the antimicrobial compound reuterin from glycerol, which inhibits a broad spectrum of bacterial species, including *E. coli* (Spinler *et al.*, 2008; Talarico *et al.*, 1988). Furthermore, lactic acid produced by *L. casei* Shirota and *L. acidophilus* YIT 0070 has bacteriostatic and bactericidal effects on EHEC (Ogawa *et al.*, 2001). However in our study, EPEC adherence was not affected by *L. reuteri*-conditioned medium, which suggests that neither lactic acid nor reuterin are responsible for the suppression of EPEC binding. We further investigated the interaction between *L. reuteri* and EPEC in the absence of HT-29 cells, to determine the effect of *L. reuteri* on EPEC viability. We observed that co-incubation with ATCC 53608 wildtype and a MUB-deficient mutant decreased EPEC viability independently of secreted products. With these initial experiments, we were unable to identify a specific mechanism behind this reduction in EPEC viability. However, further studies could investigate whether this effect is dependent on live bacteria and whether this effect is constitutive or a specific response to competitor bacteria.

Competitive binding of probiotics to pathogen cell surface receptors has also been suggested as an inhibitory mechanism. One study found that EPEC

adherence to HT-29 cells was inhibited by *L. plantarum* 299v, but not by an *L. plantarum* 299v mutant deficient in a mannose-specific adhesin (Mack *et al.*, 2003). The loss of this adhesin may have prevented the competitive exclusion of EPEC from shared receptors, as EPEC produces mannose-sensitive type I fimbriae (Adlerberth *et al.*, 1996; Knutton *et al.*, 1987). However, it should be noted that the role of type I fimbriae in EPEC host cell binding is currently undefined (Elliott & Kaper, 1997). In contrast to these previous findings, during our short-term protection assays, *L. reuteri* bound to the epithelium were unable to reduce EPEC adherence. As we only observed a protective effect when non-adherent *L. reuteri* were maintained, these findings suggest that *L. reuteri* exclude EPEC by steric hindrance, where *L. reuteri* cells impede EPEC access to the epithelial surface, during short-term pre-incubations (Chan *et al.*, 1985).

In contrast to HT-29 cells, *L. reuteri* did not affect EPEC adhesion to LS174T cells during short-term protection assays. Previous studies on pathogen-probiotic interaction within mucus have shown that a 1 hour pre-incubation of *L. casei* Shirota and *L. johnsonii* LJ1 reduced *S.* Typhimurium adhesion to immobilised human mucus (Tuomola *et al.*, 1999). This effect appeared to be species-specific as a selection of *L. rhamnosus* strains had either no effect or significantly increased *S.* Typhimurium adhesion (Tuomola *et al.*, 1999). Similarly, the probiotic *E. coli* Nissle 1917 reduced EHEC binding when co-cultured for 2 hours with LS174T cells (Rund *et al.*, 2013). This difference to our observations is likely due to alternative experimental conditions, such as the probiotic species investigated and the models used. It is currently not known why *L. reuteri* did not inhibit EPEC access to LS174T cells although the presence of additional binding sites in the secreted mucus is one potential explanation.

As we observed low levels of *L. reuteri* binding to the epithelium during short-term protection assays, we modified the cell culture model to investigate the impact of adherent *L. reuteri* on EPEC binding by increasing the pre-incubation period to 4 hours, and removing non-bound *L. reuteri*. Interestingly, we observed a reduction in EPEC adherence to HT-29 cells after pre-incubation with ATCC PTA 6475, but not ATCC 53608. This difference may be associated

with the strain-specific adherence characteristics. While ATCC PTA 6475 binding was uniformly distributed across the epithelial surface, aggregates of ATCC 53608 were associated with fewer cells thus potentially blocking fewer EPEC binding sites.

Surprisingly, neither *L. reuteri* strain inhibited EPEC binding to LS174T cells when pre-incubated for 4 h, despite higher adherence to this cell line. As LS174T cells produce mucus, EPEC may access additional binding sites within the mucus layer, and the EPEC flagellum has previously been shown to increase binding to bovine mucus (Erdem *et al.*, 2007). To differentiate between EPEC binding to mucus and the cell surface epithelium, we adapted the infection protocol to enable actin pedestal formation, a marker of epithelial adhesion, which can be detected by fluorescence microscopy (Moon *et al.*, 1983; Viswanathan *et al.*, 2009). However, we found no significant difference in the number of cell-bound EPEC on LS174T cells pre-treated with either *L. reuteri* strain compared to non-treated controls. These findings suggest that ATCC PTA 6475 and ATCC 53608 do not reduce EPEC binding to the LS174T cell surface. This could be associated with the secreted mucus layer, as MUC2 may reduce *L. reuteri* access to epithelial receptors which are shared with EPEC, reducing competition for mutual binding sites.

Interestingly, we observed a significant increase in the number of EPEC contained in microcolonies on LS174T cells pre-incubated with ATCC PTA 6475 compared to both ATCC 53608-treated and non-treated controls, which may be associated with different *L. reuteri* binding characteristics. Although the adhesion of both *L. reuteri* strains to LS174T cells appeared similar, we were unable to differentiate between mucus and cell-bound *L. reuteri*. As such, adherence to the LS174T cell surface may differ between *L. reuteri* strains, as we observed on HT-29 cells. Thus, the adhesion of ATCC PTA 6475 to LS174T cells may inhibit the cell-cell spread of EPEC, resulting in microcolony formation. Further investigation with CmbA-deficient ATCC PTA 6475 as well as removal of the LS174T mucus layer (through inhibition of mucus synthesis or mucus degradation) could be utilised to investigate ATCC PTA 6475 binding to LS174T cells.

3.3.3. Influence of *L. reuteri* on EPEC colonisation *ex vivo*

Investigations into probiotic-pathogen interactions have generally used cell-line based models to identify potential beneficial outcomes. However, cancer cell lines consist of clonal cell populations whereas *in vivo,* the intestinal epithelium is comprised of a mixture of different cell types (including enterocytes and goblet cells) with a defined mucus layer and host microbiota (Johansson & Hansson, 2011c; Noah *et al.*, 2011). To address this difference between the *in vitro* and *in vivo* situation, we further investigated the interaction of *L. reuteri* and EPEC by IVOC of human duodenal biopsies. IVOC is regarded as the current gold standard for investigating interactions of EPEC with the human intestinal mucosa (Fang *et al.*, 2013; Knutton *et al.*, 1987). Yet, IVOC has not been used to investigate probiotic effects against pathogens at the human epithelial surface. However, a previous study has shown that pre-incubation with *L. plantarum* JC1 did not reduce *S.* Typhimurium adhesion to porcine jejunal and colonic intestinal tissue (Collins *et al.*, 2010). In contrast, pre-incubation with *L. reuteri* LM1 and *L. salivarius* LM2 inhibited *Brachyspira pilosicoli* colonisation of chicken caecal tissue (Mappley *et al.*, 2011).

In this study, we showed that *L. reuteri* ATCC PTA 6475 and ATCC 53608 were localised in the mucus layer and were unable to access the epithelium of human duodenal biopsies. Previous studies have identified epithelial binding of lactobacilli to human and porcine intestinal tissue samples, in the absence of the mucus layer (Bogovič Matijašić *et al.*, 2006; Tsilingiri *et al.*, 2012). Our findings, alongside these previous studies, suggest that lactobacilli do not access the epithelium when the mucus layer is intact. Thus, investigations into the effects of probiotics on mucus-free models may only represent conditions where the mucus layer is either absent or non-functional, such as IBD (Sheng *et al.*, 2012). Furthermore, the exclusion of *L. reuteri* from the duodenal epithelium may also be due to anti-microbial products secreted by enterocytes and Paneth cells into the mucus layer (Ouellette, 1997). The mouse antimicrobial lectin RegIIIγ (a homologue of human Hepatocarcinoma-intestine-pancreas/pancreatic associated protein (HIP/PAP)) has previously been identified as an inhibitor of Gram-positive bacteria which is secreted by

villous by enterocytes (Cash et al., 2006; Vaishnava et al., 2011). The secretion of RegIIIγ, as well as other antimicrobials such as β-defensins, is thought to maintain an exclusion zone between the small intestinal epithelium and the microbiota (McGuckin *et al.*, 2011; Ouellette, 2004; Porter *et al.*, 1997). Thus, it is probable that the production of antimicrobial products and the secretion of the mucus layer exclude *L. reuteri* from the duodenal epithelium.

Interestingly, pre-incubation with *L. reuteri* significantly reduced EPEC binding to the epithelium of duodenal biopsies. As *L. reuteri* were restricted to the mucus layer, inhibition of EPEC binding to the epithelium must occur at the mucus interface, e.g. by steric inhibition of EPEC penetration of the mucus layer. These data contrast with our observations in mucus-producing LS174T cells where *L. reuteri* did not reduce EPEC adhesion. This could be due to differences in mucus composition, as the secreted mucus layer of the duodenum is mainly comprised of MUC2, whereas LS174T cells produce MUC2, MUC5AC (usually expressed in the stomach), and gallbladder mucin (Atuma *et al.*, 2001; van Klinken *et al.*, 1996). Therefore, future studies should utilise physiologically relevant model systems, such as IVOC, to assess probiotic characteristics.

3.4. Summary

In this study, we have demonstrated strain-specific protective effects of *L. reuteri* against EPEC adhesion to intestinal cell lines and duodenal biopsies. These protective mechanisms include competitive exclusion, both within the mucus layer and at the epithelial surface, as well as potential inhibition of EPEC dispersion across the epithelium. Furthermore, we observed that inhibition was dependent on the model system used. Therefore, these findings emphasise the importance of model and strain selection when investigating probiotic characteristics.

CHAPTER FOUR

Bacterial modulation of mucus production *in vitro* and *ex vivo*

4.1. Introduction and objectives of study

The mucus layer is the first line of defence against invading pathogens of the GIT. Mucus is structurally composed of mucins, heavily glycosylated proteins with tandem repeats of PTS residues, which form a mesh-like structure enabling colonisation by commensal bacteria and impeding microbial access to the epithelium (Johansson *et al.*, 2011b; Juge, 2012). The mucus of the colon forms two layers, the outer loose layer which facilitates microbial colonisation and the inner adherent layer which excludes the microbiota from the epithelium (Johansson *et al.*, 2008). In contrast, the mucus of the small intestine forms a single loose layer, which is colonised by microbes, to facilitate nutrient absorption (Johansson *et al.*, 2011b). The colonic and small intestinal mucus layers are composed of MUC2, the major secreted mucin in the human intestine (Johansson *et al.*, 2011b). In addition to the secreted mucus layer, intestinal epithelial cells are covered by the glycocalyx, which is composed of the cell-bound mucin MUC3 (Kim & Ho, 2010). The glycocalyx and secreted mucus impede bacterial movement, as flagella of EPEC and EHEC bind to mucin *O*-glycans (Erdem *et al.*, 2007; Ye *et al.*, 2015). Furthermore, mucins act as decoy receptors for pathogen adhesion, as the binding of *H. pylori* and EPEC to cell-bound mucins MUC1 and MUC3 inhibits pathogen access to the epithelial cell surface (Lindén *et al.*, 2009; Mack *et al.*, 2003). In addition to the physical mucus barrier, bacterial access to the intestinal epithelium is inhibited by the secretion of antimicrobial compounds, such as defensins and lectins, by intestinal epithelial cells into the mucus layer (Johansson & Hansson, 2011c; Vaishnava *et al.*, 2011).

Pathogens have evolved multiple strategies to circumvent the host mucus layer. The stomach pathogen *H. pylori* secretes cytotoxins CagA and VacA, which reduce mucin synthesis by inhibiting intracellular processing of glycoproteins (Beil *et al.*, 2000; Byrd *et al.*, 2000). Alternatively, pathogenic *E. coli* produce mucin-degrading enzymes such as StcE (EHEC, Grys *et al.* (2005)), SslE (secreted and surface-associated lipoprotein from *E. coli*; EPEC, ETEC, and ExPEC, Luo *et al.* (2014), Nesta *et al.* (2014)), and Pic (Dutta *et al.*, 2002), which reduce the viscosity of the mucus layer and facilitate

penetration to the epithelial surface. Therefore, enhancing the mucus layer could inhibit EPEC colonisation and probiotics offer a potential mechanism, as previous studies have identified modulation of host mucin expression by *Lactobacillus* isolates. While production of cell-associated MUC3 is enhanced by *L. rhamnosus* GG and *L. plantarum* 299v in HT-29 cells, secreted *L. rhamnosus* GG protein p40 has been associated with an increase in MUC2 expression in LS174T cells and murine intestinal models (Caballero-Franco *et al.*, 2007; Mack *et al.*, 2003; Wang *et al.*, 2014a). Thus, stimulation of host mucin production by probiotics may offer a therapeutic strategy to reduce pathogen binding to the epithelium.

In this study, we aimed to investigate the effect of EPEC and *L. reuteri* strains ATCC PTA 6475 and ATCC 53608 on mucin dynamics in mucus-secreting LS174T cells and human duodenal biopsies.

4.2. Results

4.2.1. *MUC5AC* expression in LS174T cells is significantly increased by *L. reuteri* ATCC 53608 and EPEC

To investigate the effects of *L. reuteri* and EPEC on mucin gene expression, LS174T cells were incubated with *L. reuteri* or EPEC for different time periods, and expression of intestinal mucins *MUC1*, *MUC2*, *MUC3A*, *MUC5AC*, *MUC6*, *MUC12*, *MUC13*, and *MUC17* was determined by relative qPCR analysis, using the ΔΔCt method (Livak & Schmittgen, 2001). Prior to gene expression studies, specificity and amplification efficiency of all primers was determined by Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and qPCR (Figure 4.1). Specificity of each primer set was determined by the presence of a single peak in the dissociation curve (Figure 4.1A) and the presence of a single band at the predicted size on an agarose gel (Figure 4.1B). The formation of a primer dimer was noted during *MUC13* amplification, as a single peak was identified on the dissociation curve, but two bands were observed by gel electrophoresis (Figure 4.1A and B). We excluded *MUC3A* from further analysis as only one in three *MUC3A* primer sets demonstrated amplification

of a single product. Furthermore, when the amplicon was examined by gel electrophoresis, only a faint band was observed (Figure 4.1B).

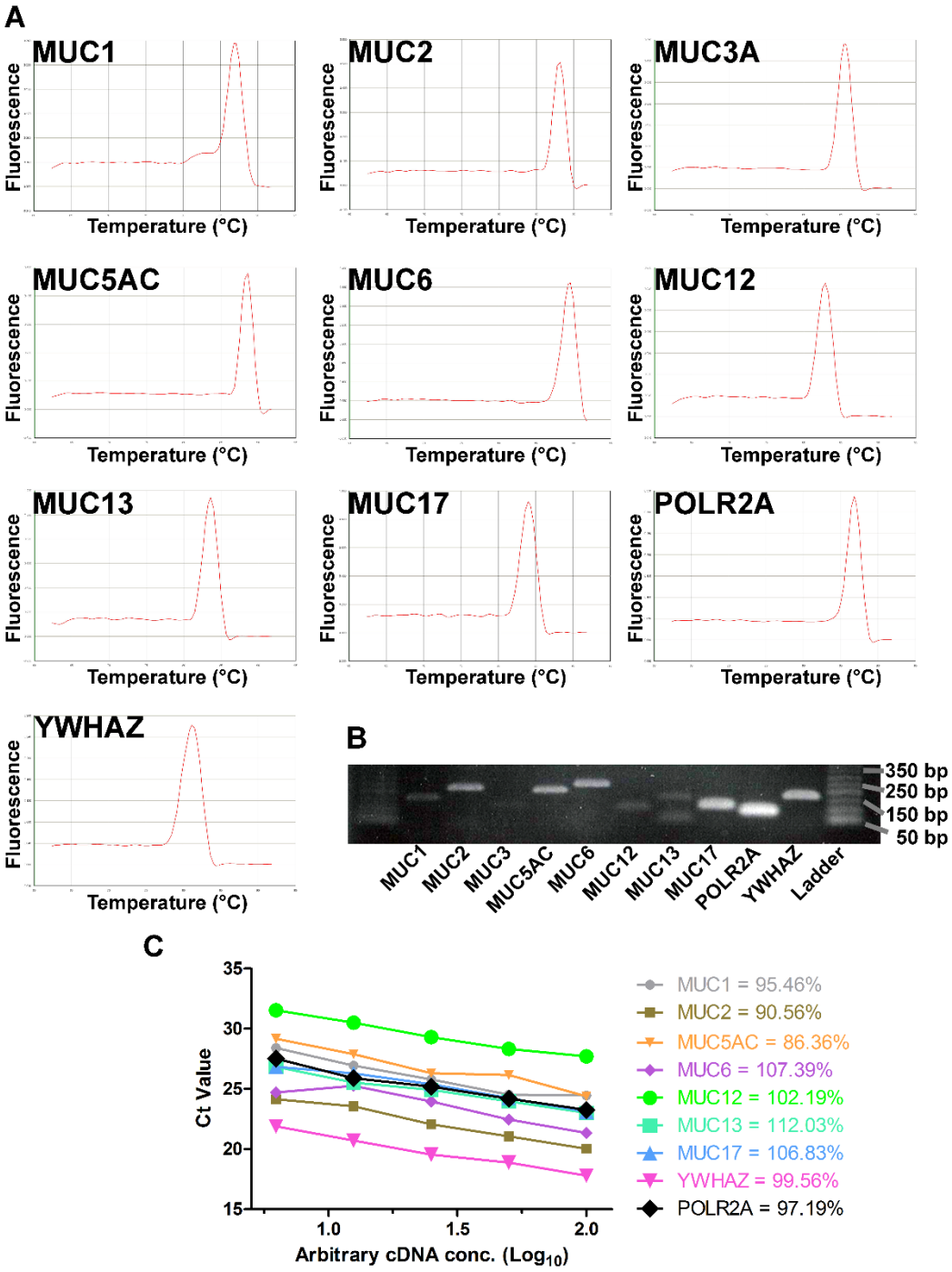

Figure 4.1: Validation of primer specificity and amplification efficiency

(A) Dissociation curves were generated for each primer pair. The presence of a single peak indicated primer specificity to a single target. (B) Agarose gel

electrophoresis analysis of qPCR products to determine amplicon product size. Representative image from two experiments (C) Amplification efficiency of primer pairs. Primers were tested in a qPCR assay with cDNA which had been serially diluted two-fold. Ct values of each dilution were plotted on a scatter graph and the slope was used to determine the primer efficiency of each primer set. Data are representative of four independent experiments.

To normalise our qPCR data, we selected the housekeeper genes tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*, Jacob *et al.* (2013)) and RNA polymerase II (*POLR2A*, Schüller *et al.* (2009)), as the expression of these genes did not change during treatment with either ATCC PTA 6475 (Figure 4.2A) or EPEC (Figure 4.2B) and non-treated cells.

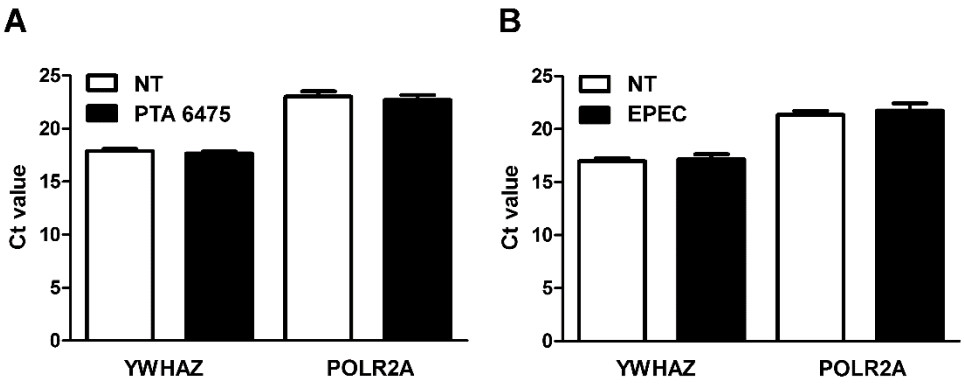

Figure 4.2: Housekeeper genes YWHAZ and POLR2A demonstrate stable expression under treated and non-treated conditions

LS174T cells were treated with *L. reuteri* ATCC PTA 6475 (A), EPEC (B) or left non-treated (NT) for 6 h and expression of *YWHAZ* and *POL2RA* was determined by qPCR. Data are shown as means of Ct ± SE of three experiments performed in duplicate.

Subsequent qPCR analysis demonstrated that incubation with *L. reuteri* ATCC 53608 significantly increased *MUC5AC* and *MUC1* gene expression at 1-4 h and 1 h, respectively (Figure 4.3). In contrast, expression of *MUC6*, *MUC12*, *MUC13* and *MUC17* did not significantly change after 1 to 6 h incubation with either EPEC or *L. reuteri* (Figures 4.3–4.5).

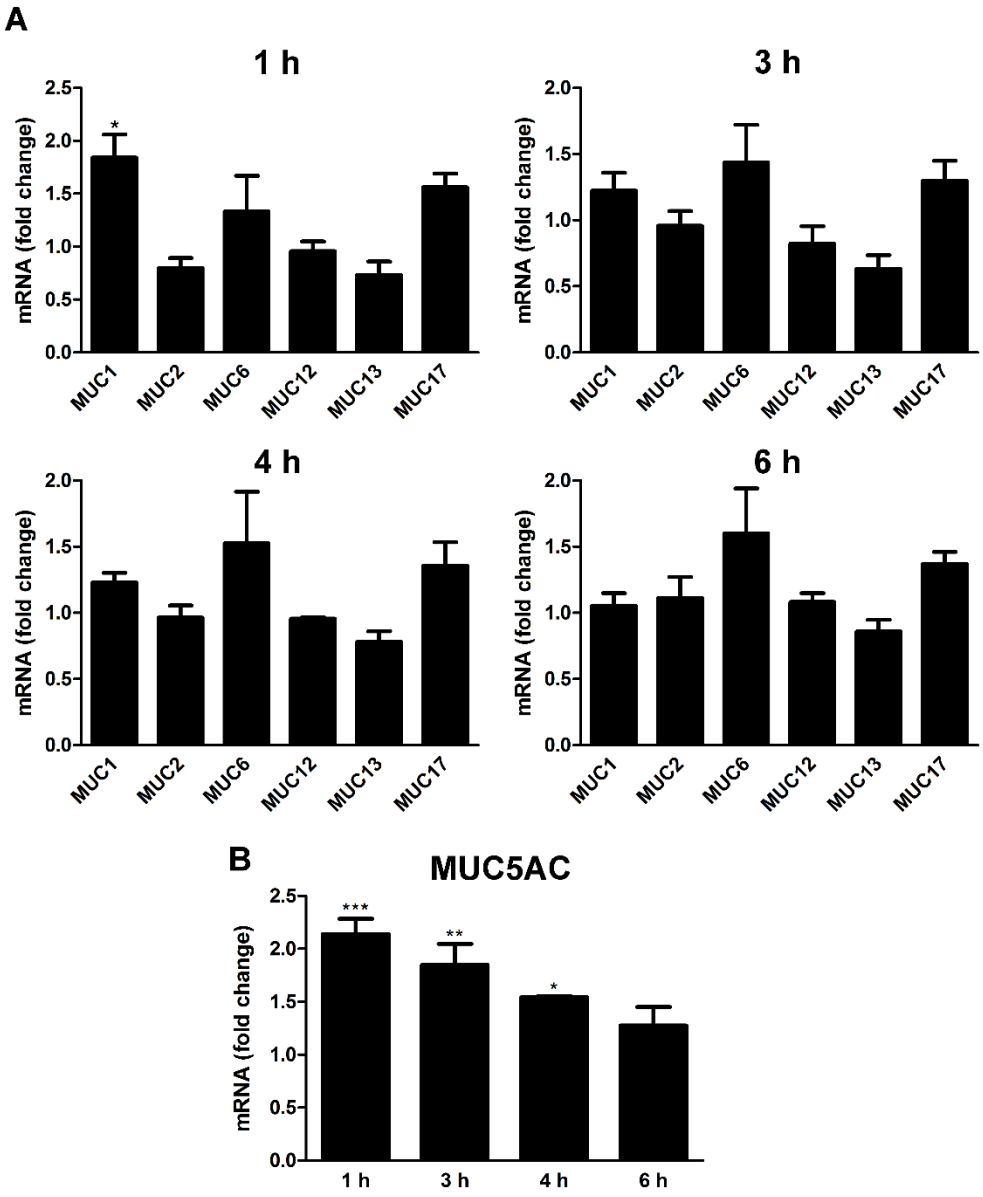

Figure 4.3: Incubation with *L. reuteri* ATCC 53608 significantly increases *MUC5AC* and *MUC1* gene expression in LS174T cells

LS174T cells were incubated with ATCC 53608 for 1, 3, 4, and 6 h or left non-treated for 6 h. (A) Expression of mucin genes which did not significantly change, or were only significantly increased at one time point, relative to NT controls. (B) Fold change in *MUC5AC* gene expression at 1, 3, 4, and 6 h, relative to non-treated control. Data are shown as means ± SE of three independent experiments performed in duplicate. * = P <0.05; ** = P <0.01; *** = P <0.001.

In contrast to *L. reuteri* ATCC 53608, incubation of LS174T cells with ATCC PTA 6475 did not significantly change mucin gene expression at any time point (Figure 4.4).

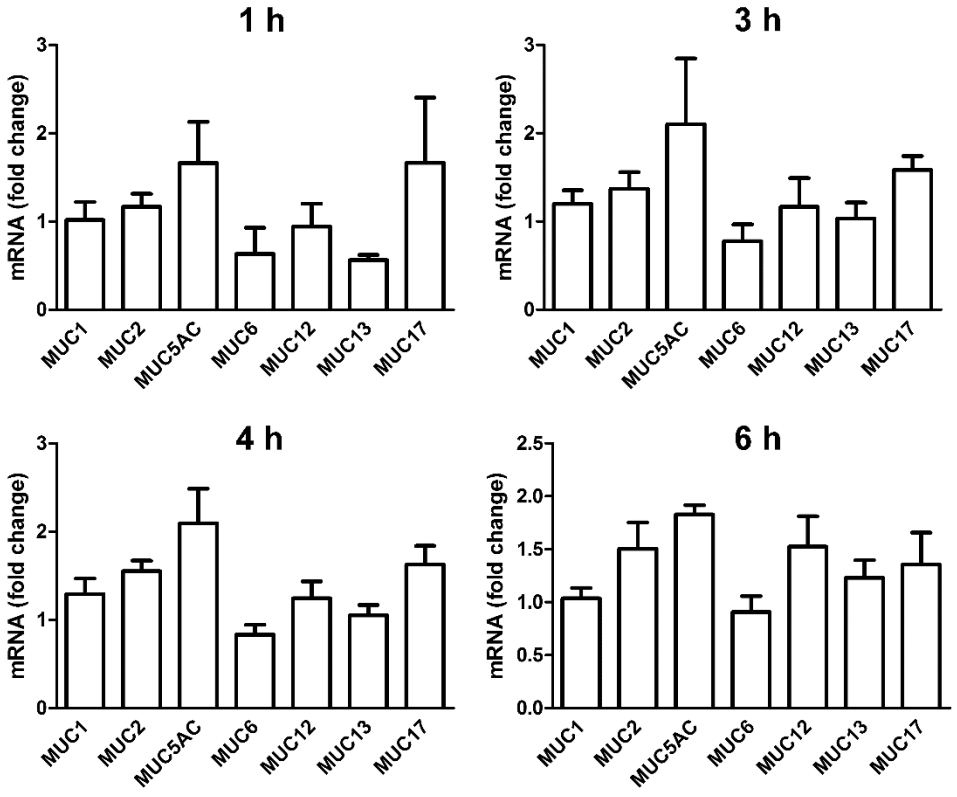

Figure 4.4: ATCC PTA 6475 does not significantly induce mucin gene expression in LS174T cells

LS174T cells were incubated with ATCC PTA 6475 for 1, 3, 4, and 6 h or left non-treated for 6 h, and expression of mucin genes was determined relative to non-treated controls. Data are shown as means ± SE of three independent experiments performed in duplicate.

Interestingly, infection of LS174T cells with EPEC significantly upregulated expression of *MUC5AC* at 1-6 h and *MUC2* at 1 and 6 h post-infection (Figure 4.5B and C).

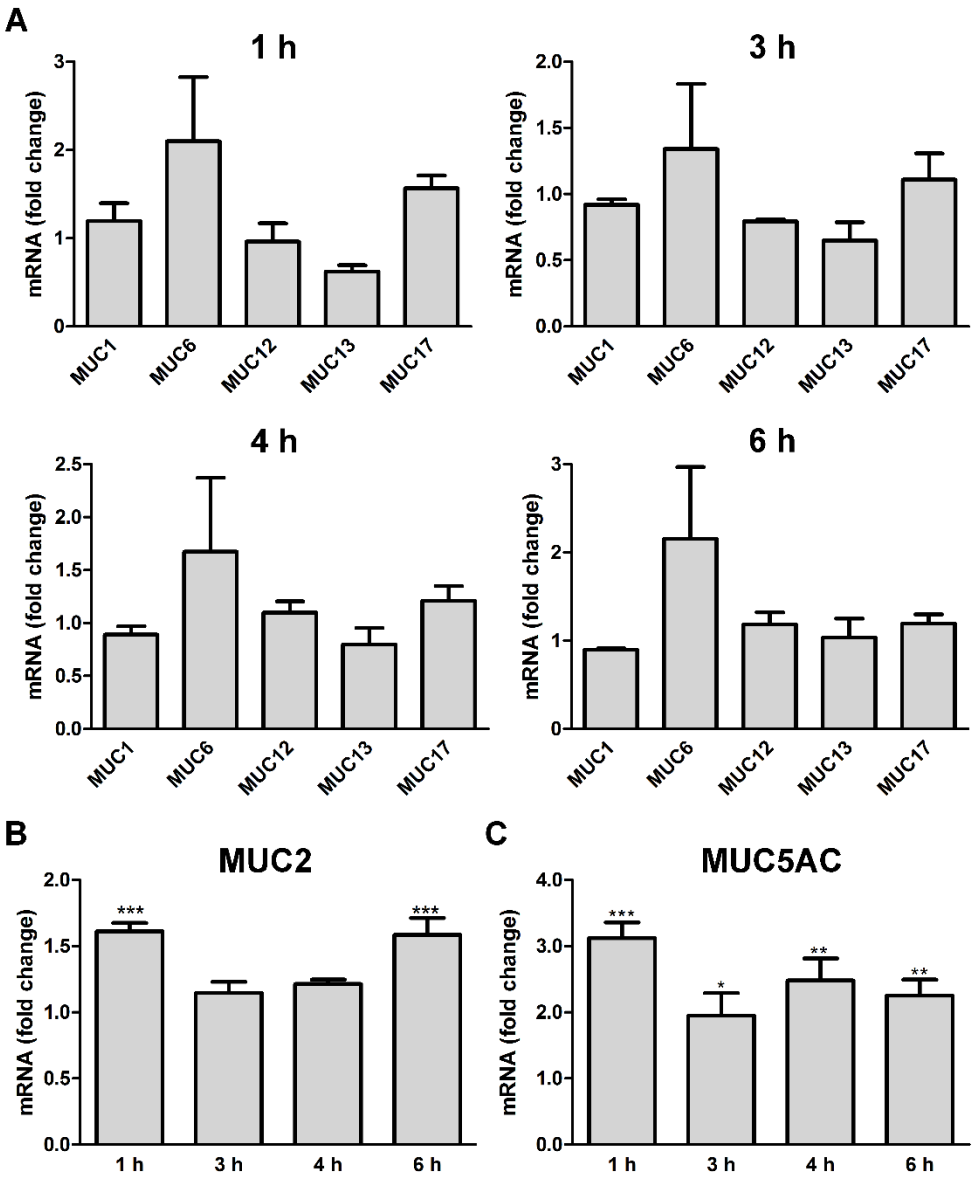

Figure 4.5: EPEC significantly induces *MUC2* and *MUC5AC* gene expression in LS174T cells

LS174T cells were incubated with EPEC for 1, 3, 4, and 6 h or left untreated for 6 h. (A) Expression of mucin genes which did not significantly change relative to non-treated controls. (B) Fold change in *MUC2* gene expression at 1, 3, 4, and 6 h, relative to non-treated controls. (C) Fold change in *MUC5AC* gene expression at 1, 3, 4, and 6 h, relative to non-treated controls. Data are shown as means ± SE of three independent experiments performed in duplicate. * = P <0.05; ** = P <0.01; *** = P <0.001.

4.2.2. MUC2 protein levels in LS174T cells are significantly altered by *L. reuteri* ATCC PTA 6475 and EPEC

As we observed an increase in *MUC2* gene expression by EPEC as well as an upregulation of *MUC5AC* by both ATCC 53608 and EPEC, we sought to confirm these findings at the protein level. Therefore, LS174T cells were incubated with EPEC, ATCC 53608, ATCC PTA 6475, or left untreated for 6 h and MUC2 and MUC5AC protein expression was investigated by immunofluorescence staining. Fluorescence was subsequently quantified by integrated density using ImageJ software (Wayne Rasband, NIH).

MUC5AC immunofluorescence staining of non-treated cells demonstrated mucin localisation on distinct cells (Figure 4.6A). In contrast to our qPCR findings, neither *L. reuteri* nor EPEC significantly affected the total amount of MUC5AC protein relative to NT controls (Figure 4.6B-D).

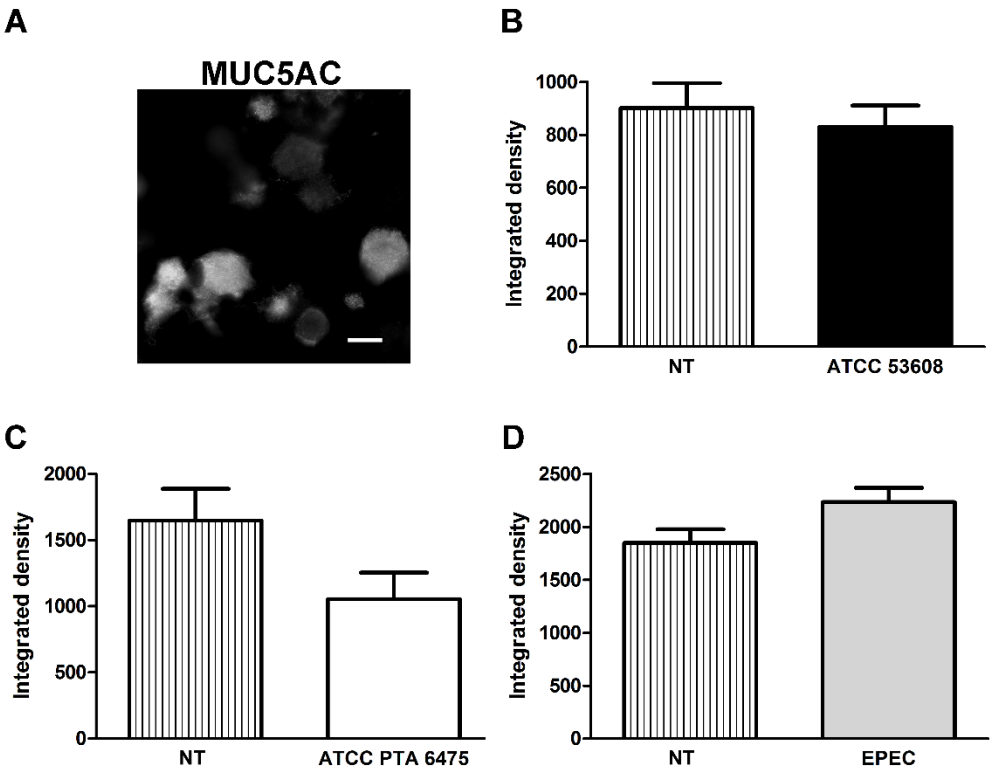

Figure 4.6: MUC5AC protein content in LS174T cells was not altered by incubation with EPEC or *L. reuteri*

(A) MUC5AC immunofluorescence staining in LS174T cells. Image is representative of three independent experiments. Scale bar = 10 µm. (B-D) Integrated density quantification of MUC5AC immunofluorescence staining in LS174T cells incubated with (B) ATCC 53608, (C) ATCC PTA 6475, or (D) EPEC for 6 h. Data were compared to the NT control and are shown as means ± SE of three independent experiments performed in duplicate.

Immunofluorescence staining of MUC2 in non-treated LS174T cells demonstrated a distinct cellular localisation on the majority of cells (Figure 4.7A). Whereas incubation of LS174T cells with ATCC 53608 did not significantly change MUC2 protein expression (Figure 4.7B), treatment with ATCC PTA 6475 significantly increased MUC2 protein levels relative to NT controls (Figure 4.7C). In contrast, infection with EPEC significantly decreased the MUC2 fluorescence density in LS174T cells (Figure 4.7D).

To determine whether incubation with *L. reuteri* could restore MUC2 levels in EPEC-infected LS174T cells, equivalent numbers of ATCC PTA 6475 and EPEC were added to LS174T cells for 6 h. As shown in Figure 4.7E, co-incubation of ATCC PTA 6475 with EPEC restored MUC2 protein in LS174T cells to non-treated levels.

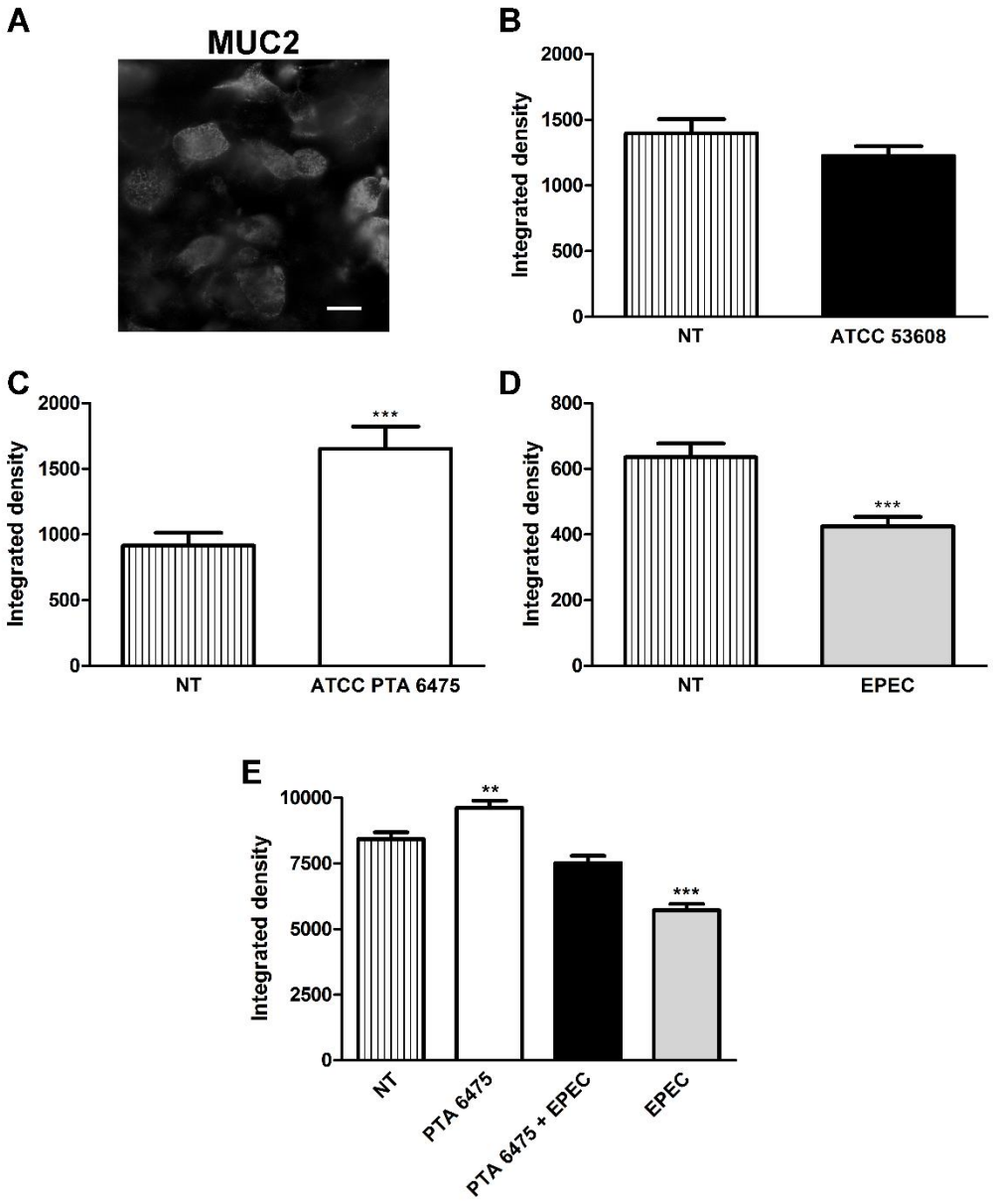

Figure 4.7: MUC2 protein levels in LS174T cells are modulated by ATCC PTA 6475 and EPEC

(A) Immunofluorescence staining of MUC2 protein in LS174T cells. Image is representative of five independent experiments. Scale bar = 10 µm. (B-E) Integrated density quantification of MUC2 immunofluorescence staining in LS174T cells incubated with (B) ATCC 53608, (C) ATCC PTA 6475, (D) EPEC, or (E) ATCC PTA 6475 and EPEC for 6 h. Data are shown as means ± SE of either three (A-D) or five (E) independent experiments performed in duplicate. * = P <0.05; ** = P <0.01; *** = P <0.001 versus NT controls.

4.2.3. Mucus analysis in duodenal biopsies

As we observed differences between mucin gene and protein expression in LS174T cells, we investigated mucus protein levels in duodenal biopsies. This represented a substantial technical challenge, as the mucus layer collapses if the sample becomes dehydrated, due to the high water content which supports the mucus structure (Johansson & Hansson, 2012; Johansson *et al.*, 2008). To optimise mucus preservation in human duodenal biopsies, cryosections were fixed with MeOH/ acetone (1:1) or processed without fixation and stained for MUC2 and cell nuclei. Cryosections fixed with MeOH/ acetone demonstrated discernible MUC2 staining in goblet cells, but not staining of the mucus layer, whereas an external mucus layer was observed in non-fixed samples (Figure 4.8A). Therefore, further experiments were performed on non-fixed samples.

To investigate whether *L. reuteri* or EPEC altered mucus production by human tissue, as observed in LS174T cells, duodenal biopsies were incubated with *L. reuteri* or EPEC for 8 h. Cryosections were stained for MUC2 and cell nuclei, and *L. reuteri* and EPEC were detected by using antisera specific to CmbA, MUB, or *E. coli*. Mucus thickness was examined at numerous points along the villous. However, mucus preservation was inconsistent along the biopsy surface, ranging from the presence of an intact mucus layer (Figure 3.8B) to small patches of mucus covering the epithelium (Figure 3.8C). Therefore, this method was unsuitable for assessing changes in mucus thickness due to *L. reuteri* and EPEC.

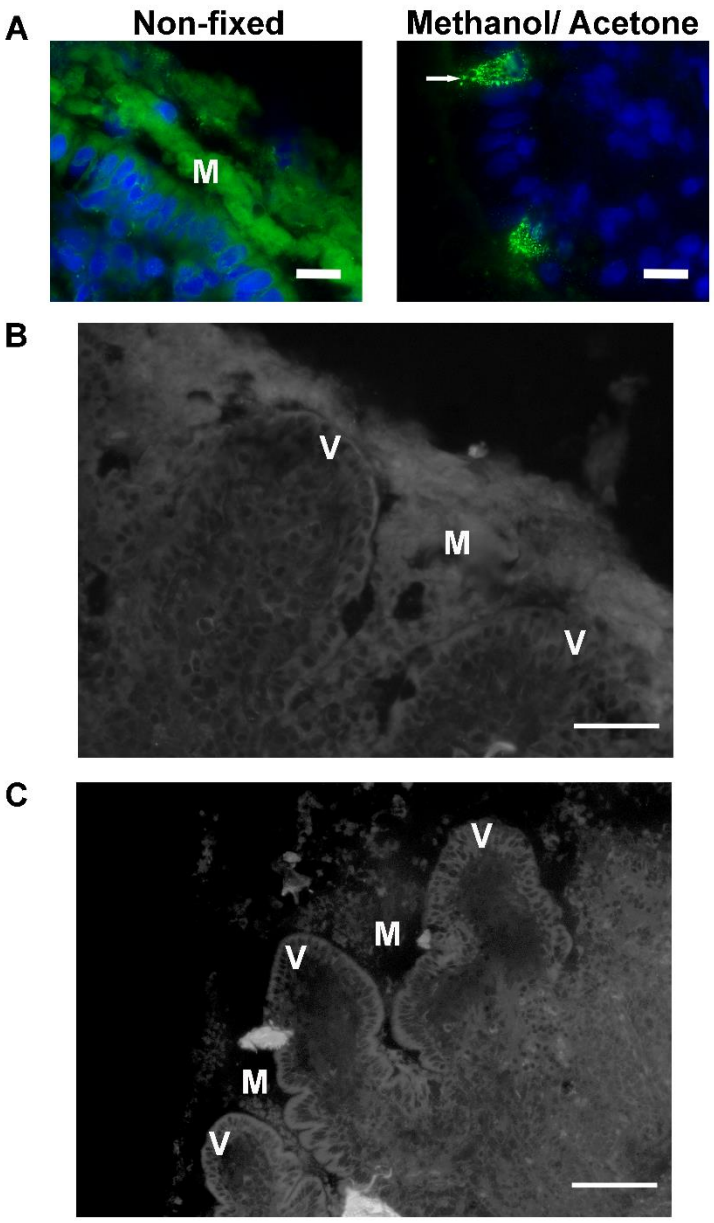

Figure 4.8: Preservation of MUC2 protein in biopsy cryosections

(A) MUC2 staining (green) of cryosections of duodenal biopsies without fixation or fixed in MeOH/ acetone. Cell nuclei were labelled in blue. White arrows indicate goblet cells, M indicates the external mucus layer. Images are representative of two independent experiments performed in duplicate. Scale bar = 10 µm. (B-C) MUC2 fluorescence staining of cryosections showing a well-preserved mucus layer overlaying the villus epithelium (B) or mucus detachment (C). Mucus and villous tips are indicated by M and V, respectively. Images are representative of three independent experiments performed in duplicate. Scale bars = 50 µm (B) and 100 µm (C).

4.3. Discussion

The mucus layer is a dynamic environment which acts as a habitat and food source to commensal bacteria, as well as providing a barrier to infection. Disruption of the mucus layer to gain access to the epithelium is a key process during GI infection by a number of pathogens thus, restoration of the mucus layer through probiotics may offer therapeutic solution to inhibit infection.

In this study, we have analysed the modulatory effects of *L. reuteri* and EPEC on mucus production using the MUC2-producing LS174T cell line. Our results suggest that EPEC reduces MUC2 protein levels and that this effect can be mitigated through co-incubation with *L. reuteri* ATCC PTA 6475.

4.3.1. Mucin gene expression does not directly correlate with protein levels

To investigate the impact of *L. reuteri* and EPEC on mucin production, we analysed the expression of major intestinal mucin genes and MUC2 and MUC5AC protein levels in LS174T cells. While *MUC5AC* gene expression was significantly upregulated by ATCC 53608 and EPEC, no significant changes in MUC5AC protein levels were detected. In addition, incubation with EPEC significantly upregulated *MUC2* gene expression, but resulted in a significant decrease in MUC2 protein levels. Ultimately, treatment with ATCC PTA 6475 caused a significant increase in MUC2 protein levels despite absent effect on *MUC2* gene expression. Taken together, these results show a lack of correlation between mucin gene expression and protein levels. This has been previously demonstrated in mice with cystic fibrosis, where *Muc2* gene expression was decreased in the ileum of mice with cystic fibrosis compared to WT mice, but no change in mucin quantity was detected (Parmley & Gendler, 1998). Differences in mucin gene and protein levels have also been reported during *C. rodentium* infection of mice, where *Muc1* and *Muc2* gene expression in the distal colon was highest during the early stage of infection, while increased mucus thickness was only observed 10 days later (Gustafsson *et al.*, 2013). These findings demonstrate that mucin gene expression is not

directly correlated with protein levels and suggest different stages of post-translational regulation, as outlined below.

As the biosynthesis of mucin is a multi-step process, there are numerous opportunities for pathogens to disrupt mucin protein production and secretion. After protein synthesis, apomucins are *N*-glycosylated and dimerized in the rough endoplasmic reticulum before being translocated into the Golgi apparatus, where *O*-glycosylation occurs (Asker *et al.*, 1998). *H. pylori* LPS reduces mucin production in rat gastric biopsies by inhibiting sulfation and *O*-glycosylation of apomucins (Liau *et al.*, 1992). After glycosylation, mucins are packed into granules and secreted into the lumen. Mucin secretion can be initiated by secondary messengers, such as Ca^{2+} and cyclic AMP (Ambort *et al.*, 2012a; Takahashi *et al.*, 1999) or bacterial proteins such as the potent secretagogue Cholera toxin (Forstner *et al.*, 1981; Lencer *et al.*, 1990). In contrast, *H*. *pylori* reduces mucus turnover in the mouse stomach by increasing the time from mucin synthesis to secretion (Navabi *et al.*, 2013b).

4.3.2. Influence of *L. reuteri* on mucin expression

In this study, we determined that *L. reuteri* ATCC PTA 6475 significantly increased MUC2 protein levels in LS174T cells relative to non-treated controls. This agrees with similar observations reported for other *Lactobacillus* species. *L. rhamnosus* GG secreted protein p40 increases MUC2 gene and protein expression in LS174T cells and mouse colonic epithelium, by activating EGF receptor (Wang *et al.*, 2014a). Modulation of MUC2 gene expression by *L. rhamnosus* GG has also been demonstrated in Caco-2 cells (Mattar *et al.*, 2002). However, as Caco-2 cells do not secrete MUC2 protein (van Klinken *et al.*, 1996), the biological relevance of these findings are questionable. In addition, *L. rhamnosus* GG and *L. plantarum* 299v stimulate expression of cell-associated MUC3 protein in HT-29 cells (Mack *et al.*, 2003). While we did not detect MUC3A mRNA in LS174T cells, future experiments could investigate the expression of MUC3A protein at the intestinal epithelium using human duodenal biopsies, to determine whether MUC3A is modulated by *L. reuteri*

and whether mucin expression was associated with decreased microbial binding.

Mucus secretion from goblet cells into the gut lumen is a continuous process, excluding microbes from the epithelial surface and providing lubrication for the movement of digested material (Deplancke & Gaskins, 2001). This process can be induced by secretagogues such as carbachol or calcium ionophores, thereby increasing the thickness of the mucus layer and reducing pathogen access to the epithelium (Deplancke & Gaskins, 2001; McCool *et al.*, 1990). Previous studies have shown that administration of the probiotic VSL#3, containing a mixture of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus spp.,* increased *Muc1*, *Muc2*, and *Muc3* gene expression and total mucin secretion in the rat intestine (Caballero-Franco *et al.*, 2007). Furthermore, conditioned medium of the individual VSL#3 *Lactobacillus* species stimulated mucin secretion by LS174T cells (Caballero-Franco *et al.*, 2007). Unfortunately, the secretagogues were not identified in the study and thus, we do not know whether this effect is strain-specific or a generic feature of *Lactobacillus*.

In our investigation, we did not determine whether the increase in MUC2 protein levels in LS174T cells was caused by stimulation of MUC2 production or exocytosis. Mucin synthesis could be determined by pulse-chase experiments using labelled mucins (GalNAz or [^{3}H] glucosamine) (Byrd *et al.*, 2000; Cole *et al.*, 2013). Alternatively, mucin synthesis could be inhibited with benzyl-α-GalNAc and total MUC2 levels could be compared between non-treated, *L. reuteri*-treated, and secretagogue-treated LS174T cells (Kuan *et al.*, 1989; McCool *et al.*, 1990).

4.3.3. Influence of EPEC on mucin expression

In this study, we determined that EPEC reduced MUC2 protein levels in LS174T cells. While previous studies have investigated the interaction of EPEC with mucus, these reports have mainly focused on microbial binding rather than the effect of EPEC on mucin protein levels. One study showed a

significant increase in expression of MUC2 and MUC5AC protein in HT-29 MTX cells infected with atypical EPEC, but not EPEC E2348/69 used in our study (Vieira *et al.*, 2010). This discrepancy may be due to the difference cell lines used as HT-29 MTX cells predominantly secrete MUC5AC, whereas LS174T cells primarily produce MUC2 (Hennebicq-Reig *et al.*, 1998).

Further studies have investigated the effect of related pathogens on mucus production *in vivo*. Infection of rabbits with a rabbit EPEC strain induced mucus secretion and increased the number of ileal goblet cells (Ramirez *et al.*, 2005). Similarly, infection of mice with *C. rodentium* increased mucus secretion (Bergstrom *et al.*, 2008; Lindén *et al.*, 2008b). Interestingly, these effects were dependent on the adaptive immune response, as mucus secretion in immunocompromised mice was unaffected by *C. rodentium* infection (Bergstrom et al., 2008). In contrast, our study focussed on the localised rather than systemic response during the early stages of infection and in the absence of an adaptive immune response. While we did not determine the mechanisms underlying reduced MUC2 protein levels, we can suggest potential explanations and future experiments to test these hypotheses. Firstly, EPEC infection could inhibit mucin synthesis as shown for *H. pylori*, which suppresses MUC5AC and MUC1 synthesis in gastric cells and MUC1 synthesis in the stomach of infected mice (Byrd *et al.*, 2000; Navabi *et al.*, 2013b). These effects were also mediated by bacterial lysates and could be caused by the cytotoxins CagA and VacA, which inhibit mucin production (Beil *et al.*, 2000; Byrd *et al.*, 2000). No homologous toxins have been identified in EPEC, but other proteins might mediate a similar effect. To further investigate this hypothesis, pulse-chase experiments with labelled mucins could be performed as previously described (4.3.2).

An alternative mechanism could be degradation of MUC2 protein by secreted enzymes. The extracellular *E. coli* serine protease autotransporter of the *Enterobacteriaceae* (SPATE) proteins have previously been associated with mucin degradation (Dutta et al., 2002). In particular, the class II SPATE Pic (produced by EAEC and *S. flexneri*) and Tsh (produced by avian pathogenic *E. coli*), but not the EPEC class I SPATE protein EspC, degrade bovine submaxillary mucus (Dutta *et al.*, 2002). While class I SPATEs have not

previously been associated with glycoprotein degradation (Ruiz-Perez & Nataro, 2014), it is important to note that the impact of EspC on intestinal MUC2 has not been investigated thus far. Specificity of SPATEs to particular mucins has previously been demonstrated for the class II SPATE EatA produced by ETEC, which degraded MUC2, but not bovine submaxillary mucus (Kumar *et al.*, 2014). In addition, the secreted serine proteases from the nematode *Trichuris muris* selectively degrade murine Muc2, but not Muc5ac (Hasnain *et al.*, 2012).

Alternative secreted enzymes produced by *E. coli* pathotypes have also been associated with mucin degradation, such as the EHEC extracellular zinc metalloprotease StcE, which degrades salivary MUC7 (Grys *et al.*, 2005). StcE has been suggested to enhance intimate attachment to the epithelium by removing cell-bound mucins, as an isogenic *stcE* mutant formed fewer actin pedestals on Hep-2 cells than the wildtype strain, despite similar levels of adhesion (Grys *et al.*, 2005). Surprisingly, recombinant StcE did not degrade intestinal MUC2 or MUC5AC in cell lysates (Grys *et al.*, 2006). In contrast, the *E. coli* metalloprotease SslE degrades both bovine submaxillary mucus and human MUC2 (Luo *et al.*, 2014; Nesta *et al.*, 2014; Valeri *et al.*, 2015). SslE (previously referred to as YghJ) is produced by ETEC, ExPEC, and EPEC and can be expressed on the cell surface or secreted (Baldi *et al.*, 2012; Iguchi *et al.*, 2009; Luo *et al.*, 2014). SslE promotes rabbit EPEC infection, as an isogenic *sslE* mutant demonstrated reduced colonisation and pathogenesis (Baldi *et al.*, 2012). Thus, SslE and EspC represent potential candidates and their role in MUC2 degradation during EPEC infection should be further investigated by construction of isogenic deletion mutants.

Our study has also demonstrated that incubation with *L. reuteri* ATCC PTA 6475 inhibits the reduction in MUC2 protein levels induced by EPEC. Our findings in Chapter 3 demonstrate that *L. reuteri* ATCC PTA 6475 does not affect EPEC viability or inhibit binding to LS174T cells. It is therefore unlikely that inhibition of EPEC binding would explain the effect of *L. reuteri* on MUC2 levels in LS174T cells co-incubated with EPEC. As ATCC PTA 6475 enhanced MUC2 protein amounts when incubated with LS174T cells alone, this suggests that the increase in MUC2 induced by *L. reuteri* counteracts the decrease

induced by EPEC. Thus, further investigation is required to identify the molecular determinants responsible for EPEC and ATCC PTA 6475-mediated modulation of MUC2 protein levels.

4.3.4. Investigating mucus production in intestinal biopsies

In addition to the studies on LS174T cells described above, we also used human duodenal biopsies to investigate the effects of *L. reuteri* and EPEC on mucus production *ex vivo*. Unfortunately, we were unable to reliably quantify mucus thickness due to lack of consistent mucus preservation on biopsy samples. Preservation of the mucus layer has previously been described as problematic due to high water content, as dehydration of this layer causes the mucus layer to collapse (Johansson & Hansson, 2012).

Non-fixed tissue samples have been used to assess the mucus thickness of the human stomach (Jordan *et al.*, 1998) and the colon (Fyderek *et al.*, 2009) using the histochemical stains Alcian Blue and Periodic Acid Schiff, which detect acidic and neutral mucins, respectively (Matsuo *et al.*, 1997). Comparison of mucus thickness by evaluation of stained cryosections and *in vivo* measurements in the rat distal colon, has produced similar results for the inner adherent mucus layer, while the outer loose mucus layer was lost during histological processing (Strugala *et al.*, 2003). In contrast to the stomach and the colon, the mucus layer of the small intestine is loose and can be easily removed (Atuma *et al.*, 2001; Gustafsson *et al.*, 2012). Thus, determining the thickness of the small intestinal mucus layer presents a substantial technical challenge. Fixation with Carnoy's solution (60% dry ethanol, 30% chloroform, and 10% glacial acetic acid) and paraffin embedding has demonstrated excellent preservation of both the loose outer and the adherent inner mucus layer of the murine colon (Johansson *et al.*, 2008; Johansson *et al.*, 2009). However, this protocol required the preservation of a whole piece of murine colon with the faecal pellet *in situ*, to prevent mucus layer detachment (Johansson *et al.*, 2011a). As we used human duodenal biopsies in our study,

it is uncertain whether comparable preservation of the small intestinal mucus layer would have been achieved with Carnoy's fixation.

Measurements of mucus layer thickness in the murine small intestine have previously been achieved by visualising the mucus with inactivated charcoal particles (Atuma *et al.*, 2001; Gustafsson *et al.*, 2012). Mucus thickness was subsequently determined *in situ* by measuring the mucus with a micromanipulator connected to a microscope (Atuma *et al.*, 2001; Gustafsson *et al.*, 2012). The advantage of this technique is the lack of additional processing steps as well as real-time measurements of changes in the mucus layer. However, specialist equipment and method adaption for human duodenal samples would be required.

An alternative method to assess the impact of EPEC and *L. reuteri* on mucus production *ex vivo* would be to analyse *de novo* mucin synthesis, as previously described in 4.3.2. This technique has previously been used on resected colonic tissue and colonic biopsies from patients with ulcerative colitis and thus could potentially be adapted for IVOC (Finnie *et al.*, 1995; Tytgat *et al.*, 1996).

4.4. Summary

In this study, we identified that *L. reuteri* ATCC PTA 6475 and EPEC enhanced and reduced MUC2 protein levels in LS174T cells, respectively. Furthermore, when these strains were co-incubated with LS174T cells, ATCC PTA 6475 counteracted the decrease in MUC2 protein content induced by EPEC. While the specific mechanisms behind these changes still remain to be investigated, these data suggest that both EPEC and *L. reuteri* ATCC PTA 6475 affect mucus production in the human GIT.

As the mucus layer is the first line of defence against infection, the development of probiotics supporting the mucus layer may have applications for reducing enteric infections and alleviating diseases with a compromised mucus layer, such as IBD. As mucus-stimulating characteristics are strain-specific, further research is required to identify the mechanisms behind these

effects as well as the bacterial strains capable of modulating mucus production. Furthering our understanding of these processes may contribute to the development of new therapies and vaccines against EPEC infection.

CHAPTER FIVE

Modulation of the innate immune response

5.1. Introduction and objectives of study

EPEC infection of the intestinal mucosa is characterised by moderate inflammation and the recruitment of neutrophils to the site of infection (Savkovic *et al.*, 1996). This response is modulated by both pro- and anti-inflammatory signals generated by EPEC (Savkovic *et al.*, 1996; Sharma *et al.*, 2006). The initial immune response against EPEC is dependent on the detection of bacterial flagellin by host TLR5, which activates NF-κB and induces the translocation of this transcription factor into the nucleus (Khan *et al.*, 2008; Ruchaud-Sparagano *et al.*, 2007; Schüller *et al.*, 2009; Sharma *et al.*, 2006; Zhou *et al.*, 2003). Binding of NF-κB to inflammatory genes subsequently initiates the production of pro-inflammatory cytokines such as the neutrophil chemoattractant IL-8 (Kunsch *et al.*, 1994; Savkovic *et al.*, 1996). However, EPEC downregulate the immune response by T3S effector proteins, inhibiting NF-κB translocation into the nucleus and counteracting other signalling pathways, such as AP-1 (Hartland *et al.*, 2000; Jarvis *et al.*, 1995; Knutton *et al.*, 1998; Wong *et al.*, 2011). The reduced inflammatory response inhibits EPEC clearance from the host (Wong *et al.*, 2011).

Probiotics can restore immune homeostasis to the intestinal milieu and specific strains exhibit anti-inflammatory or immuno-stimulatory characteristics in *in vitro* and *in vivo* systems (Díaz-Ropero *et al.*, 2007). For example, the anti-inflammatory probiotic mixture VSL#3 induced and maintained remission in patients with ulcerative colitis (Bibiloni *et al.*, 2005; Huynh *et al.*, 2009; Miele *et al.*, 2009; Sood *et al.*, 2009; Tursi *et al.*, 2010). Immunomodulation has also been demonstrated for *L. reuteri* as strains ATCC 55730 and ATCC PTA 5289 reduced pro-inflammatory cytokine expression in patients with gingivitis (Twetman *et al.*, 2009). Furthermore, ATCC PTA 4659 and ATCC 55730-derived strain DSM 17938 reduced the incidence of LPS-induced necrotising enterocolitis by modulation of TLR4 and NF-κB signalling (Liu *et al.*, 2012). As inflammation contributes to the development of diarrhoea, anti-inflammatory probiotics could be applied to reduce diarrhoeal symptoms (Viswanathan *et al.*, 2009). A promising candidate probiotic is *L. reuteri* ATCC PTA 6475, which produces histamine that suppresses the pro-inflammatory response (Jones &

Versalovic, 2009; Lin *et al.*, 2008; Liu *et al.*, 2010; McCabe *et al.*, 2013; Preidis *et al.*, 2012; Thomas *et al.*, 2012). In line with these studies, we investigated the influence of *L. reuteri* ATCC PTA 6475 and ATCC 53608 on IL-8 cytokine production during EPEC infection of intestinal cell lines and human duodenal biopsies.

5.2. Results

5.2.1. *L. reuteri* inhibits EPEC-induced IL-8 production by HT-29 cells

To investigate the effects of *L. reuteri* on IL-8 protein production, HT-29 cells were incubated with *L. reuteri* ATCC PTA 6475, ATCC 53608, EPEC, or left non-treated for 6 h, and IL-8 protein levels in cell culture media were determined by ELISA. As shown in Figure 5.1A, IL-8 production by HT-29 cells was significantly induced by EPEC infection relative to non-treated controls. In contrast, neither *L. reuteri* isolate induced the production of IL-8. To determine whether these strains demonstrated anti-inflammatory effects against EPEC-induced IL-8 production, HT-29 cells were co-incubated with equivalent numbers of EPEC and either ATCC PTA 6475 or ATCC 53608 for 6 h. We identified that co-incubation with *L. reuteri* significantly reduced IL-8 levels relative to HT-29 cells incubated with EPEC alone, though this did not reach baseline levels (Figure 5.1B).

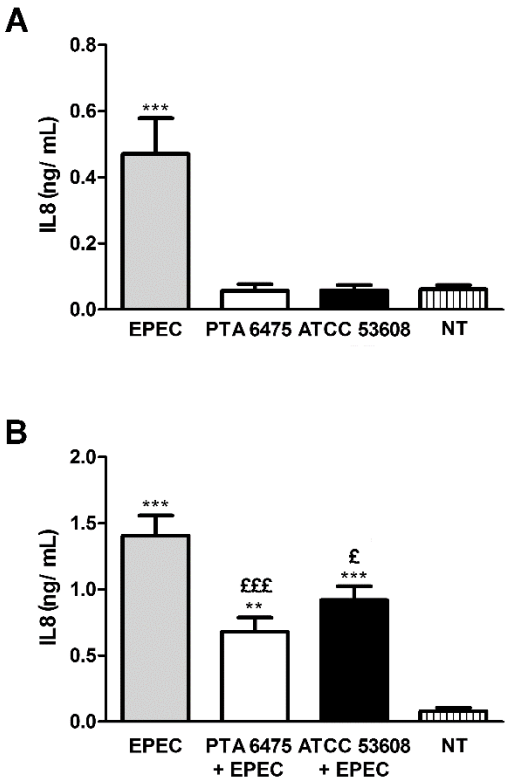

Figure 5.1: Co-incubation with *L. reuteri* ATCC PTA 6475 and ATCC 53608 inhibits EPEC-induced IL-8 production by HT-29 cells

(A) HT-29 cells were incubated with *L. reuteri* ATCC PTA 6475, ATCC 53608, EPEC, or left non-treated (NT) for 6 h. (B) HT-29 cells were co-incubated with equivalent numbers of EPEC and ATCC PTA 6475, ATCC 35608, or EPEC alone for 6 h. IL-8 protein levels in cell culture media were assessed by ELISA. Data are shown as means ± SE of three independent experiments performed in duplicate. ** = P <0.01; *** = P <0.001 versus non-treated controls. £ = P <0.05; £££ = P <0.001 versus EPEC-infected samples.

To determine the kinetics of IL-8 production in mucus-producing LS174T cells, incubations with *L. reuteri* or EPEC were performed for 3, 4 and 6 h, and IL-8 was determined as previously described. In contrast to HT-29 cells, incubation with *L. reuteri* ATCC PTA 6475 and ATCC 53608 induced IL-8 production in LS174T cells, suggesting pro-inflammatory effects in this cell line (Figure 5.2), therefore HT-29 cells were used for further experiments.

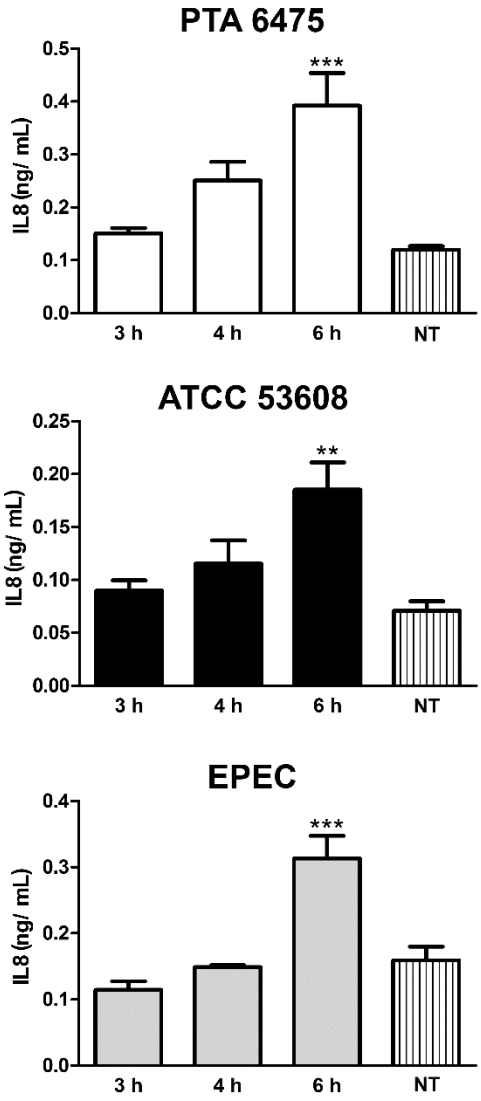

Figure 5.2: *L. reuteri* and EPEC induce IL-8 protein production by LS174T cells

LS174T cells were incubated with ATCC PTA 6475, ATCC 53608, EPEC for 3, 4, or 6 h or left non-treated (NT) for 6 h. IL-8 levels in cell culture media were assessed by ELISA. Data are shown as means ± SE of three independent experiments performed in duplicate. ** = P<0.01; *** = P <0.001 versus NT.

5.2.2. EPEC supernatant induces IL-8 production in HT-29 cells

Previous studies have shown that EPEC-induced IL-8 response *in vitro* and *ex vivo* is predominantly associated with flagellin, although other factors have been described (Khan *et al.*, 2008; Schüller *et al.*, 2009; Sharma *et al.*, 2006; Zhou *et al.*, 2003). To determine whether EPEC flagellin caused increased IL-8 production in HT-29 cells, 6 h incubation were performed with wildtype EPEC, an isogenic *fliC* mutant (AGT01), or filter sterilised supernatant from EPEC overnight cultures (SN). As shown in Figure 5.3, incubation with wildtype EPEC and EPEC SN significantly increased IL-8 production relative to HT-29 cells treated with the *fliC* mutant or the NT control.

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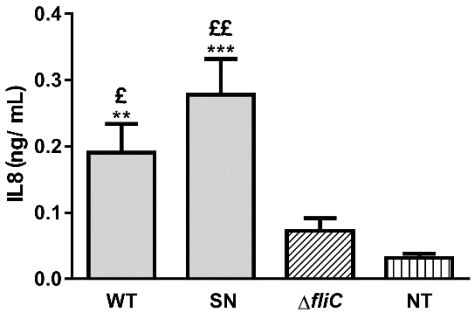

Figure 5.3: EPEC supernatant induces IL-8 production in HT-29 cells

HT-29 cells were incubated with wildtype EPEC (WT), EPEC supernatant (SN), isogenic *fliC* mutant strain AGT01 (Δ*fliC*), or left non-treated (NT) for 6 h. IL-8 protein levels in cell culture media were assessed by ELISA. Data are shown as means ± SE of either three (WT SN) or six independent experiments performed in duplicate. ** = $P<0.01$; *** = $P<0.001$ versus NT. £ = $P<0.05$; ££ = $P<0.01$ versus isogenic *fliC* mutant.

5.2.3. *L. reuteri* ATCC PTA 6475 inhibits EPEC supernatant-induced IL-8 production in HT-29 cells

As EPEC SN induced a comparable inflammatory response to EPEC, we investigated the impact of *L. reuteri* on IL-8 induction in HT-29 cells treated with EPEC SN or SN diluted 10- to 1000-fold. We identified that non-diluted and 10 to 100-fold diluted EPEC SN significantly increased IL-8 production, and that ATCC PTA 6475 inhibited IL-8 production in HT-29 cells treated with 100-fold diluted EPEC SN (data not shown). Therefore, EPEC SN was diluted 100-fold for further experiments. As anti-inflammatory characteristics have been linked to secreted products, *L. reuteri*-conditioned medium (cell culture medium treated with *L. reuteri* for 6 h) and EPEC SN were co-incubated with HT-29 cells for 6 h. As shown in Figure 5.4, only incubation with ATCC PTA 6475 significantly reduced IL-8 production stimulated by EPEC SN, whereas treatment with ATCC 53608 or *L. reuteri*-conditioned medium had no effect.

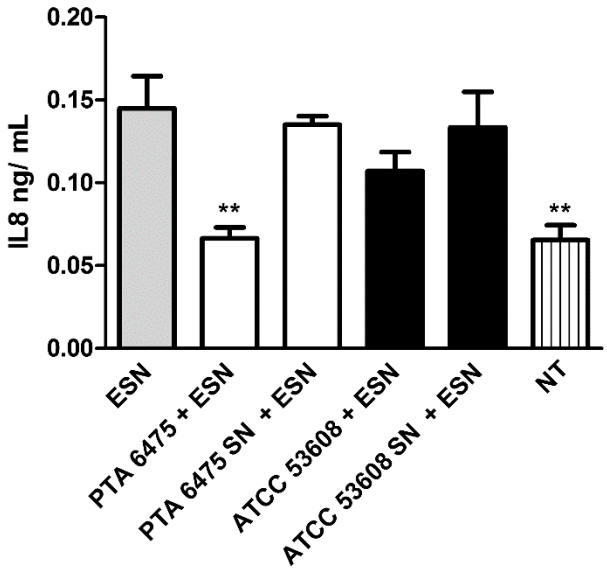

Figure 5.4: ATCC PTA 6475-mediated inhibition of IL-8 production is independent of secreted products

HT-29 cells were incubated with EPEC supernatant (ESN) and *L. reuteri* ATCC PTA 6475, ATCC 53608 or *L. reuteri*-conditioned medium, or left non-treated (NT) for 6 h. IL-8 levels in cell culture media were assessed by ELISA. Data are shown as means ± SE of three independent experiments performed in duplicate. ** = $P<0.01$ versus ESN.

5.2.4. NF-κB translocation in HT-29 cells

The translocation of NF-κB from the cytoplasm to the cell nucleus is a key step in the upregulation of IL-8 protein in response to EPEC infection (Savkovic *et al.*, 1997). To determine whether this process was modulated by *L. reuteri*, a protocol for establishing NF-κB p65 (RelA) translocation into the nucleus was required. For this purpose, HT-29 cells were incubated with EPEC for 1 and 2 h or treated with 10 ng/ mL TNF-α for 1 and 4 h. Non-confluent HT-29 cells were used to improve visual acuity, and short time points were chosen to reduce NF-κB suppression by EPEC effector proteins.

While we observed cytoplasmic staining of p65 in all samples, we did not detect nuclear translocation of NF-κB in EPEC- or TNF-α-treated HT-29 cells (Figure 5.5). This finding suggests that alternative techniques are required to investigate changes in NF-κB localisation in HT-29 cells.

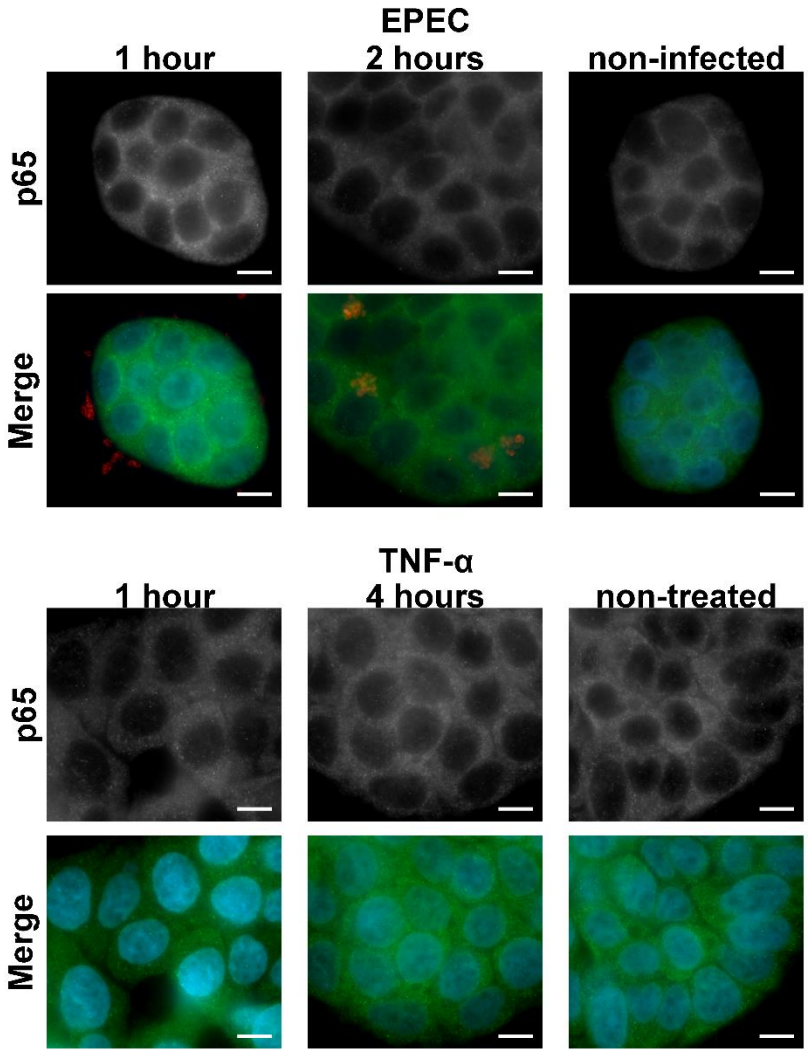

Figure 5.5: Anti-p65 immunofluorescence staining did not detect NF-kB nuclear translocation in HT-29 cells

HT-29 cells were infected with EPEC for 1-2 h, treated with 10 ng/ mL TNF-α for 1-4 h, or remained non-treated. Cells were stained for p65 (green), *E. coli* (red), and cell nuclei (blue). Black and white images of the green channel is shown to improve clarity. Images are representative of either two (EPEC) or

three (TNF-α) independent experiments performed in duplicate. Scale bars = 10 µm.

5.2.5. Influence of *L. reuteri* on IL-8 production in EPEC-infected duodenal biopsies

As *L. reuteri* inhibited EPEC-induced IL-8 production in HT-29 cells, we further investigated the effects of *L. reuteri* and EPEC on IL-8 protein levels in duodenal tissue using pIVOC and evaluated by ELISA. As biopsies were of variable size, IL-8 protein levels were adjusted against total protein content of the biopsy lysate. As shown in Figure 5.6, there was no significant difference in IL-8 protein levels between *L. reuteri* and non-treated controls, whereas EPEC infection significantly increased IL-8 protein content. In contrast to HT-29 cells, co-incubation with *L. reuteri* did not significantly affect IL-8 production in EPEC-infected tissues. (Figure 5.6).

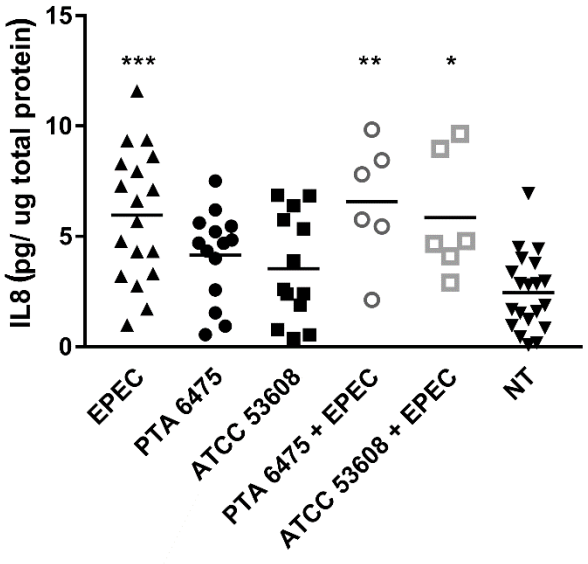

Figure 5.6: *L. reuteri* does not affect EPEC-induced IL-8 production in duodenal biopsies

Duodenal biopsies were exposed to *L. reuteri* ATCC PTA 6475, ATCC 53608, EPEC, or left non-treated (NT) for 6 h. In addition, co-incubations of equivalent numbers of *L. reuteri* and EPEC were performed. Each data point represents an individual biopsy sample. The median is indicated by a line. * = P <0.05; ** = P <0.01; *** = P <0.001 versus NT.

5.2.6. The IL-8 response is not suppressed by EPEC during infection of duodenal biopsies

As we did not observe *L. reuteri* modulation of the IL-8 response in EPEC-infected biopsies, we investigated whether EPEC suppression of the immune response described in cell lines may have obscured any protective effects of *L. reuteri*. Therefore, HT-29 cells were incubated with wildtype EPEC or a T3SS-deficient *escN* mutant (CVD452; Jarvis *et al*, 1995) for up to 8 h. As shown in Figure 5.7A, HT-29 cells produced significantly more IL-8 when infected with Δ*escN* mutant versus wildtype EPEC after 4-8 h of incubation.

Interestingly, no immunosuppressive effect was observed in human duodenal as incubation with T3SS-deficient mutants Δ*escN* and Δ*espB* (UMD864; Donnenberg *et al,* 1993a) induced comparable IL-8 levels to wildtype EPEC (Figure 5.7B). In addition, infection with a *fliC* mutant did not induce a significant increase in IL-8 levels compared to NT controls.

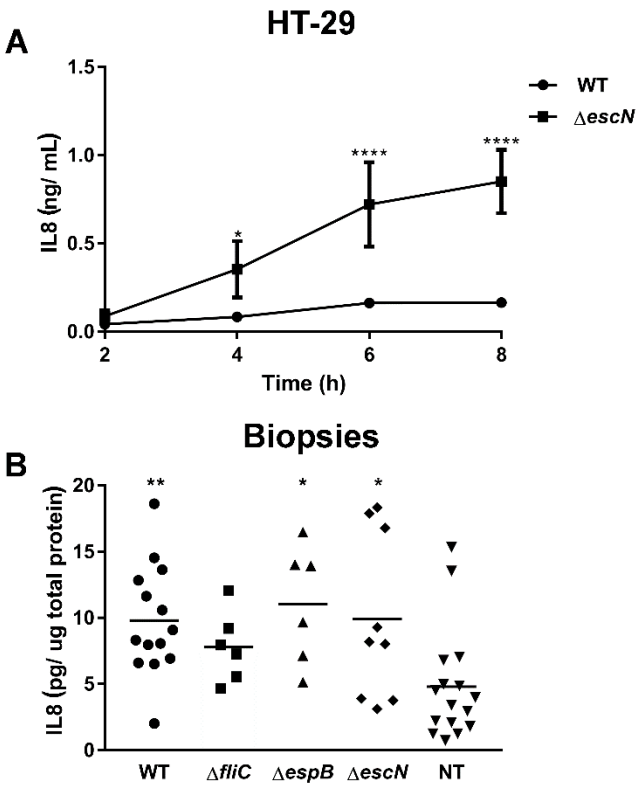

Figure 5.7: The IL-8 response is not downregulated by EPEC during infection of duodenal biopsies

(A) HT-29 cells were incubated with wildtype EPEC (WT) or isogenic *escN* mutant strain CVD452 (Δ*escN*) for 2-8 h. IL-8 levels in cell culture media were assessed by ELISA. Data are shown as means ± SE of three independent experiments performed in duplicate. * = $P<0.05$; **** = $P<0.0001$ versus WT. (B) Duodenal biopsies were infected with either wildtype EPEC, isogenic mutants deficient in *fliC* (AGT01), *espB* (UMD864), *escN* (CVD452), or left non-treated (NT) for 6 h. IL-8 protein was determined from biopsy lysates and is displayed relative to the total protein content of each biopsy. Each data point represents an individual biopsy sample. The median is indicated by a line. * = $P<0.05$; ** = $P<0.01$ versus NT.

5.3. Discussion

The innate immune system is a key component of the host response which is responsible for the initial defence against invading pathogens. While the innate immune response prevents the vast majority of potential insults, some GI pathogens have evolved mechanisms to inhibit inflammation. The weakened immune response contributes to the production of diarrhoea in the host. Probiotics may offer a solution to reduce the incidence of diarrhoea, as certain bacterial strains have been identified as having immunomodulatory characteristics.

In this investigation, we have explored the immunomodulatory effects of *L. reuteri* on EPEC-induced IL-8 protein production using both *in vitro* and *ex vivo* models. Furthermore, we have examined the role of the T3SS in IL-8 production during EPEC infection of the small intestinal epithelium.

5.3.1. Role of flagellin and the T3SS in IL-8 induction during EPEC infection

In this study, we determined that EPEC induced the production of the immune cytokine IL-8 by HT-29 cells and human duodenal biopsies. As HT-29 cells showed a diminished IL-8 response when infected with an isogenic *fliC* mutant, this suggests that flagellin is a major inducer of the IL-8 response during EPEC infection. This finding is consistent with previous reports which have identified EPEC flagellin as a key inducer of IL-8 production by intestinal HT-29, T84, and Caco-2 cells (Khan *et al.*, 2008; Ruchaud-Sparagano *et al.*, 2007; Sharma *et al.*, 2006; Zhou *et al.*, 2003). The role of flagellin in IL-8 induction has also been demonstrated in paediatric duodenal biopsies, as both wildtype EPEC and purified flagella induced IL-8 protein production (Schüller *et al.*, 2009). Similar to our own results in duodenal biopsies, infection with a *fliC* deletion mutant resulted in a diminished, although not completely abrogated, IL-8 response, which suggests other inflammatory factors may be produced by EPEC. The induction of IL-8 by a FliC-independent mechanism has been shown in non-polarised HT-29 and Caco-2 cells, although the exact

mechanism has not been clarified (Khan *et al.*, 2008; Schüller *et al.*, 2009; Sharma *et al.*, 2006).

While EPEC flagellin induces IL-8 protein expression, the T3SS has been implicated in down-regulating the innate immune response (Ruchaud-Sparagano *et al.*, 2007). This effect has been attributed to several effector proteins encoded outside the LEE region (Nle) (Nadler *et al.*, 2010; Newton *et al.*, 2010; Vossenkämper *et al.*, 2010). NleE prevents the phosphorylation of Iκβ kinase (IKK), and thus reduces the degradation of the NF-κB inhibitor IκB, inhibiting activation of NF-κB (Nadler *et al.*, 2010). This effect has been demonstrated in HeLa and Caco-2 cells as well as isolated dendritic cells and has been associated with a diminished IL-8 response (Nadler *et al.*, 2010; Newton *et al.*, 2010; Vossenkämper *et al.*, 2010). The inhibitory action of NleE is further enhanced by the effector protein NleB, which inhibits the activation of NF-κB by reducing GAPDH-mediated ubiquitination of TRAF2, a protein involved in TNF-α mediated NF-κB activation (Gao *et al.*, 2013; Nadler *et al.*, 2010). Furthermore, the effector protein NleC degrades the p65 subunit of the NF-κB complex (Pearson *et al.*, 2011; Yen *et al.*, 2010). Notably, the roles of these effector proteins have primarily been documented in cancer cell lines, and their effect in the *in vivo* situation remains largely unknown. While we observed a T3SS-dependent immunosuppressive effect in EPEC-infected HT-29 cells, this was not evident during infection of human duodenal biopsies. A similar lack of immunosuppression has recently been demonstrated during EHEC infection of human colonic biopsies, suggesting a delay or absence of this mechanism in human intestinal tissues (Lewis *et al.*, 2016).

5.3.2. Impact of *L. reuteri* on the EPEC-induced IL-8 response *in vitro*

In our study, we showed that *L. reuteri* strains ATCC PTA 6475 and ATCC 53608 induced IL-8 production in LS174T, but not HT-29 cells. This difference could be due to cell line-specific features, such as cell type (goblet cell versus enterocyte) and the genetic background of the host, which can impact on

probiotic effects (van Baarlen *et al.*, 2013). This has previously been observed in *L. rhamnosus* GG, which stimulated IL-8 production in HT-29 cells infected with *S.* Typhimurium, but reduced IL-8 protein in Caco-2 cells treated with flagellin, despite similar methodologies (Lopez *et al.*, 2008; Pinto *et al.*, 2009). While the immune stimulus was different in each of the prior studies (*S.* Typhimurium versus isolated flagellin from *Pseudomonas aeruginosa*) which may have influenced probiotic effects, these findings demonstrate that thorough evaluation of probiotics on relevant model systems is necessary to determine immunomodulatory characteristics of specific *Lactobacillus* strains.

Probiotic immunomodulation of pathogen-induced immune responses by intestinal epithelial cell lines has previously been reported, and strain-specific anti-inflammatory and immunostimulatory characteristics have been shown. In this study, we found that co-incubation with *L. reuteri* ATCC PTA 6475 and ATCC 53608 significantly reduced IL-8 production in EPEC-infected HT-29 cells. Similar findings have been reported for other *Lactobacillus* species, such as the porcine isolate *Lactobacillus sobrius* DSM 16698, which upregulated anti-inflammatory IL-10 and reduced IL-8 protein content in ETEC-infected porcine IECs (Roselli *et al.*, 2007). In addition, pre-incubation of T84 cells with *L. rhamnosus* JB-1 or mixed probiotic VSL#3 prior to infection with *Salmonella,* reduced IL-8 production (Ma *et al.*, 2004; Madsen *et al.*, 2001). In contrast, lactobacilli can sensitise cells to pathogenic insult, as the pre-treatment of HT-29 cells with *L. plantarum* BFE or *L. rhamnosus* GG increased the IL-8 response to *S.* Typhimurium (Pinto *et al.*, 2009). This effect may be due to increased production of TLR2 and TLR5, which detect lipoteichoic acid and flagellin, respectively (Pinto *et al.*, 2009).

As flagellin is a potent inducer of the pro-inflammatory response to many pathogens, the effect of lactobacilli on flagellin-induced IL-8 production has been investigated in previous studies. Pre-treatment with *L. rhamnosus* GG attenuated IL-8 induction in Caco-2 cells stimulated with flagellin from *P. aeruginosa* (Lopez *et al.*, 2008). Interestingly, anti-inflammatory effects of probiotics against a pathogen do not imply reduction of the immune response to particular bacterial components, as *L. salivarius* UCC118 attenuated IL-8 production in HT-29 cells treated with *S.* Typhimurium but not with *Salmonella*

flagella (O'Hara *et al.*, 2006). These findings are similar to our own, as *L. reuteri* ATCC 53608 significantly reduced the production of IL-8 by HT-29 cells when co-cultured with EPEC. However, there was no reduction in IL-8 protein when HT-29 cells were treated with ATCC 53608 and EPEC SN. In contrast, *L. reuteri* ATCC PTA 6475 significantly reduced the IL-8 response to both EPEC and EPEC SN. The mechanism behind this strain-specific effect remains to be defined but potential explanations will be discussed.

While EPEC SN contains EPEC flagella (Girón *et al.*, 2002; Zhou *et al.*, 2003), it may also comprise additional pro-inflammatory factors, such as LPS, which induce the production of IL-8 (Cario *et al.*, 2000; Hoshino *et al.*, 1999; Poltorak *et al.*, 1998). Probiotics have previously demonstrated anti-inflammatory effects against LPS-induced inflammation, as VSL#3, *L. paracasei* 1602, and *L. reuteri* 6798 decreased the production of TNF-α in LPS-treated ileal and colonic explants from IL-10-deficient mice (Madsen *et al.*, 2001; Peña *et al.*, 2005). Interestingly, *L. reuteri* ATCC PTA 6475, ATCC PTA 4659, and ATCC PTA 5289 inhibited LPS-induced IL-8 production in porcine IECs and rat ileum (Liu *et al.*, 2010). As we utilised SN rather than EPEC-derived LPS or flagellin, further experimentation is required to determine whether *L. reuteri* inhibits IL-8 production induced by these specific components. These findings would elucidate the host signalling pathways through which *L. reuteri* modulates the innate immune response. Additionally, as EPEC-derived flagella induce IL-8 production in duodenal biopsies (Schüller *et al.*, 2009), it would be interesting to investigate whether *L. reuteri* inhibited flagellum-induced IL-8 production in duodenal samples.

As mentioned previously, production of IL-8 and other pro-inflammatory cytokines is mediated by the transcription factor NF-κB. A number of *Lactobacillus* strains, such as *L. rhamnosus* GG and JB-1, as well as *L. plantarum* ATCC 8014, have been shown to inhibit NF-κB activation by preventing IκB degradation in HeLa, T84, and Caco-2 cells (Ko *et al.*, 2007; Lopez *et al.*, 2008; Ma *et al.*, 2004). Unfortunately, we were unable to determine whether ATCC PTA 6475-mediated IL-8 reduction in EPEC-infected HT-29 cells was dependent on NF-κB activation, as immunofluorescence staining of NF-κB was unsuitable to detect nuclear

translocation in HT-29 cells treated with EPEC or TNF-α. This was consistent with a previous study reporting the absence of nuclear NF-κB immunofluorescence staining in IL-1β-treated HT-29 cells, whereas Caco-2 cells demonstrated distinct NF-κB nuclear localisation (Jobin *et al.*, 1997). However, use of electrophoretic mobility shift assays and ELISA showed nuclear localisation in IL-1β-treated HT-29 cells, although at lower levels than treated Caco-2 cells (Jobin *et al.*, 1997; Sibartie *et al.*, 2009). These findings suggest that immunofluorescence microscopy is unsuitable to determine changes in NF-κB localisation in HT-29 cells, and alternative methods such as mobility shift assays or ELISA should be used.

Some *Lactobacillus* strains secrete factors inhibiting immune stimulation by pathogens. The mechanism behind this inhibition is often undefined, and possibly dependent on bactericidal effects. However, pre-incubation of *Salmonella* with supernatant from *L. acidophilus* LB, *L. casei* 2756, *L. curvatus* 2775, or *L. plantarum* 2142 reduced IL-8 induction in Caco-2 cells (Coconnier *et al.*, 2000; Nemeth *et al.*, 2006). Alternatively, some *Lactobacillus* strains secrete products which directly impact on the immune state of the host cell. For example, *L. reuteri* ATCC PTA 6475 SN reduced production of TNF-α by LPS-treated monocytes and macrophages from children with active Crohn's disease (Jones & Versalovic, 2009; Lin *et al.*, 2008). This has been linked to the production of the immunoregulatory compound histamine, which is derived from the essential amino acid L-histidine (Thomas *et al.*, 2012). ATCC PTA 6475-derived histamine activates the histamine H_2 receptor, which reduces the production of TNF-α via inhibition of the MAPK pathway in monocytoid cells (Thomas *et al.*, 2012). These findings contrast our own observations, as the conditioned medium of ATCC PTA 6475 did not attenuate the production of IL-8 by HT-29 cells stimulated with EPEC SN. These differences may be due to differences in cell type (IECs versus monocytes), cytokines investigated (IL-8 versus TNF-α), or bacterial culture conditions (as histamine may not have been produced during our experiments). Taken together, our findings demonstrate that *L. reuteri* ATCC PTA 6475 and ATCC 53608 have immunomodulatory effects on IECs. These effects are independent of secreted products and thus may be dependent on the presence of the bacteria

at the epithelium.. Further investigation is required to define the mechanism behind the reduction in IL-8 protein levels in *L. reuteri* ATCC PTA 6475- and ATCC 53608-treated IECs, such as the impact of *L. reuteri* cell surface proteins on the innate immune response. An improved understanding of the mechanisms behind this effect will further validate the probiotic potential of these anti-inflammatory bacteria on the inflamed epithelium.

5.3.3. The effect of *L. reuteri* on the EPEC-induced IL-8 response *ex vivo*

In contrast to findings in HT-29 cells, incubation with *L. reuteri* did not reduce IL-8 production in EPEC-infected duodenal biopsies. As *L. reuteri* did not penetrate the mucus layer, this could imply that adhesion to the epithelium is necessary for modulation of the immune response. This has previously been demonstrated by *L. rhamnosus* GG, which required the SpaCBA pilus for epithelial binding and inhibition of IL-8 expression in Caco-2 cells (Lebeer *et al.*, 2012). Similarly, adherent live *L. rhamnosus* JB1 were required to inhibit IL-8 production in stimulated T84 and HT-29 cells (Ma *et al.*, 2004). It is important to note that trauma of tissue removal induced inflammation in biopsies, as non-treated samples produced elevated levels of IL-8. The induction of the innate immune response due to tissue removal is a technical limitation of pIVOC when investigating the innate immune response. Therefore, it would be interesting to assess the anti-inflammatory effects of *L. reuteri* using an *in vivo* model, which would not present with background inflammation.

Additionally, the immune status of the host can also impact on probiotic immunomodulation, as *L. rhamnosus* GG downregulated the immune response to milk in hypersensitive patients, but increased the expression of immunostimulatory receptors in the control group (Pelto *et al.*, 1998). Active inflammation can also sensitise the epithelium to probiotics, as TNF-α-treated HT-29 cells showed enhanced IL-8 protein levels when subsequently exposed to *L. plantarum* BFE 1685 or *L. rhamnosus* GG, whereas neither strain induced

IL-8 production in non-treated cells (Pinto *et al.*, 2009). Furthermore, the incubation of *L. rhamnosus* GG, *L. plantarum* NCIMB8826, and *L. paracasei* B21060 with tissue samples from patients with UC and CD caused severe degradation of biopsy tissue, whereas incubation with healthy samples did not alter tissue structure (Tsilingiri *et al.*, 2012).

Moreover, it is important to note that the immunomodulatory effects of probiotics may be cell-specific, as *L. rhamnosus* GG and *L. plantarum* NCIMB8826 did not induce IL-8 production in Caco-2 cells, but stimulated IL-12 and TNF-α expression in human peripheral blood monocytes (Mileti *et al.*, 2009). Furthermore, *L. rhamnosus* GG and *L. plantarum* NCIMB8826 amplified the progression of colitis in DSS-treated mice (Mileti *et al.*, 2009). Crucially, these characteristics are strain-specific, as *L. paracasei* B21060 reduced the symptoms of DSS-induced colitis versus mice without the probiotic (Mileti *et al.*, 2009). In addition to chemically-induced inflammation, lactobacilli also decreased inflammation induced by pathogens. *L. johnsonii* La1 reduced keratinocyte-derived protein chemokine (KC; mouse cytokine homologous to human IL-8) serum levels in mice with ongoing *H. pylori* infection, and *L. paracasei ssp*. *paracasei* NTU 101 decreased IL-1β and IL-6 expression in EHEC-infected mice (Sgouras *et al.*, 2005; Tsai *et al.*, 2010). Anti-inflammatory effects have also been described in human studies, as *L. reuteri* ATCC 55730 and ATCC PTA 5289 decreased IL-8 and TNF-α levels in patients with active gingivitis (Twetman *et al.*, 2009).

As previous studies have demonstrated that probiotics can deliver anti-inflammatory effects during acute inflammation events, it would be interesting to investigate the impact of ATCC PTA 6475 and ATCC 53608 on inflamed tissue samples using the pIVOC model; to determine whether the anti-inflammatory characteristics we observed were applicable to an inflamed milieu. Furthermore, future study into the role of epithelial binding on immunomodulation of intestinal samples would be beneficial for developing our understanding of how probiotics modulate the innate immune response. Aspiration of the mucus layer has been demonstrated on murine duodenal tissue (Atuma *et al.*, 2001; Gustafsson *et al.*, 2012). Therefore, this approach could be applied to human duodenal biopsies, to investigate whether epithelial

contact with the epithelium is necessary for *L. reuteri* immunomodulation. Additionally, it would be interesting to assess the impact of *L. reuteri* on EPEC-induced IL-8 production in paediatric biopsies. As the immune system matures with age, the inflammatory response to EPEC infection may differ between adult and paediatric samples (Levy, 2007).

5.4. Summary

In this part of the study, we demonstrated that *L. reuteri* ATCC PTA 6475 and ATCC 53608 inhibited IL-8 induction in EPEC-infected HT-29 cells. This effect appeared to be dependent on *L. reuteri* adhesion to the host epithelium, as we did not observe IL-8 inhibition by *L. reuteri* supernatants or biopsy samples with an intact mucus layer. While the specific mechanisms behind *L. reuteri* immune modulation remain undefined, our findings demonstrate that ATCC PTA 6475 and ATCC 53608 can modulate the innate immune response induced by EPEC infection.

The innate immune system provides a key barrier against EPEC infection of the intestinal epithelium. The secretion of T3S proteins weakens the inflammatory response which inhibits EPEC clearance but contributes to the production of diarrhoea in the host. Modulation of the inflammatory response by anti-inflammatory probiotics could reduce diarrhoeal symptoms in these patients. Therefore, the identification of probiotics with anti-inflammatory characteristics is an important area of research for developing therapies to reduce the burden of EPEC-induced diarrhoea.

CHAPTER SIX

Conclusions

The aim of this PhD project was to evaluate the potential of *Lactobacillus reuteri* to reduce pathogenesis of enteropathogenic *Escherichia coli* infection. In particular, the effects of *L. reuteri* on 1) EPEC binding to the intestinal epithelium, 2) mucus production, and 3) the innate immune response were characterised. To investigate these effects, mucus- and non-mucus producing intestinal epithelial cells (HT-29 and LS174T cells, respectively) and duodenal biopsies were utilised as models of the human intestinal epithelium.

In the first part of this study, we identified that *L. reuteri* inhibited EPEC binding to intestinal epithelium at the mucus and epithelial level. These effects were strain-specific and included competition for binding receptors and the inhibition of EPEC dispersal across the epithelial surface. The protective effects observed were also dependent on the intestinal epithelial model used, which emphasised the requirement for physiologically relevant model systems. Therefore, future studies should investigate whether *L. reuteri* ATCC PTA 6475 and ATCC 53608 inhibit EPEC colonisation of the intestinal epithelium in animal trials and clinical studies.

A limitation of this study was the use of non-polarised IECs, as the human intestinal epithelium demonstrates distinct apical and basolateral polarisation of cell surface receptors. Thus, it would be interesting to assess the efficiency of *L. reuteri* binding to polarised cells which may have an alternative distribution of receptors on the apical surface than non-polarised cells. Additionally, polarised cells could be used to investigate the impact of *L. reuteri* on EPEC-induced changes to TJ integrity, a key mechanism behind the induction of diarrhoea *in vivo*. However, it is not possible to polarise HT-29 cells, thus alternative small intestinal models, such as Caco-2 cells, xenografts, or intestinal biopsies, would be required. Furthermore, while we assessed the impact of *L. reuteri* monocultures on EPEC infection, mixed probiotic cultures have been suggested to deliver greater benefits to the host. Therefore, it would be interesting to investigate the impact of *L. reuteri* co-cultures on EPEC pathogenesis.

We also examined the impact of *L. reuteri* and EPEC on mucus production and determined that these bacteria significantly increased and decreased

MUC2 protein levels in LS174T cells, respectively. The effect of probiotic bacteria on the production of mucus has previously been poorly investigated, thus our data provide evidence that certain *L. reuteri* strains influence GI mucin levels. A limitation of this study was that we determined relative changes in fluorescence rather than total change in mucin protein. Therefore, it would be beneficial to confirm that the change in fluorescence which we identified corresponded with a significant change in MUC2 protein, by analysis of *de novo* mucin synthesis or Western blot. Future research should investigate the mechanism behind *L. reuteri*-induced change in MUC2 levels and determine whether ATCC PTA 6475 induced *de novo* mucin synthesis or enhanced mucus secretion, as well as the role of *L. reuteri* epithelial binding, cell surface proteins, and secreted products on mucus production.

In the final part of this study, we determined that *L. reuteri* inhibited IL-8 production by EPEC-infected intestinal epithelial cells. This is the first study to identify that *L. reuteri* ATCC PTA 6475 and ATCC 53608 can modulate the production of IL-8 protein by enterocytes in response to infection. As these anti-inflammatory effects appear to be dependent on epithelial binding, it would be interesting to investigate whether *L. reuteri* modify the innate immune response of intestinal samples with a compromised mucus layer. Additionally, future studies should assess the impact of *L. reuteri* on other pro- and anti-inflammatory markers, such as IL-1β, IL-6, and IL-10 to further elucidate the mechanisms behind *L. reuteri* immunomodulation.

In summary, the findings from this PhD demonstrate that *L. reuteri* ATCC PTA 6475 and ATCC 53608 can protect the human intestinal epithelium against EPEC infection. These effects were particularly pertinent to ATCC PTA 6475, which inhibited EPEC microcolony dispersal, enhanced the levels of MUC2, and inhibited EPEC-induced IL-8 protein production. The characterisation of *L. reuteri* protective effects provides further evidence for the selection of these lactobacilli in the prevention of EPEC-induced acute infectious diarrhoea.

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APPENDIX 1

Norwich Biorepository

Information Sheet for Patients and Consent Form – Adult

The Norwich Biorepository

The donation, collection, storage and use of samples of tissue and/or fluids and/or other material from an adult for research

Information sheet for patients - Version 15 (21 February 2014)

Thank you for considering giving a sample for biomedical research. This information sheet provides a brief summary to help you to understand what this means and involves.

There is a consent form after the information sheet. It is important that you complete and sign it, if you decide to give us a sample. Please complete all parts of the consent form.

Doctors and other health professionals often take samples (which may be blood, small biopsies, or something else) from patients to help tell us what is wrong and how best to treat it. Larger pieces of tissue or whole organs may be removed by surgeons from some patients as part of their treatment.

Quite often, some of the test sample or surgical tissue is left over at the end. The left-over sample can be used by other doctors or researchers in special experiments to learn more about illness, how it happens and how to treat it, and sometimes to help make new medicines.

We would like to do some experiments on any left-over sample or surgical tissue, once your tests are finished. To do that, we need your permission and signed consent.

Samples donated (given) to the Norwich Biorepository are not:

- Normally used in animal research. It will be made clear to you if animal research is an integral part of the project for which we are seeking a donation.
- Used in cloning experiments. However, the Biorepository would consider the use of donations in non-reproductive cloning experiments based on their scientific value and in the context of prevailing law and ethical standards. It will be made clear to you if cloning experiments are part of the research project for which we are seeking a donation.

If you want to help us, please sign the form at the end of this document.

Continued.....

If you give permission for a sample to be taken –

- The Hospital will own the sample.
- The sample may be stored, usually in a deep freezer, until it is used. The freezer is referred to as a tissue bank in the consent form.
- Nobody involved in the research will know where the sample has come from.
- The sample will be used only in experiments that are ethical and to help other people. Please see the section entitled 'Scientific and ethical approval' below to understand what we mean by *ethical*.
- Your donated sample(s) and any genetic material derived from it (them) may be stored for an indefinite amount of time for future research projects, which may include whole genome sequencing. *Whole genome sequencing* means reading your total DNA code (your genetic blueprint) in a single assessment.
- We might give some of or all the sample to other doctors or researchers for their experiments, if they are ethical and to help other people. Some of these people might work in companies in this country or abroad.
- Data derived from your sample(s) may be placed anonymously in an international database to be used in future research. While we will take all possible steps to maintain your anonymity and protect your privacy, there is a very small risk that genetic information produced in the research and stored on databases could lead to your identification by being linked to other stored information.
- We will keep some facts about you on our Biorepository database.
- Although these facts might be given to the research doctors or scientists to help their experiments, we will NOT tell them your name or other details that would let them know who you are.
- Doctors in the Hospital might also read your hospital records to help them understand what the doctors or scientists find out in the experiments. This is possible because your hospital records can be linked to the anonymous research sample without loss of confidentiality as far as the researchers are concerned. If the research results are important for you, it might be possible using this linkage to feed back the information to your doctor, so that any appropriate action can be considered.

Your treatment will not be affected in any way, if you do not feel able to say yes to our request for a research sample.

The next sections give you some more detailed information. If you have any questions, please do not hesitate to ask the person who is seeking your permission.

WHAT WILL HAPPEN

Tissue, blood or other samples taken from you for diagnosis and/or treatment of your condition will be sent to the Pathology Laboratories, where they will be tested to decide exactly what they are and whether any further treatment will be necessary. This is a standard part of treatment. **Only as much tissue or fluid as is needed will be removed.**

Continued.....

MEDICAL RESEARCH AND WHY THIS PROGRAMME IS IMPORTANT
When all the routine tests have been done, if any samples are left over, with your consent, they could be donated (given) for use in medical research. As part of a research programme which now includes the Norfolk and Norwich University Hospitals NHS Foundation Trust, the James Paget University Hospitals NHS Foundation Trust, the University of East Anglia (UEA), and the Institute of Food Research (IFR), some of the sample or material extracted from it will be stored in the Norwich Biorepository (usually in a special deep freezer) for use by ourselves or by researchers from other centres at a later date. Some of this research may involve an assessment of genetic material (DNA and/or RNA) to help us understand the genetic basis of health and disease.

The purpose of this research is to understand more about human health and disease, and to develop new methods of prevention or new treatments for the benefit of future patient care. Some of these research programmes could lead to the development of new products and processes, which may be developed commercially for the improvement of patient care, in which case there would be no financial benefit to you.

Medically qualified doctors or other suitably qualified staff at the hospital may need to review your hospital records, including case notes, as part of this research in order to relate the research findings to the clinical outcome. It is important to be able see how you progress after the tissue or other samples you have donated (given) have been used in the research project(s). They will not give your name to those doing the research.

The research may also involve training doctors and researchers in scientific medicine, and may lead to higher qualifications for them (e.g., PhD or MD degrees). This is important for future research into diseases and for looking for new, more effective, treatments for them.

LINKS WITH OTHER ORGANISATIONS
If you agree, we may send stored material or products derived from it to other approved tissue banks or companies in this country or abroad to support their research programmes or the research programmes of those companies' clients. Such outside organisations will provide financial support for the Norwich Biorepository (our tissue bank), to help it recover its operating costs. We are not, however, allowed to sell tissue or other samples in order to make any financial profit from these commercial links.

SCIENTIFIC AND ETHICAL APPROVAL
The Norwich Biorepository acts as a custodian of the samples it holds. It releases them only to individuals or organisations that have an acceptable scientific background and work to high ethical standards. We require that all such medical research has been approved by a properly constituted Research Governance Committee before it starts. It must also be approved by a Research Ethics Committee or on behalf of the Research Ethics Committee that oversees the work of the Norwich Biorepository under the terms of the Biorepository's own Research Ethics Committee approval. That committee is the Cambridge East Research Ethics Committee. These committees look particularly at the purpose and validity of the research proposal, the welfare of any participants and issues of consent and confidentiality. We will release samples to commercial companies only if they work to appropriate ethical and scientific standards.

Continued.....

DONATING EXTRA SAMPLES FOR RESEARCH

In certain circumstances you may be asked by the doctor treating you (or by a doctor, research nurse or nurse practitioner working with him/her) to consider donating (giving) tissue or other samples in addition to those to be removed as part of your diagnostic investigation or treatment. **Such extra samples will be taken only if you give your consent and if their removal does not cause you any harmful effects now or in the future.**

YOUR RIGHTS

If your samples are stored, information about your case will be kept on a computer in the Norwich Biorepository. This will help us understand what your illness was like and relate what we find in experiments to what happens to patients. Under the Data Protection Act, you are entitled to ask to see what is recorded about you by applying to the Chairman of the Norwich Biorepository Committee, Norfolk & Norwich University Hospital, c/o Dept. of Cellular Pathology, The Cotman Centre, Colney Lane, Norwich, NR4 7UB. No one other than you has the right to see these records and any information needed for research purposes will be made anonymous before it is given to the researcher.

The researchers will not be able to find out your name or any personal details about you from the information that they receive.

You will have the opportunity to discuss with a doctor issues relating to the possible use of your samples for research purposes. He or she will answer any questions you may have.

MAKING A DONATION (GIFT) OF TISSUE AND/OR OTHER MATERIAL FOR RESEARCH

If you decide that you want your tissue, etc., to be stored in the Norwich Biorepository and used for research purposes, you will be asked to sign a special Consent Form confirming your decision and stating that you have read and understood this sheet. When you sign the form you will give the ownership of the tissue or other samples to the Norfolk & Norwich University Hospitals NHS Foundation Trust. The tissue or other samples will then belong to the Trust, which will store it for an indefinite period of time and will able to decide how it should be used for research. It will also have the right to dispose of unused stored material in an appropriate legal and ethical manner following normal procedures.

If you do not want your tissue to be stored in the Norwich Biorepository, please tell us and do NOT sign the special Consent Form.

If you do not sign this form, the tissue or other samples will still be sent to the laboratory to undergo those tests that are necessary for your care but they will not be used for research purposes. All unused tissue from your procedure will be disposed of using normal hospital methods. We will respect your decision and it will not affect in any way the treatment you receive.

Continued.....

Our Vision
To provide every patient with the care we want for those we love the most

Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

Affix an addressograph label here or complete the following details:

Patient's name.....

Date of birth.....

Hospital no. .....

The Norwich Biorepository

Consent for the collection, storage and release of human samples for research

I agree (Please initial small box) that the following tissue or other material may be used for research, including genetic (DNA and/or RNA) studies and for the possible development of commercial products for the improvement of patient care, from which I would receive no financial benefit:

List samples for research:	☐

I also agree that (Please initial small boxes, as appropriate):

These samples become the property of the Norfolk & Norwich University Hospitals NHS Foundation Trust ("the Trust")	☐
The Trust may store these samples in a tissue bank / biorepository	☐
The Trust may use these samples at its discretion in properly approved research programmes	☐
The Trust may pass on these samples to other approved tissue banks and/or companies, which may be in this country or abroad, in properly approved research programmes	Yes / No
My genetic material and donated sample(s) may be stored for an indefinite amount of time for future research projects, which may include whole genome sequencing	☐
Information about my case may be kept on the Norwich Biorepository database	☐
Anonymous data derived from my sample(s) may be placed in an international database for future research	☐
Such information may be passed in an anonymous form to persons outside the Trust In connection with research and may be published with any research findings	☐
I agree that appropriately qualified staff employed by the Trust may review my hospital Medical records, including case notes, as appropriate, for the purposes of research using the donated samples	☐
These samples may be used in ethically approved animal research	Yes / No
These samples may be used in ethically approved cloning research	Yes / No

Continued.....

Affix an addressograph label here or complete the following details:

Patient's name.....

Date of birth.....

Hospital no. .....

The Norwich Biorepository

Consent for the collection, storage and release of human samples for research

I confirm that:

1) **I have read and understand the Information Sheet for Patients, Version 15, dated 21 February 2014**
2) **The issues have been explained to me, and that I have had the opportunity to ask questions.**

Signed _____ (Patient) Date _____

I have explained the request for tissue for research purposes and have answered such questions as the patient has asked.

Signed_____ Print name _____
Medical / Nursing Practitioner

Date _____

CONSENT FOR TAKING EXTRA SAMPLES FOR RESEARCH

Please initial the appropriate box for each item:	**YES**	**NO**
In addition to the removal of tissue, blood or other fluid samples as a necessary part of my procedure, I also consent to the removal of additional tissue, blood or other fluid samples from the operation site during my procedure PROVIDED THAT SUCH REMOVAL CAUSES ME NO HARM now or in the future, is limited to what I and the doctor treating me (or a research nurse or nurse practitioner delegated by him/her) have discussed and agreed, and which is specified below. * ***Please initial appropriate box***		
***Please list additional samples for research:**		

Signed _____ (Patient) Date _____

I have explained the request for the donation (gift) of extra tissue and/or other samples for research purposes and have answered such questions as the patient has asked.

Signed_____ Print name_____
Medical / Nursing Practitioner

Date _____

APPENDIX 2

Publication containing data presented in Chapter Three

frontiers in Microbiology

ORIGINAL RESEARCH
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Lactobacillus reuteri Inhibition of Enteropathogenic *Escherichia coli* Adherence to Human Intestinal Epithelium

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Enteropathogenic *Escherichia coli* (EPEC) is a major cause of diarrheal infant death in developing countries, and probiotic bacteria have been shown to provide health benefits in gastrointestinal infections. In this study, we have investigated the influence of the gut symbiont *Lactobacillus reuteri* on EPEC adherence to the human intestinal epithelium. Different host cell model systems including non-mucus-producing HT-29 and mucus-producing LS174T intestinal epithelial cell lines as well as human small intestinal biopsies were used. Adherence of *L. reuteri* to HT-29 cells was strain-specific, and the mucus-binding proteins CmbA and MUB increased binding to both HT-29 and LS174T cells. *L. reuteri* ATCC PTA 6475 and ATCC 53608 significantly inhibited EPEC binding to HT-29 but not LS174T cells. While pre-incubation of LS174T cells with ATCC PTA 6475 did not affect EPEC attaching/effacing (A/E) lesion formation, it increased the size of EPEC microcolonies. ATCC PTA 6475 and ATCC 53608 binding to the mucus layer resulted in decreased EPEC adherence to small intestinal biopsy epithelium. Our findings show that *L. reuteri* reduction of EPEC adhesion is strain-specific and has the potential to target either the epithelium or the mucus layer, providing further rationale for the selection of probiotic strains.

Keywords: *L. reuteri*, EPEC, diarrhea, probiotic, human intestinal epithelium, adherence, mucus

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INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) was first reported by Bray (1945) as a cause of infant summer diarrhea in the UK, and has since remained an important pathogen, particularly in the developing world (Nataro and Kaper, 1998). According to a recent systematic review, EPEC is the second most common cause (after rotavirus) of diarrheal death in children <5 years in the world (Lanata et al., 2013). One of the major virulence traits of EPEC is its ability to adhere to small intestinal epithelium by forming attaching/effacing (A/E) lesions. These are characterized by intimate bacterial attachment to the host cell membrane, effacement of underlying microvilli and polymerization of filamentous actin underneath adherent bacteria (Moon et al., 1983; Knutton et al., 1989). A/E lesion formation causes a loss of absorptive surface and, together with other

effects mediated by type 3 secreted EPEC effector proteins, leads to the development of diarrhea (Viswanathan et al., 2009; Wong et al., 2011).

Clinical studies have shown that probiotic bacteria, such as lactobacilli can protect against intestinal infection (Sazawal et al., 2006). In particular, *Lactobacillus reuteri*, a natural inhabitant of the gastrointestinal tract of many mammals and birds (Casas and Dobrogosz, 2000), is effective as a therapeutic agent in acute rotavirus diarrhea in children (Shornikova et al., 1997a,b) and has recently been shown to protect against EPEC infection in infants (Savino et al., 2015). Several mechanisms of how probiotic bacteria protect against pathogens have been suggested and confirmed by *in vitro* studies. These include pathogen inhibition through microbe–microbe interactions (e.g., competition for nutrients and binding sites, production of antimicrobials), enhancement of epithelial barrier function (e.g., induction of mucins and preservation of tight junctions), and modulation of immune responses (e.g., regulation of cytokine expression, phagocyte, and T cell function; Lebeer et al., 2008). Competition for binding sites, also referred to as competitive exclusion, has been the focus of many *in vitro* studies (Bernet et al., 1994; Forestier et al., 2001; Sherman et al., 2005) and forms part of the rationale why adherence to intestinal mucosa is considered a desirable trait for probiotic bacteria (Havenaar et al., 1992; Tuomola et al., 2001). However, most of these studies have been performed on human enterocyte-derived cell lines (e.g., Caco-2, HT-29, T84) which do not produce a secreted mucus layer (van Klinken et al., 1996; Navabi et al., 2013). While this approach might be suitable to study the effects of probiotics on inflammatory bowel disease or other conditions with a compromised mucus layer (Sheng et al., 2012), it remains unknown whether findings on non-mucus producing epithelial cell lines can be translated to a normal intestinal mucosa where the epithelium is protected by a thick mucus layer (Juge, 2012).

In this study, we have investigated the effect of *L. reuteri* on EPEC adherence to mucus- and non-mucus-secreting human intestinal epithelial cell lines (LS174T and HT-29 cells, respectively) and to human small intestinal explants in an *in vitro* organ culture model.

MATERIALS AND METHODS

Cell Culture

Human colon carcinoma HT-29 (ATCC HTB-38) and LS174T cells (ATCC CL-188) were cultured in DMEM medium (Sigma) supplemented with 10% fetal bovine serum (Sigma) and used between passage 5 to 20 and passage 7 to 27, respectively. Cells were grown at 37°C in a 5% CO_2 atmosphere.

Bacterial Strains and Culture Conditions

Bacterial strains used in this study are listed in **Table 1**. EPEC was grown standing in LB broth overnight at 37°C. *L. reuteri* was cultured standing in an anaerobic cabinet (5% CO_2, 10% H_2, and 85% N_2) overnight at 37°C in MRS broth. Bacteria were spun down and suspended in an equivalent volume of serum-free DMEM medium prior to infection.

Adherence Assays

Cells were seeded out in 24 well plates at a density of 10^5 cells per well and grown for 6 days (HT-29) or 8 days (LS174T) to full confluence reaching a number of approximately 10^6 cells per well. For adherence assays, cell monolayers were inoculated with around 5×10^7 *L. reuteri* or EPEC (50 µl of a standing overnight culture of approximately 1×10^9 colony forming units/ml), equaling a multiplicity of infection (MOI) of around 50 bacteria per cell. After incubation for 1 h, cells were washed three times in PBS to remove non-adherent bacteria. Adherent bacteria were quantified by lysing cell monolayers in 1% Triton X-100 for 15 min and plating out serial dilutions of lysates on MRS (*L. reuteri*) or LB agar plates (EPEC). In addition, bacterial inocula and total numbers of EPEC after 1 h of incubation were determined by plating out dilutions of the inocula and combined cell supernatants/lysates, respectively. MRS plates were incubated anaerobically, and LB plates were incubated aerobically at 37°C. Colony forming units (CFU) were determined next day. Adherence was calculated according to the following equation: % adhesion = (number of adherent bacteria/number of inoculated bacteria) × 100.

Protection Assays

Two different protocols were applied for protection assays. For short-term assays without removal of non-adherent lactobacilli, cell monolayers were pre-incubated with 5×10^6 to 5×10^8 (equal numbers to 100-fold excess, MOI of 5 to 500) *L. reuteri* for 1 or 3 h before 5×10^6 EPEC (MOI of 5) were added for 1 h. To assess protection by adherent lactobacilli, cells were pre-incubated with 5×10^8 *L. reuteri* (MOI of 500) for 4 h, non-adherent bacteria were removed by washing, and cells were further incubated with 5×10^6 EPEC for 1 or 3 h. At the end of the experiment, cell viability and pH of the culture medium were evaluated by Trypan blue stain (0.2%, Sigma) and pH indicator sticks (pH range 4.5–10.0 at 0.5 intervals, Fisher), respectively.

Polarized *In Vitro* Organ Culture (pIVOC)

This study was performed with approval from the University of East Anglia Faculty of Medicine and Health Ethics Committee (ref 2010/11-030). All samples were provided through the Norwich Biorepository, which has NRES approval (ref 08/h0304/85+5). Biopsy samples from the second part of the duodenum were obtained with informed consent during routine endoscopy of 11 adult patients (48–82 years old). Samples were taken from macroscopically normal areas, transported to the laboratory in IVOC medium and processed within 1 h. Polarized IVOC was performed as described previously (Schüller et al., 2009). Briefly, biopsies were mounted on a membrane filter with the mucosal side facing upward and sandwiched between two Perspex disks with a central aperture. This assembly was then inserted into a Snapwell support (Corning Costar). For adherence assays, biopsies were inoculated with 2×10^7 *L. reuteri* or EPEC on the mucosal side and incubated for 6 h in a 5% CO_2 atmosphere at 37°C on a rocking platform. For protection assays, biopsies were inoculated with 10^9 *L. reuteri*

TABLE 1 | Bacterial strains.

Strain	Host	Reference
Lactobacillus reuteri		
ATCC PTA 6475	Human	Oh et al., 2010
ATCC PTA 6475 CmbA$^-$	Human	Etzold et al., 2014b
ATCC 55730 (SD2112)	Human	Oh et al., 2010
LMS11-3	Human	Oh et al., 2010
DSM 20016	Human	Oh et al., 2010
ATCC 53608 (1063)	Pig	Oh et al., 2010
1063N	Pig	MacKenzie et al., 2010
100-23C	Rat	Oh et al., 2010
LB54	Chicken	Oh et al., 2010
EPEC		
O127:H6 E2348/69	Human	Levine et al., 1978

for 2 h, non-adherent lactobacilli were removed, and 2×10^7 EPEC were applied for 4 h. At the end of the experiment, biopsies were removed from the Snapwell support, washed three times in PBS to remove non-adherent bacteria and mucus, unless indicated otherwise, and processed for further analysis. For determination of adherent bacteria, biopsies were homogenized with a sterile pestle and lysed as described above.

Immunofluorescence Staining

Biopsy samples with preserved mucus layer were embedded in OCT compound (Sakura), snap-frozen in a dry ice/ethanol bath and stored at −70°C until use. Serial sections of 7 μm were cut with a Microm HM550 cryostat (Thermo Scientific), picked up on poly L-lysine-coated slides and air-dried. Tissue sections were blocked with 0.5% bovine serum albumin (BSA) in PBS for 20 min. Cells on coverslips were fixed in 3.7% formaldehyde in PBS for 10 min or in methanol/acetone (1:1) for 4 min on ice (for mucus staining) and blocked/permeabilized with 0.1% Tx-100 and 0.5% BSA for 20 min. Cells and cryosections were incubated with primary antibodies (rabbit anti-CmbA (Etzold et al., 2014b), rabbit anti-MUB (MacKenzie et al., 2010), rabbit anti-SRR (kind gift from Donald MacKenzie), goat anti-*E. coli* from abcam, mouse anti-MUC2 from Santa Cruz) for 60 min, washed and incubated with Alexa Fluor-conjugated secondary antibodies (Life Technologies) for 30 min. Cell nuclei and filamentous actin were stained with DAPI (Roche) and FITC-conjugated phalloidin (Sigma), respectively. Samples were mounted in Vectashield (Vector Laboratories) and analyzed using a fluorescence light microscope (Axiovert 200M, Zeiss). Formation of EPEC actin pedestals or microcolonies was quantified from ten random fields of view containing around 70 cells for each experimental condition.

Scanning Electron Microscopy

Biopsies were either washed in PBS to remove the mucus layer or fixed immediately with preserved mucus layer in 2.5% glutaraldehyde in PBS. Samples were dehydrated through a graded acetone series, dried using tetramethylsilane (Sigma), mounted on aluminum stubs, sputter-coated with gold (Polaron SC7640 sputter coater, Quorum Technologies), and viewed with a JEOL JSM 4900 LV scanning electron microscope.

Statistical Analysis

All data are shown as means ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism software (version 5). One-way ANOVA with Tukey's multiple comparisons test was used to determine differences between multiple groups. A P-value of <0.05 was considered significant.

RESULTS

Adherence of *L. reuteri* to Intestinal Epithelial Cells is Host Strain-Specific

In order to investigate the adherence properties of *L. reuteri* strains isolated from different hosts and select highest binders, HT-29 cells were first incubated with different *L. reuteri* strains from human, pig, chicken and rat (**Table 1**), or with EPEC prototype strain E2348/69, and adherence was quantified after 1 h. No EPEC replication was detected during this short time period, and 97.0 ± 2.6% of inoculated bacteria ($n = 5$) were recovered after incubation as evaluated by CFU counting. As shown in **Figure 1A**, the *L. reuteri* pig isolate ATCC 53608 showed the highest level of adherence followed by human isolates ATCC PTA 6475 and DSM 20016, which demonstrated comparable binding levels to EPEC. In contrast, the human isolate ATCC 55730 and the LB54 strain from chicken demonstrated low binding, whereas the human strain LMS11-3 exhibited an intermediate adherence potential. Furthermore, the rat isolate 100-23C showed low binding to HT-29 cells compared to ATCC PTA 6475, DSM 20016, LMS11-3, and ATCC 53608 as evaluated by immunofluorescence staining (**Figure 1B**, images shown for ATCC PTA 6475 and 100-23C only) and was therefore not included in the quantitative adherence assay shown in **Figure 1A**. In these assays, it was also noted that ATCC 53608 formed extensive biofilm-like aggregates on cell surfaces (data not shown), as previously reported *in vitro* (MacKenzie et al., 2010). Based on these results, ATCC PTA 6475 (human) and ATCC 53608 (pig) were selected for further studies as they both showed high adherence but different binding phenotypes.

Lactobacillus reuteri binding of these strains to the host is mediated by adhesins on the bacterial surface (Etzold and Juge, 2014) with CmbA (also known as Lar_0958) being present in human isolates (Etzold et al., 2014b; Jensen et al., 2014) whereas MUB is specific for pig isolates (Roos and Jonsson, 2002; MacKenzie et al., 2010; Etzold et al., 2014a). In order to investigate the effect of these adhesins on the binding of the selected strains (ATCC PTA 6475 and ATCC 53608) to intestinal epithelial cells, an ATCC PTA 6475 knockout mutant in CmbA (Etzold et al., 2014b) and an ATCC 53608-derived strain expressing truncated MUB (1063N) (MacKenzie et al., 2010) were compared to the wild-type strains. Here, both HT-29 cells (which do not secrete mucus) and the goblet cell-derived cell line LS174T secreting intestinal mucin MUC2 (**Figure 2A**) were used to assess binding. Quantification of bacterial adherence indicated

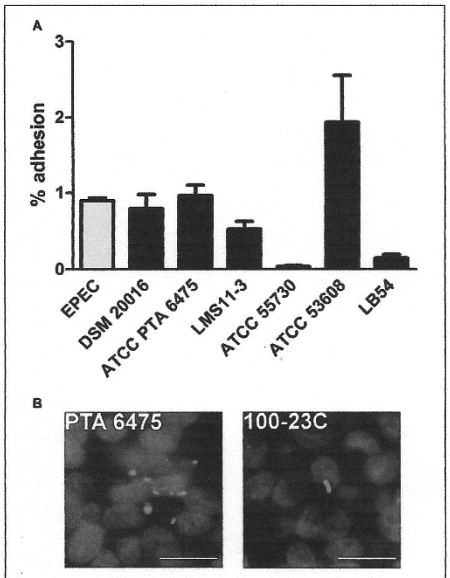

FIGURE 1 | Adhesion of *L. reuteri* to HT-29 cells is host strain-specific. HT-29 cells were incubated with *L. reuteri* or EPEC for 1 h, and adherent bacteria were quantified by CFU **(A)** and/or visualized by immunofluorescence staining **(B)**. **(A)** Adhesion is expressed as percentage of adherent bacteria relative to the inoculum. Data are shown as means ⊥ SEM from three independent experiments performed in duplicate. **(B)** *L. reuteri* ATCC PTA 6475 (left) and 100-23C (right) were stained with anti-CmbA and anti-SRR (red), respectively. Cell nuclei were counterstained with DAPI in blue. Shown are representative images from three independent experiments performed in duplicate. Bar = 10 μm.

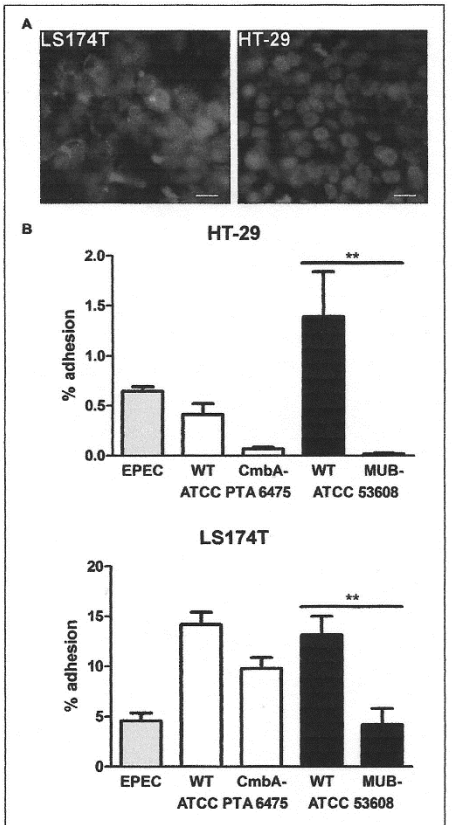

FIGURE 2 | Mucus binding proteins CmbA and MUB increase *L. reuteri* adhesion to HT-29 and LS174T cells. (A) Immunofluorescence staining demonstrating MUC2 expression (green) by LS174T but not HT-29 cells. Cell nuclei were labeled in blue. Shown are representative images from three independent experiments performed in duplicate. Bar = 10 μm. **(B)** HT-29 and LS174T cells were incubated with EPEC, or *L. reuteri* strains ATCC PTA 6475 or ATCC 53608 for 1 h. In addition to wild-type strains (WT), isogenic mutants deficient in expression of the adhesin CmbA (ATCC PTA 6475 CmbA-) or MUB (ATCC 53608 MUB-) were included for comparison. Adhesion is expressed as percentage of adherent bacteria relative to the inoculum. Data are shown as means ± SEM from three independent experiments performed in duplicate. $^{**}P < 0.01$.

impaired binding of MUB- and CmbA-deficient strains to both HT-29 and LS174T cells as compared to their respective wild-type strains (**Figure 2B**). In addition, adhesion of all strains tested (including EPEC) was considerably higher on mucus-producing LS174T cells compared to HT-29 cells (**Figure 2B**). Cell viability and pH of the culture medium (pH 7.5) were maintained during all incubations (data not shown).

Lactobacillus reuteri Inhibits EPEC Binding in a Strain- and Cell-Dependent Manner

To examine whether pre-incubation with *L. reuteri* reduces intestinal EPEC adhesion, HT-29 cells were incubated with a 100-fold excess of *L. reuteri* ATCC PTA 6475 or ATCC 53608 for 1 or 3 h before EPEC was added for another hour. This resulted in significantly reduced EPEC adherence to HT-29 cells (**Figure 3A**). No significant inhibition of EPEC binding was observed using a 10-fold excess or equal numbers of ATCC PTA 6475 or ATCC 53608 (**Figure 3B**). Similar experiments were performed with LS174T cells, and although pre-incubation with a 100-fold excess of *L. reuteri* decreased EPEC adherence to some

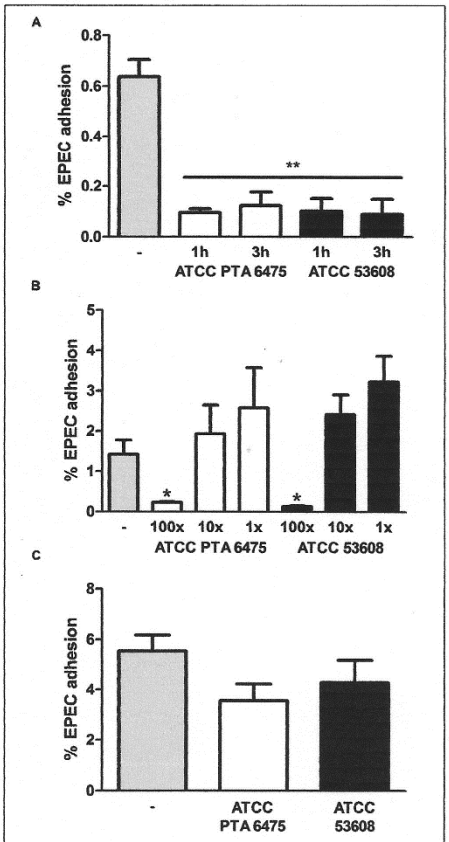

FIGURE 3 | Pre-incubation with *L. reuteri* ATCC PTA 6475 and ATCC 53608 inhibits EPEC adherence to HT-29 but not LS174T cells. **(A)** HT-29 cells were pre-incubated with a 100-fold excess of *L. reuteri* ATCC PTA 6475, ATCC 53608, or left untreated (–) for 1 or 3 h before addition of EPEC for 1 h. **(B)** HT-29 cells were pre-incubated with a 100-fold (100x) or 10-fold (10x) excess or equal numbers (1x) of *L. reuteri* ATCC PTA 6475, ATCC 53608, or left untreated (–) for 1 h before addition of EPEC for 1 h. **(C)** LS174T cells were pre-incubated with a 100-fold excess of ATCC PTA 6475, ATCC 53608 or left untreated (–) for 1 h before addition of EPEC for 1 h. Cell-associated EPEC were quantified by CFU, and adhesion is expressed as percentage of adherent bacteria relative to the inoculum. Data are shown as means ± SEM from three independent experiments performed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$.

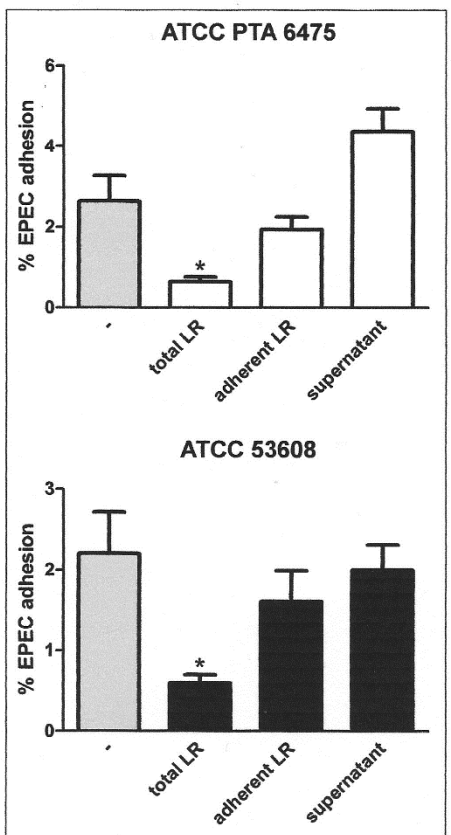

FIGURE 4 | Reduced EPEC binding to HT-29 cells is independent of *L. reuteri* adherence and secreted molecules. HT-29 cells were subjected to the following treatments before addition of EPEC for 1 h: pre-incubation with *L. reuteri* for 1 h (total LR), pre-incubation with *L. reuteri* for 2 h and subsequent removal of non-adherent bacteria (adherent LR), pre-incubation with *L. reuteri*-conditioned medium for 2 h (supernatant), or no treatment (–). Cell-associated EPEC were quantified by CFU, and adhesion is expressed as percentage of adherent bacteria relative to the inoculum. Data are shown as means ± SEM from three independent experiments performed in duplicate. $^{*}P < 0.05$.

extent, this was not as pronounced as observed with HT-29 cells and did not reach significance (**Figure 3C**).

Adhesion of bacteria to the mucosa is generally considered as one of the desirable criteria for the selection of probiotics as it promotes intestinal persistence and pathogen exclusion despite peristaltic flow (Servin, 2004). To determine whether reduced EPEC binding was mediated by *L. reuteri* adhering to

the epithelium, HT-29 cells were first incubated with a 100-fold excess of *L. reuteri* ATCC PTA 6475 or ATCC 53608 for 2 h, and in contrast to experiments described above, non-adherent bacteria were removed by washing before addition of EPEC for 1 h. In addition, to test the potential impact of released antimicrobial components in the supernatant, HT-29 cells were incubated with *L. reuteri*-conditioned medium (cultured with HT-29 cells for 2 h) and EPEC for 1 h. As shown in **Figure 4**, none of these treatments resulted in significant reduction of EPEC adherence.

Increasing the pre-incubation period to 4 h promoted *L. reuteri* adherence as shown by immunofluorescence staining of ATCC PTA 6475 and ATCC 53608 bound to HT-29 and LS174T cells (**Figure 5A**). Non-adherent bacteria were removed by washing, and cells were subsequently incubated with EPEC for 1 h. Adherent ATCC PTA 6475 but not ATCC 53608 significantly reduced EPEC binding to HT-29 cells (**Figure 5B**). In contrast, no significant inhibition of EPEC adhesion was observed using LS174T cells (**Figures 5A,B**) despite the higher *L. reuteri* binding to this cell type as reported above. As LS174T cells secrete mucus, it is likely that a large proportion of EPEC is located within the mucus layer rather than bound to the epithelial cell membrane. Therefore, CFU from cell lysates might not accurately reflect the number of EPEC bacteria adhering to the cell surface. To this aim, the protection assay was modified, and the incubation time with EPEC was extended to 3 h to allow the formation of A/E lesions. Cell-bound EPEC were subsequently identified by fluorescent actin staining (**Figure 6A**), and the number of EPEC associated with actin pedestals was determined. Quantification of actin-linked versus total cell-associated bacteria revealed that there was no significant difference in A/E lesion formation on cells pre-incubated with *L. reuteri* and non-treated controls (**Figure 6B**). However, larger clusters of A/E bacteria were observed on LS174T cells pre-incubated with ATCC PTA 6475 (**Figure 6C**), and quantification of microcolony formation (five or more bacteria per colony) at a higher magnification confirmed that pre-incubation with ATCC PTA 6475 significantly increased the number of A/E microcolonies as compared to ATCC 53608 and non-treated controls (**Figure 6D**). Cell viability and pH of the culture medium (pH 7.5) were maintained during all incubations (data not shown).

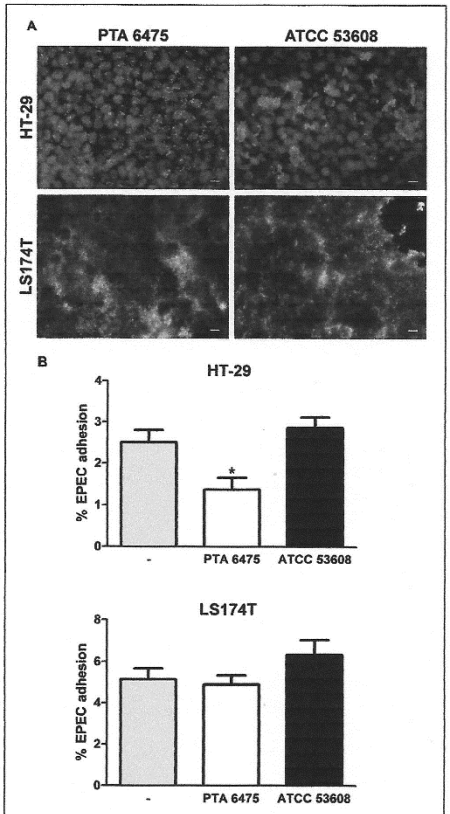

FIGURE 5 | Adherent *L. reuteri* ATCC PTA 6475 inhibits EPEC binding to HT-29 but not LS174T cells. (A) HT-29 and LS174T cells were incubated with *L. reuteri* ATCC PTA 6475 or ATCC 53608 for 4 h. Adherent *L. reuteri* were visualized by immunofluorescence staining with anti-CmbA (PTA 6475) or anti-MUB (ATCC 53608) in green. Cell nuclei were counterstained with DAPI (blue). Merged color images of both channels are shown for HT-29 cells, whereas black-and-white images of the green channel (*L. reuteri* staining) are displayed for confluent LS174T cells for greater clarity. Shown are representative images from three independent experiments performed in duplicate. Bar = 10 μm. **(B)** HT-29 and LS174T cells were pre-incubated with ATCC PTA 6475, ATCC 53608, or left untreated (-) for 4 h, non-adherent bacteria were removed, and EPEC was added for 1 h. Cell-associated EPEC were quantified by CFU, and adhesion is expressed as percentage of adherent bacteria relative to the inoculum. Data are shown as means ± SEM from three independent experiments performed in duplicate. $^*P < 0.05$.

Lactobacillus reuteri Binds to the Mucus Layer and Decreases EPEC Adherence to Human Small Intestinal Biopsies

To examine whether the results obtained on cancer-derived cell lines could be translated to human intestinal tissue, a polarized *ex vivo* model using human small intestinal biopsies was employed. Polarized *in vitro* organ culture (pIVOC) restricts bacterial access to a defined mucosal surface area and therefore allows quantification of bacterial adhesion (Schüller et al., 2009). Adult endoscopic biopsies from the duodenum were incubated with *L. reuteri* ATCC PTA 6475, ATCC 53608, or EPEC and incubated for 6 h. Biopsies were then either fixed with mucus layer or washed first to remove mucus and expose the epithelium, and bacterial adherence was visualized by scanning electron microscopy. As shown in **Figure 7A**, *L. reuteri* was observed in the mucus layer but not on the

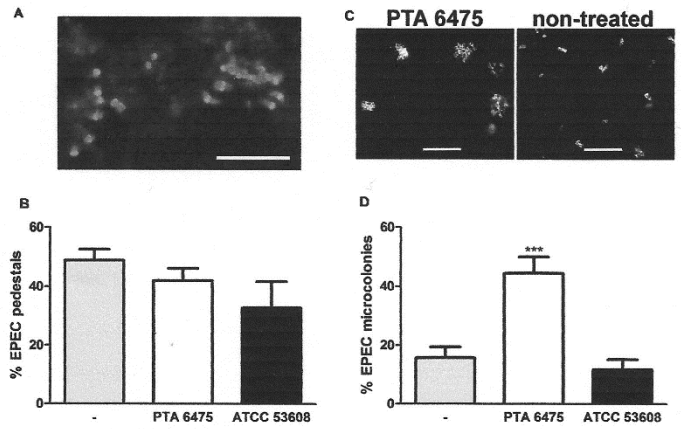

FIGURE 6 | Adherent *L. reuteri* ATCC PTA 6475 enhances EPEC microcolony formation on LS174T cells. LS174T cells were pre-incubated with *L. reuteri* ATCC PTA 6475, ATCC 53608, or left untreated (-) for 4 h, non-adherent bacteria were removed, and EPEC was added for 3 h. **(A)** EPEC forms actin pedestals on LS174T cells as visualized by immunofluorescence staining (EPEC stained in red and polymerized actin in green). Representative image from four independent experiments performed in duplicate. Bar = 10 μm. **(B)** Quantification of EPEC bacteria associated with actin pedestals by counting. EPEC pedestal formation is expressed as percentage of bacteria associated with polymerized actin relative to the total number of adherent EPEC observed. Data are shown as means + SEM from four independent experiments performed in duplicate. **(C)** Pre-incubation with *L. reuteri* ATCC PTA 6475 results in enhanced EPEC microcolony formation compared with non-treated samples. Adherent EPEC were visualized by immunofluorescence staining. Representative images from four independent experiments performed in duplicate. Bar = 10 μm. **(D)** Quantification of EPEC microcolony formation (five or more bacteria) by counting. Microcolony formation is expressed as percentage of bacteria associated with microcolonies relative to the total number of adherent EPEC observed. Data are shown as means ± SEM from four independent experiments performed in duplicate. ***P <0.001.

epithelium (images shown for ATCC PTA 6475 only), whereas EPEC was present in both the mucus layer and on the epithelium where A/E lesions were formed. None of the treatments visibly affected epithelial integrity, and no cell extrusion was observed (**Figure 7A**). The confinement of *L. reuteri* to the mucus layer was also confirmed by immunofluorescence staining of biopsy cryosections (**Figure 7B**, images shown for ATCC PTA 6475 only). For protection assays, biopsies were pre-incubated with a 50-fold excess of *L. reuteri* for 2 h, non-adherent bacteria were removed, and EPEC was added for another 4 h to enable penetration of the mucus layer and epithelial binding. At the end of the experiment, the mucus layer was removed by washing, and EPEC adherence to the epithelium was quantified. As demonstrated in **Figure 7C**, pre-incubation with ATCC PTA 6475 or ATCC 53608 significantly reduced EPEC adherence to duodenal biopsy epithelium.

DISCUSSION

The gut symbiont *L. reuteri* is unique among probiotic bacteria in that it resides in a range of hosts (including mammals and birds) and can protect against disease in the species of origin (Casas and Dobrogosz, 2000). Previous studies have demonstrated host adaptation of *L. reuteri* with evolution of host-specific binding proteins which enable rodent but not human, pig, or chicken isolates to colonize the gut of mice (Frese et al., 2011). Similarly, our results showed good adherence of human and pig isolates versus reduced binding of rat and chicken isolates to human HT-29 cells, with the exception of the human isolate ATCC 55730. This was surprising as ATCC 55730 and its plasmid-free derivative DSM17938 have been widely used in probiotic trials (Szajewska et al., 2014), and colonization of the human stomach and small intestine has been shown (Valeur et al., 2004).

Lactobacillus reuteri expresses several adhesins such as the mucus-binding proteins MUB (Roos and Jonsson, 2002), CmbA (Etzold et al., 2014b; Jensen et al., 2014), and MapA (Miyoshi et al., 2006) with the latter two also mediating adherence to Caco-2 cells. Here, we show that MUB and CmbA increase *L. reuteri* adherence to HT-29 cells which, like Caco-2 cells, do not produce MUC2, the main secreted mucin in the human intestine (van Klinken et al., 1996; Johansson et al., 2011). A similar effect, although less pronounced, was also observed in MUC2-secreting LS174T cells indicating binding of MUB and CmbA to both human epithelial cell surface molecules and secreted mucins. Interestingly, *L. reuteri* strains and EPEC adhered considerably better to LS174T compared with HT-29 cells. This might reflect a higher bacterial binding

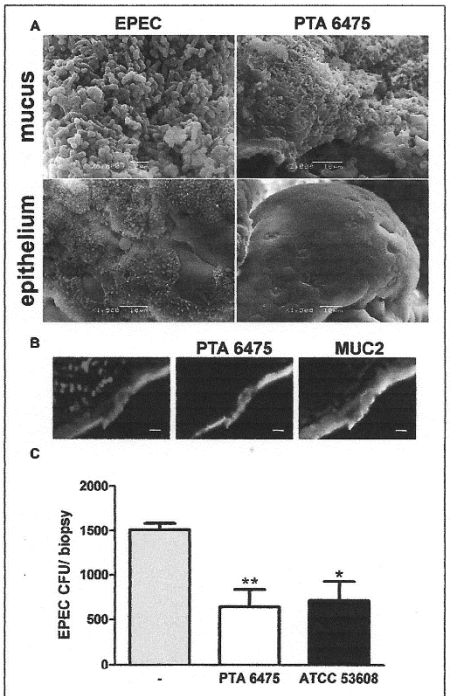

FIGURE 7 | *L. reuteri* is localized in the mucus layer and inhibits EPEC binding to human duodenal biopsy epithelium. (A) Scanning electron microscopy of duodenal biopsies incubated with EPEC or *L. reuteri* ATCC PTA 6475 for 6 h. Biopsies were either fixed with mucus layer or washed beforehand to expose the epithelium. Shown are representative images from three independent experiments performed in duplicate. **(B)** Immunofluorescence staining of duodenal biopsies incubated with *L. reuteri* ATCC PTA 6475 for 6 h. Cryosections were stained for MUC2 (green), lactobacilli (anti-CmbA, red) and cell nuclei (blue). Shown are representative images from four independent experiments performed in duplicate. Bar = 10 μm. **(C)** Duodenal biopsies were pre-incubated with *L. reuteri* ATCC PTA 6475, ATCC 53608, or left untreated (–) for 2 h, non-adherent bacteria were removed, and EPEC was added for 4 h. Cell-bound EPEC were quantified by CFU, and adherence is expressed as CFU per biopsy. Data are shown as means ± SEM from four independent experiments performed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$.

affinity to mucus compared with the epithelial surface, as previously demonstrated for commonly used probiotic strains on human intestinal tissue pieces and mucus (Ouwehand et al., 2002).

One suggested mechanism of probiotic protection is competitive exclusion where probiotics and pathogens compete for binding sites on the epithelial surface. This generally requires pre-incubation (preventive treatment) as pathogen displacement by lactobacilli is seldom observed (Lebeer et al., 2008). While no such studies have been performed on *L. reuteri* and EPEC so far, *L. acidophilus* has been shown to inhibit EPEC binding to Caco-2 cells when pre-incubated at equal numbers (Bernet et al., 1994). Similarly, pre-incubation with *L. rhamnosus* or *L. acidophilus* at a 1,000-fold or 10-fold excess, respectively, reduced EPEC adherence to polarized T84 cells (Sherman et al., 2005), and *L. rhamnosus* inhibited EPEC adherence to Caco-2 cells when used at a 10-fold excess (Forestier et al., 2001). In addition, pre-incubation with *L. acidophilus* or *L. rhamnosus* inhibited adherence of the related A/E pathogen enterohemorrhagic *E. coli* to HT-29 cells at a 1,000-fold excess (Kim et al., 2008). Therefore, our findings that a pre-incubation with *L. reuteri* at a 100-fold excess decreases EPEC adherence to HT-29 cells, are in agreement with previous studies on other *Lactobacillus* species. In the conditions of the short term protection assay, the effect was not solely reliant on the adhesion of *L. reuteri* strains to the cell surface, suggesting that steric hindrance may also play a role (Lebeer et al., 2008).

Other probiotic mechanisms of microbe–microbe interactions apart from competitive exclusion include production of antimicrobial compounds. Previous studies have demonstrated that lactic acid produced by lactobacilli has a bactericidal effect against EHEC and *Salmonella* Typhimurium (Ogawa et al., 2001; De Keersmaecker et al., 2006). In addition to lactic acid, some *L. reuteri* strains produce the antimicrobial substance reuterin during glycerol fermentation (Talarico et al., 1988). These compounds did not appear to be involved in the observed inhibitory effect in our experimental model as *L. reuteri*-conditioned media were not sufficient to reduce EPEC adherence.

In contrast to static cell culture models, peristaltic movement and flow rapidly clear non-adherent bacteria from the gut (Servin, 2004). Modification of the cell culture model to allow efficient adherence of *L. reuteri* supported two mechanisms to reduce EPEC infection which were evident for strain ATCC PTA 6475 only. Firstly, adherent ATCC PTA 6475 reduced EPEC adhesion to HT-29 cells suggesting blockage of specific binding sites. This effect was not observed for ATCC 53608 which showed aggregative binding to distinct cells, whereas ATCC PTA 6475 was uniformly distributed. Auto-aggregation of ATCC 53608 might prevent efficient spread and blockage of cell surface receptors in contrast to non-aggregating ATCC PTA 6475. In contrast to HT-29 cells, no inhibition of EPEC binding was observed on LS174T cells. This difference could be explained by direct contact of *L. reuteri* with epithelial EPEC receptors on HT-29 cells while contact with the mucus layer on LS174T cells would not directly compete with EPEC adherence to the epithelium. However, a significantly higher number of EPEC microcolonies was observed with ATCC PTA 6475 compared with LS174T cells pre-incubated with ATCC 53608 and the non-treated control. Although both *L. reuteri* strains appeared to bind similarly to LS174T cells, different adherence to the epithelial surface (as opposed to the overlying mucus), as observed on HT-29 cells, may provide a second mechanism by which ATCC PTA

6475 could potentially inhibit EPEC microcolony dispersal and cell-to-cell spread by blockage of cell surface receptors.

Furthermore, both ATCC PTA 6475 and ATCC 53608 significantly inhibited EPEC binding to duodenal biopsy epithelium. As both *L. reuteri* strains were shown to be confined to the mucus layer, this suggests that, in presence of a preserved mucus layer, the inhibitory effect of *L. reuteri* strains is exerted at the mucus interface rather than the epithelial surface. The exact mechanism of this effect still remains to be elucidated, but it is conceivable that *L. reuteri* binding to the mucus layer could result in a stronger physical barrier against EPEC infection. Contrasting results in LS174T cells and duodenal biopsy tissue might be explained by differences in mucus organization and composition. Notably, LS174T cells present a "goblet cell-like" phenotype and produce MUC2 but also demonstrate an aberrant secretion of human gallbladder mucin and gastric MUC5AC often associated with colon cancer (van Klinken et al., 1996; Bartman et al., 1999). This is in contrast to human duodenal biopsy mucosa which presents a single layer of secreted MUC2.

In summary, our studies have demonstrated that *L. reuteri* can reduce EPEC infection by several mechanisms which include competitive exclusion at the mucus or epithelial level or *via* potential inhibition of microcolony dispersal and cell-to-cell spread. These effects were strain-specific and dependent on the intestinal model system used, highlighting the need for a careful choice of experimental models when selecting potential probiotic strains. Alterations in the mucus layer have been reported in a number of infectious and inflammatory diseases, often facilitating bacterial access to the epithelial surface. Whether individuals present with a healthy or compromised mucus layer may therefore influence the rationale for selecting specific probiotic strains.

AUTHOR CONTRIBUTIONS

AW, DM, NJ, and SS designed the study. DM and NJ provided bacterial strains, cell lines, and antibodies. VC and SW provided human tissue samples. AW and CH performed the experimental work and analyzed the data. SS and AW prepared the manuscript, and NJ and DM contributed to the final manuscript.

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