The development of colicins as novel antimicrobials against Crohn's disease associated adherent-invasive *Escherichia coli*

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Abstract

Crohn's Disease (CD) is an incurable form of inflammatory bowel disease (IBD) of complex etiology that is associated with gut dysbiosis that frequently includes colonisation by adherent-invasive *E. coli* (AIEC). AIEC are adept at forming biofilms and are able to invade host cells and stimulate the production of pro-inflammatory cytokines. A number of antibiotics have been tested in the treatment of CD but at best show limited efficacy. Additionally, broad spectrum antibiotics are themselves strong drivers of dysbiosis and a number of studies have linked the administration of antibiotics with an increased risk of the development of CD. An alternative approach to the eradication of pathogenic bacteria in a complex community is to use species-specific antibiotics such as the colicin-like bacteriocins. Alongside their narrow spectrum killing, colicins also demonstrate extreme potency, are active against bacterial biofilms and can be readily isolated from clinical isolates. The aim of this work was to investigate the efficacy of these *E. coli* targeted antibiotics against AIEC. To achieve this, colicin activity against ileal CD-mucosa associated AIEC strain LF82, was tested *in vitro* and also *in vivo* using a murine model of persistent AIEC colonisation. Colicins showed good efficacy against LF82 associated with intestinal epithelial cells and growing intracellularly within macrophages. In addition, colicins do not show toxicity towards a macrophage cell line or stimulate the production of pro-inflammatory cytokines. To test the efficacy of colicins in an *in vivo* model of AIEC colonisation, methods were developed to formulate purified protein for delivery to the GI tract. However, this formulation showed only minimal efficacy in a mouse model of AIEC colonisation. In addition to investigating the efficacy of colicins, this thesis also attempts to further elucidate the role of the target pathogen, AIEC in the development of intestinal inflammation. Interestingly, we show here that AIEC associated phenotypes can be detected in commensal *E. coli* strains, undermining the current definition and classification of the AIEC pathotype.

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I can definitely say that doing a PhD has been one of the most difficult but rewarding experiences of my life. I started my PhD at University of Glasgow as a shy and insecure microbiology graduate but have developed into a confident and ambitious young woman. For this I have many people to thank.

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Author’s declaration

I declare that, except where explicit reference is made to the contribution of others, this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or at any other institution.

Carla Louise Brown
September 2015

Contribution of others

Chapter 3

LF82-GFP was constructed by Karen Smith (Walker lab)

Chapter 4

Mouse model was established by Gillian Douce and Zoe Marjenberg, who supervised work and provided experimental support for all experiments.

Data shown in Figure 4.2 was obtained by Zoe Marjenberg.

Chapter 5

All stool samples utilised in this work were collected by Gerasimidis and colleagues at Yorkhill hospital (University of Glasgow; Human Nutrition).

Isolation of faecal *E. coli* strains was performed with Clare Clark (Human Nutrition, Gerasimidis group).

Serotyping and multiplex PCR was performed at Public Health, Gastrointestinal Unit, Colindale by Holly Ciesielczuk and Dawn Hedges, respectively.

Clare Clark created Figure 5.1.

Appendix G

Purification of Ag43α and antibody production was performed by Inokentijis Josts (Walker lab).

Contents

Abbreviations

AIEC – Adherent-invasive *E. coli*

AMP – Antimicrobial peptide

ANOVA- Analysis of variance

AS- Ankylosing spondylitis

ASD – Autism spectrum disorders

ATCC- American Type Culture Collection

ATG16L1- Autophagy related 16-like 1

BFT- *Bacteriodes fragillis toxin*

BLAST – Basic local alignment search tool

bp – Base pair

CARD15- Caspase recruitment domain-containing protein 15

CAZymes- Carbohydrate active enzymes

CCR6- C-C chemokine receptor 6

CD- Crohn's disease

CDI- *Clostridium difficile* infection

CEACAM - Carcinoembryonic antigen-related cell adhesion molecule

CF – Cystic fibrosis

CFUs- Colony forming units

Cip – Ciprofloxacin

Cir – Colicin I receptor

CLBs – Colicin like bacteriocins

CRC – Colorectal cancer

CS – Corticosteroids

DAPI- 4', 6-diamidino-2-phenylindole (DAPI)

DCs – Dendritic cells

DIC - Differential Interference Contrast

DMA- Dimethylamyloride

DMEM- Dulbecco's Modified Eagle's Medium

DMSO- Dimethylsulfoxide

DSS – Dextran sodium sulphate

E. coli- Escherichia coli

ECCC- European Collection of Cell Cultures

ECL- Enzyme linked chemiluminescence

EDTA- Ethylenediaminetetraacetic acid

ELISA- Enzyme-linked immunosorbent assay

EMB – Eosin methylene blue

ERA- Enthetis-related arthritis

ETBF - Enterotoxigenic *Bacteriodes fragillis*

ExPEC- Extraintestinal pathogenic *E. coli*

FISH- Fluorescence *in situ* hybridisation

GALT – Gut-associated lymphoid tissue

gDNA- Genomic DNA

GEN- Gentamicin

GF – Germ-free

GFP – Green fluorescent protein

GI- Gastrointestinal

Gp2 – Glycoprotein 2

Gp96 – Glycoprotein 96

GWAS – Genome-wide association studies

HEPES- (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HLA-B27 – Human leukocyte antigen B27

HRP - Horseradish peroxidase

I_INV – Invasion index

I_PERS- Persistence index

I_REP- Replication index

IBD- Inflammatory bowel disease

IBS- Irritable bowel syndrome

ICOSLG- Inducible T-Cell Co-Stimulator Ligand -

IECs– Intestinal epithelial cells

IFN-γ - Interferon-gamma

IgA- Immunoglobulin A

IL-10– Interleukin-10

IL-12- Interleukin-12

IL-17A- Interleukin-17A

IL-1β- Interleukin-1β

IL-23- Interleukin-23

IL-23R- Interleukin-23 receptor

IL-6- Interleukin-6

IL-8– Interleukin 8

IM- Inner membrane

IPTG– Isopropyl β-D-1-thiogalactopyranoside

IRGM- Immunity-related GTPase family M

ITLN1- Intelectin 1

IUTD- Intrinsically unstructured translocation domain

JAK2- Janus Kinase 2

JIA- Juvenile idiopathic arthritis

KAN- Kanamycin

LB – Lysogeny broth

Lc-3- Microtubule-associated protein 1A/1B-light chain 3

LDH- Lactate dehydrogenase

LPF – Long polar fimbriae

LPS – Lipopolysaccharide

LRRK2- Leucine-rich repeat kinase 2

MAC- MacConkey

MAMP – Microbe-associated molecular pattern

MHC- Major histocompatibility complex

MIC- Minimum inhibitory concentration

MOI – Multiplicity of infection

MRSA – Methicillin-resistant *Staphylococcus aureus*

MyD88- Myeloid differentiation primary response gene 8

NF-kβ- Nuclear factor-kappa B

NOD2- Nucleotide-binding oligomerization domain-containing protein 2

NORA -New-onset rheumatoid arthritis

OM – Outer membrane

OmpA- Outer membrane protein A

OmpC – Outer membrane protein C

OmpF- Outer membrane protein F

OMVs – Outer membrane vesicles

ORFs – Open reading frames

pCol- Colcinogenic plasmid

PCR- Polymerase chain reaction

PFA- Paraformaldehyde

PRR – Pattern recognition receptor

PSA- Polysaccharide A

PTGER4- Prostaglandin E Receptor 4 (Subtype EP4))

PVP- Polyvinylpyrrolidone

RA – Rheumatoid arthritis

SCFA- Short-chain fatty acid

SD- Standard deviation

SDS- Sodium dodecyl sulphate

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel

SEM- Standard error

SpAs- Spondyloarthropathies

SPF – Specific pathogen free

STAT - Signal Transducers and Activators of Transcription

T5KO- TLR-5 knock out

Th cell- T helper cell

TLR- Toll-like receptor

TNF-α- Tumour necrosis factor alpha

UC- Ulcerative colitis

UPEC – Uropathogenic *E. coli*

UTI- Urinary tract infection

WHO – World Health Organisation

XBP1- X-box binding protein 1

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1 Introduction

"We are fast approaching a post-antibiotic era."

(World Health Organisation, 2014)

1.1 The emerging problem of antibiotic resistance

The beginning of the antibiotic era was marked by the discovery of penicillin by Alexander Fleming in 1928. This was followed by the discovery of streptomycin in 1943 and the 'golden era' of antibiotic discovery (1950-1960) during which approximately one half of the antibiotics commonly used today were produced (Aminov 2010). This 'golden era' however, lasted less than half a century and we have now returned to an era where novel antibiotics are urgently required and bacterial infections are once again recognised as a major threat (Figure 1.1).

One reason for this is that antibiotics are losing their effectiveness at an alarming rate. This is mainly due to the emergence of resistant bacteria as a result of the misuse and overuse of antibiotic therapies in human health and agriculture (Figure 1.1). The WHO have expressed great concern on the problem of antibiotic resistance and last year published a detailed report on its incidence and significance worldwide (World Health Organisation 2014). This report showed that: 1) antibiotic resistance is a global problem and is detected in every surveyed country and 2) there are high rates of resistance for many bacteria that cause important health care associated and community acquired infections (World Health Organisation 2014). Furthermore, a subsequent report on the global response to antimicrobial resistance showed that very few countries (34 /133 participating in the survey) have a comprehensive national plan to fight resistance to antibiotics (World Health Organisation 2015). Additionally, public awareness of the issue is low in all regions, with many people still believing that antibiotics are effective against viral infections (World Health Organisation 2015).

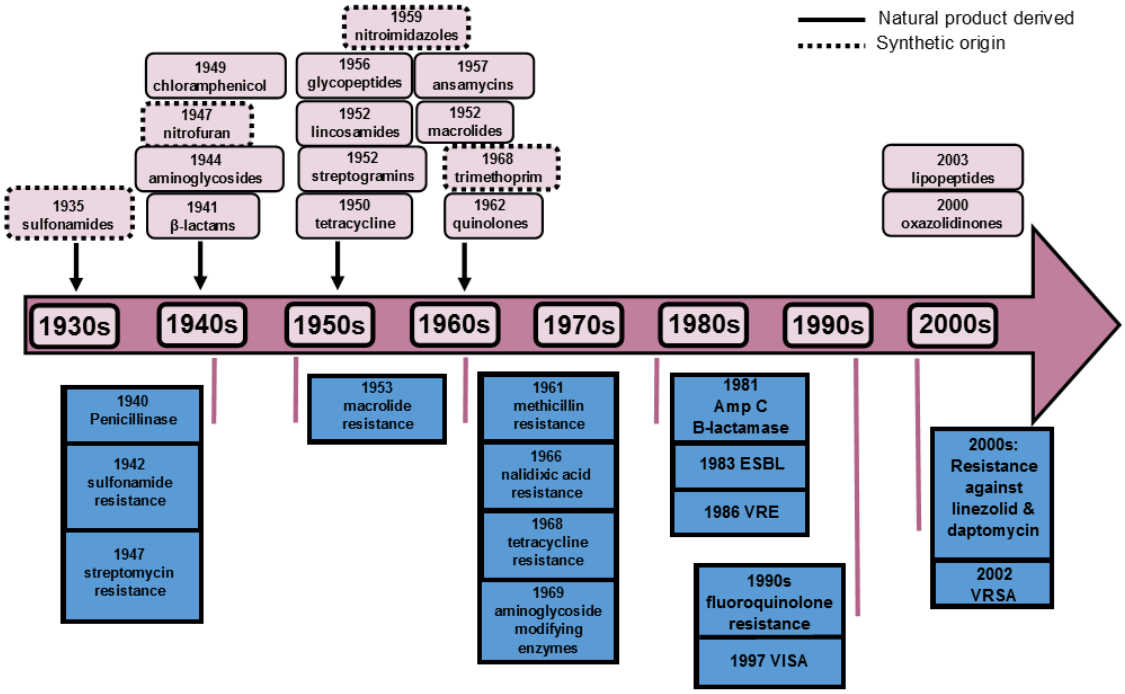


Figure 1-1: Antibiotic resistance is a major health concern.

Figure shows timeline of antibiotic discovery and the development of antibiotic resistance (1930-2000s). Timeline on top shows discovery and timeline on bottom shows emergence of resistance. ESBL; Extended-spectrum beta-lactamases, VRE; Vancomycin-resistant *Enterococci,* VISA; Vancomycin-intermediate *Staphylococcus aureus*, VRSA; Vancomycin-resistant *Staphylococcus aureus.* Image adapted from Lumibyte (LumiByte 2015).

Bacteria employ a wide range of strategies to resist antibiotics and we now face sophisticated, highly resistant and often multi-resistant pathogens including *Pseudomonas aeruginosa*, *Escherichia coli* (*E. coli)* and *Klebsiella pneumonia.* The almost absent production of new classes of antibiotics also contributes significantly to this problem. Due to the high costs and risk associated with antibiotic discovery and development, only one new class of antibiotics, the oxazolidinones (2000), has progressed to therapeutic use since the 1960s (Chien *et al.* 2000). The lipopeptides represent an old class of antibiotics, discovered over 50 years ago that include the polymyxins (Figure 1.1) (Pirri *et al.* 2009). In addition, the number of Food and Drug Administration (FDA) approved antibiotics has reduced in recent years, with only 3 antibiotics being approved between 2008 and 2012 (Figure 1.2). However, recent work has identified a novel antibiotic that kills by inhibiting cell wall synthesis, via binding to lipid II and III, suggesting that

new and effective classes of antibiotics can still be isolated from currently unculturable bacteria (Ling *et al.* 2015).

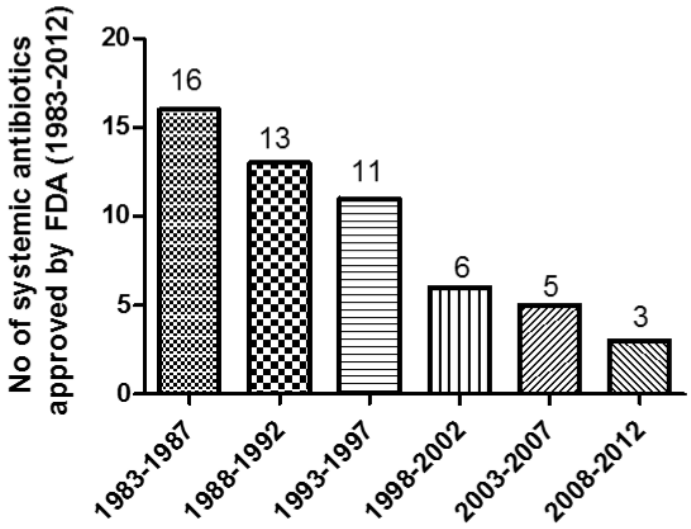


Figure 1-2: The number of new antibiotic agents approved for clinical use by FDA (1983-2012).

Data shown in figure is adapted from (Shlaes *et al.* 2013).

1.1.1 The requirement for potent and narrow spectrum antibiotics

Alongside the emerging problem of resistance, additional and unrelated factors also contribute significantly to the requirement for novel antimicrobials. For example, some bacteria cause chronic biofilm mediated infections that are highly resistant to antibiotic treatment (Costerton 1999). Key examples of this include chronic, biofilm-mediated *Pseudomonas aeruginosa* infection in the lungs of cystic fibrosis patients and recurrent UTI infections mediated by biofilm growth of uropathogenic *E. coli* (UPEC) on the urinary bladder wall (Tapiainen *et al.* 2014). Many of the antibiotics currently used in clinical practice show poor efficacy against bacterial biofilms and the mechanisms of increased tolerance to antibiotics displayed in the biofilm state are poorly understood (Høiby *et al.* 2010, Li Zhang *et al.* 2013).

Increased knowledge on the significance of the gut microbiota in health also highlights potential concerns in the use of broad spectrum antimicrobials. Several independent studies have reported prolonged antibiotic induced changes in the composition of gut bacterial populations (Jakobsson *et al.* 2010, Jernberg *et al.* 2007, Löfmark *et al.* 2006). Furthermore, antibiotic exposure in early childhood is

now being linked to an increased risk of developing chronic immune related disorders including asthma, inflammatory bowel disease (IBD), autism and obesity. This is thought to be mediated by dysfunctional immune responses induced by altered microbiota populations (Bailey *et al.* 2014, Mueller *et al.* 2015, Murk *et al.* 2011, Shannon L Russell *et al.* 2012, Ungaro *et al.* 2014). It has been suggested that increased use of antibiotics may be a contributing factor to the large increase in the incidence of immune related disorders that has occurred since the 1950s (Figure 1.3).

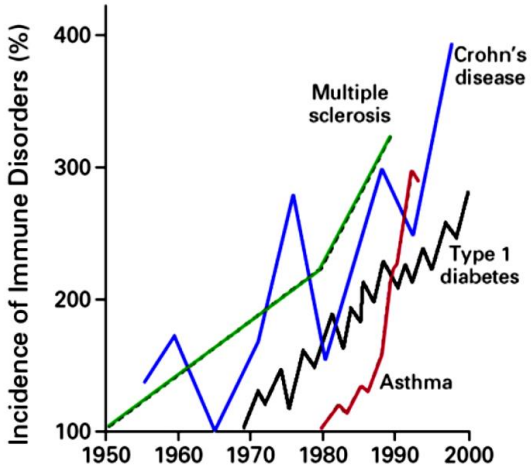


Figure 1-3: Increase in incidence of autoimmune diseases from 1950—2000.

Figure shows increase in prevalence of chronic immune disorders between 1950 and 2000 that coincides with introduction of antibiotics and increased clinical use of antibiotics. Reproduced with permission from (Bach 2002). Copyright Massachusetts Medical Society.

Alternatives to traditional antibiotics include bacteriophage, phage lysins, probiotics and antimicrobial peptides (Parisien *et al.* 2008). The ideal properties of novel antibiotics include good efficacy and potency against persistent and biofilm mediated infections. Additionally, in order to overcome the problem of resistance, novel therapeutics would employ distinct and diverse killing mechanisms so that 'combinational therapy or 'antibiotic cocktails' could be utilised. Finally, preservation of the gut microbiota composition should be recognised as a crucial component for the development of novel antimicrobials. The significance of this 'microbial organ' in human health will now be discussed.

1.2 The gut microbiota in health and disease

"No man is an island, entire of itself" — John Donne (1572–1631)

1.2.1 Stability of the gut microbiota

The human gastrointestinal tract is a multi-functional system that exhibits structural sectioning to facilitate its diverse functions. Sectioning of the gastrointestinal (GI) tract allows the digestive functions (proximal) to be separate from the vast microbial populations that inhabit and thrive within the intestinal regions (distal) (Underwood 2014).m The gut microbial community termed the microbiota includes a diverse range of microbial species (Figure 1.4). The microbiota is comprised of bacteria, viruses, fungi and archaea with 99% of total microbial gene number being bacterial (Avila *et al.* 2009, Qin *et al.* 2010). Although bacteria are recognised as the most abundant members of the gut microbiome, in some individuals bacteriophage particles are present in potentially greater numbers than bacterial cells (Reyes *et al.* 2012). The size of the bacterial population of the GI tract, that is currently estimated at 100 trillion, greatly exceeds the size of any other microbial community found in the human body (Qin *et al.* 2010). This microbial community possesses many genes that provide us with an array of additional metabolic and genetic functions (Fredrik Karlsson *et al.* 2013). Studies on germ-free (GF) animals have demonstrated the intestinal bacterial population as a multi-functional community; providing the host with nutritive, metabolic, protective and immunogenic functions (Falk *et al.* 1998, Macpherson and Uhr 2004). These include improved digestion of complex dietary polysaccharides (Martens *et al.* 2008) and through competitive colonisation of the intestinal lumen, protection of the host from invasion by pathogens (Sekirov *et al.* 2010). This protective role is further facilitated by direct interactions of the microbiota with components of the host immune system.

Analysis of *16S* rRNA sequences has shown that over 1000 bacterial species are capable of inhabiting the human gut (Human Microbiome Project Consortium 2012) with at least 160 species in each individual (Qin *et al*. 2010). Bacterial gene numbers present in the gut are proposed to outnumber the count of host genes by approximately 150-fold (Qin *et al*. 2010).

Mammals become colonised by microbes during and after birth, and in humans colonisation is from mother to infant (Palmer *et al.* 2007). The delivery mode used for birth has been shown to shape the initial intestinal microbiota composition in new-borns. *16S* rRNA pyrosequencing demonstrated that vaginally delivered babies acquired bacterial communities resembling their mother's vaginal microbiota, dominated by *Lactobacillus*, *Prevotella*, or *Sneathia* spp (Dominguez-Bello *et al.* 2010). However, infants delivered by C-section possessed bacterial gut communities resembling the mother's skin microbiota that included *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp (Dominguez-Bello *et al.* 2010). By the age of 2-5 years, the gut microbiota profile of infants will resemble that of a healthy adults in both composition and diversity (Koenig *et al.* 2011, Palmer *et al.* 2007). Although there is a considerable level of variation between individuals, the adult gut microbiota is dominated by 4 major bacterial phyla. These are the Firmicutes and Bacteroidetes, which constitute approximately 90% of the microbiota population, and Proteobacteria and Actinobacteria. (Dominguez-Bello *et al.* 2010). Additionally, it was proposed that the human gut can be classified into one of three distinct gut types, termed enterotypes. The three enterotypes are characterised by the enrichment of a single microbial genus: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2), or *Ruminococcus* (enterotype 3) (Arumugam *et al*. 2011). The existence of distinct enterotypes is still highly controversial with recent work suggesting that enterotypes exist as gradients involving additional species including *Methanobrevibacter*.

1.2.2 Individual variations in the gut microbiota

Individual variations in the gut microbiome are driven by a range of complex processes. Diet is one of the most important factors shaping microbial diversity in the gut. As different members of the gut microbiota have unique preferences for dietary substrates, there is a high level of competition for resources. Therefore, alterations in diet can result in significant changes in microbiota composition and metabolic functions. It was demonstrated that individuals on long-term diets enriched in protein and animal fat possessed a gut enterotype dominated by *Bacteroides* and carbohydrate enriched diets were associated with a *Prevotella* dominated enterotype (Wu *et al.* 2011). Similar trends were reported in European and African children, who have diets rich in protein/animal fat and carbohydrates

respectively (De Filippo *et al.* 2010). Children from rural Africa showed a gut microbiota population dominated by Bacteroidetes and EU children showed an increased abundance of Firmicutes, that were present in low numbers in African children (De Filippo *et al.* 2010). Higher microbial richness and diversity was also detected in rural African faecal samples.

Obesity is also associated with distinctions in microbiome composition. *16S* rRNA sequencing of faecal microbial communities of monozygotic and dizygotic obese / lean twin pairs showed obesity to be associated with reduced bacterial diversity and reduced representation of the Bacteroidetes (Turnbaugh *et al.* 2009). Additionally, recent work has also highlighted an association between exercise and variations in gut microbiota composition. Clarke and colleagues reported specific alterations in the microbiota composition of professional athletes compared to non-athlete controls (Clarke *et al.* 2014). Alongside increased microbial gut diversity, athlete microbiota showed a significant increase in the abundance of *Akkermansia muciniphila*, that has been shown to inversely correlate with obesity and metabolic disorders (Clarke *et al.* 2014).

Work carried out over the last decade has demonstrated the significant impact of antibiotic exposure on the gut microbiota. It is now known that administration of broad spectrum antibiotics has the capacity to induce drastic and long-term shifts in the microbiota structure (Cotter *et al.* 2012). Exposure of healthy adults to a short dosage of ciprofloxacin impacted on the relative abundance of 30% of bacterial taxa in the gut decreasing the taxonomic richness and diversity of the community (Dethlefsen *et al.* 2008). Additionally, repeated exposure to ciprofloxacin resulted in incomplete recovery of the distal gut microbiota composition with subjects showing a distinct gut profile post-treatment compared to pre-treatment profile (Dethlefsen and Relman 2011).

Enteric infection can also influence the bacterial community structure of the gut. *Clostridium difficile* infection (CDI) was shown to decrease gut microbial diversity, evenness, and species richness that involved a lowered abundance of Firmicutes sequences and enriched *Lactobacillus* and *Enterococcus* sequences (Antharam *et al.* 2013). Sequences from Gammaproteobacteria were also enriched in CDI patients compared to healthy controls. Furthermore, in CDI patients, a depletion of butyrate-producing bacteria including members of Clostridium cluster XIVa was detected (Antharam *et al.* 2013).

Chronic intestinal inflammation, experienced by inflammatory bowel disease (IBD) patients induces marked alterations in microbial composition. IBD patients consistently demonstrate reduced microbial stability and diversity in the gut microbiota compared to healthy individuals (Manichanh *et al.* 2006, Ott *et al.* 2004). A decrease in Bacteroidetes and Firmicutes frequencies and an increase in Proteobacteria and Actinobacteria frequencies has been detected in several studies using high throughput sequencing of *16S* rRNA genes (Frank *et al.* 2007, Manichanh *et al.* 2006). Additionally, patients with sporadic colorectal cancer (CRC) show perturbed gut microbiota composition involving overrepresentation of *Bacteroides* and *Fusobacterium* (Kostic *et al.* 2012, Toprak *et al.* 2006). Furthermore, an increased abundance of mucosa-associated and internalised *E. coli* is detected in the gut mucosa of inflammation-associated CRC patients compared to healthy controls (Bonnet *et al.* 2014, Martin *et al.* 2004).

Indeed, a wide range of processes are capable of influencing the structure and composition of the gut microbiome. However, a key factor in the development and stability of the intestinal microbiota population is the host immune system. The gut microbiota and host mucosal immune system co-exist as mutualistic partners creating an environment in which stability is dependent on a complex array of fine-tuned processes. This is known as intestinal homeostasis.

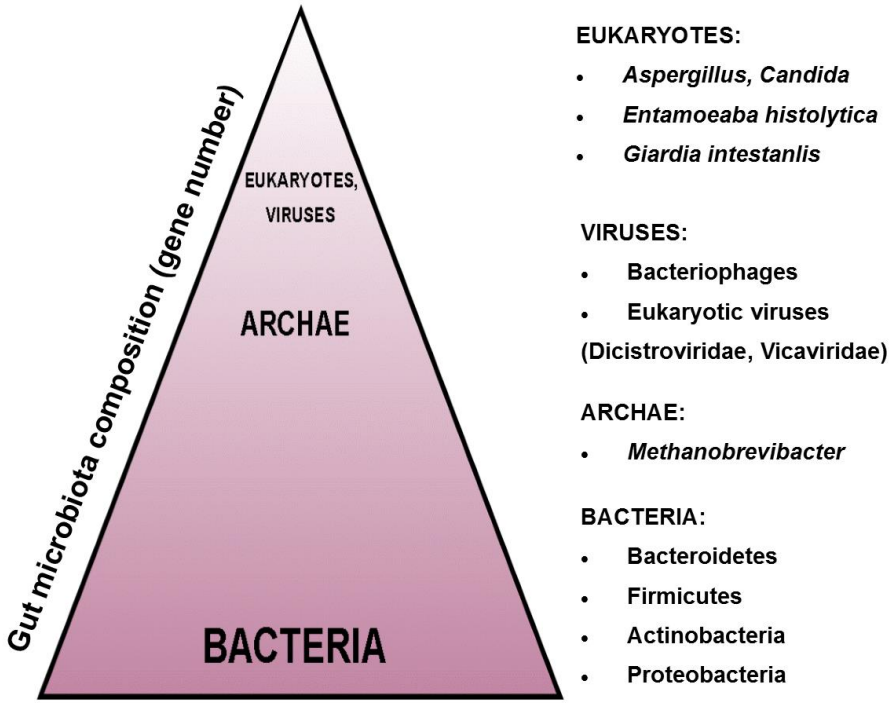


Figure 1-4: The gut microbiota is comprised of bacteria, archaea, viruses and eukaryotic microbial species.

Bacterial genes comprise 99% of total microbial genes, with the remainder being archaea and viral and eukaryotic genes (Qin *et al.* 2010). Figure shows dominant microbial species for each microbial component.

1.2.3 Intestinal homeostasis

1.2.3.1 Regulation of the intestinal microbiota by the host immune system

The intestinal microbiota is recognised or 'sensed' by the host through pattern recognition receptors (PRRs). PRRs are expressed by immune and non-immune cells and include Toll-like receptors (TLRs) and nucleotide-binding oligomerization domains (NODs) (Benoit Chassaing, Kumar, *et al.* 2014). NODs are the main receptors involved in recognition of conserved bacterial motifs termed microbe associated molecular patterns (MAMPs) (Kawai and Akira 2011). Due to the high antigenic burden present in the gut an ongoing challenge exists for the host immune system. It must ensure that resident microbiota communities remain intact without pro-inflammatory responses being induced. However, it must also remain equipped to eradicate and clear invading pathogens entering via the intestine (Benoit Chassaing, Kumar, *et al.* 2014).

To maintain intestinal homeostasis the host mucosal immune system regulates both the composition and localisation of the resident gut microbiota (Sekirov et al 2010) (Figure 1.5). These two functions are carried out by a wide range of immune cells that function in both the innate and adaptive immune system. Cross-talk between the intestinal epithelial cells and gut microbiota mediated by PRRs leads to the expression of gene products that enhance epithelial barrier function and innate immunity (Takeuchi and Akira 2010). The physical separation of microbiota and gut mucosa is mediated by myeloid differentiation primary response gene 88 (*MyD88*) signalling in intestinal epithelial cells (Benoit Chassaing, Kumar, *et al.* 2014). Cytoplasmic adaptor protein MyD88 transduces signals from TLRs that recognise bacterial products (Jobin 2010). MyD88 activation triggers a cascade of signalling events leading to nuclear localisation of NF-κB along with other inflammatory responses. MyD88 signalling also controls the production of the mucus layer and antimicrobial peptides (AMPs) in the intestine (Frantz *et al.* 2012).

Intestinal goblet cells secrete mucin glycoproteins that form a thick mucus layer at the epithelial cell surface. In the colon, this is comprised of two distinct layers with the inner layer being resistant to bacterial penetration (Vaishnava *et al.* 2011).

Mucus in both the small and large intestine is comprised of the same mucin protein, mucin 2 (MUC2). MUC2 plays a crucial role in the organisation of the intestinal mucus layers at the epithelial surface of the colon (Hansson and Johansson 2014). The inner mucus layer of the distal colon is formed from sheets of MUC2 organised into stacked layers in a stratified way that prevents bacteria to penetrate (Johansson *et al.* 2014). Mice deficient in the *Muc2* gene lack secreted mucus layers that results in contact between gut bacteria and the epithelium (Hansson and Johansson 2014). The significance of mucin production by goblet cells for intestinal homeostasis was demonstrated in *Muc2*-deficient mice that spontaneously develop colitis and show predisposition to colitis-induced colorectal cancer (Sluis and Koning 2006, Velcich *et al.* 2002).

Furthermore, in murine models of experimental colitis and in patients with active ulcerative colitis (UC) increased bacterial penetration of inner mucus layer is mediated by reduced MUC2 density in the inner mucus layer (Johansson *et al.* 2014).

In the small intestine, a less robust mucus layer is found and therefore secretion of AMPs limits penetration of bacteria (Vaishnava *et al.* 2011). Paneth cells, secretory cells in the epithelium of the small intestine, are an important source of antimicrobial peptides including α-defensins (Bevins and Salzman 2011). The significance of AMPs in the regulation of microbial composition has recently been demonstrated (Cullen *et al.* 2015). In this work, it was shown that commensal bacteria were largely resistant to inflammation associated AMPs but pathogenic species were highly susceptible (Cullen *et al.* 2015). Susceptibility to AMPs is mediated by the expression of specific lipopolysaccharide (LPS) types on the bacterial surface (Cullen *et al.* 2015). Additionally, production of secretory immunoglobulin A (IgA) in the small intestine maintains barrier function of the epithelium (Frantz *et al.* 2012) (Figure 1.5).

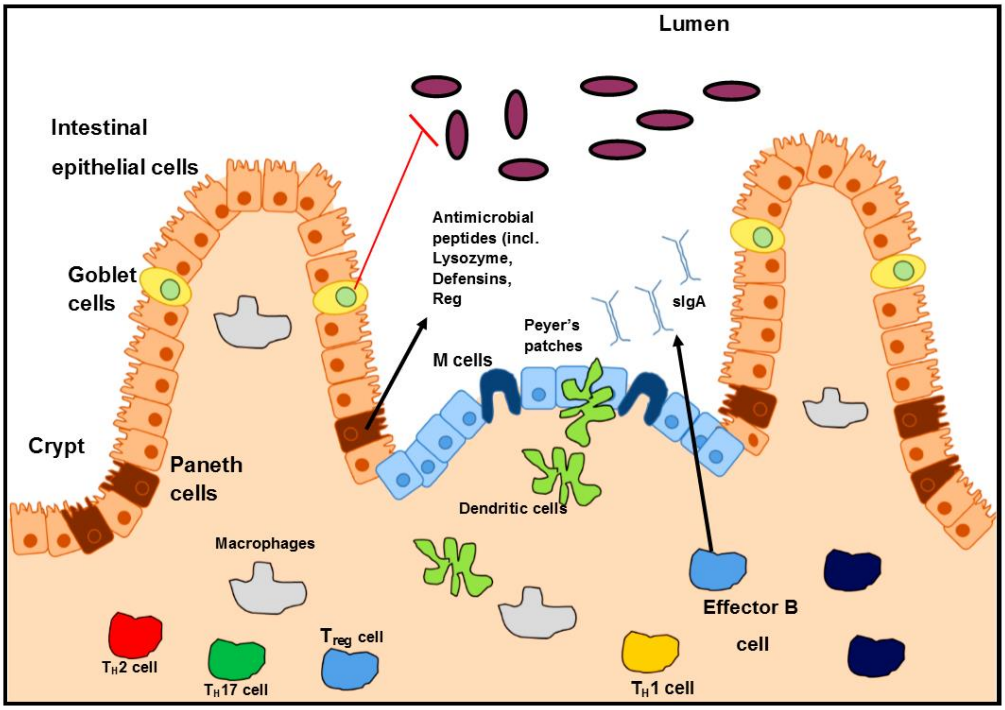


Figure 1-5: Components of the gut mucosal immune system.

In healthy individuals, PRR-microbiota interactions result in the secretion of antimicrobial peptides (AMPs) and development of gut-associated lymphoid tissue (GALT). Crosstalk between microbiota and intestinal immune system results in intestinal homeostasis that includes sIgA and defensins that maintain microbiota homeostasis and epithelial barrier integrity. Adapted from figure shown in (Benoit Chassaing, Kumar, *et al.* 2014)

1.2.3.2 Functional input of the intestinal microbiota

Maintenance of intestinal homeostasis requires a high level of functional input from the intestinal microbiota. Studies using GF animals have given us great insights into the mechanisms involved in this partnership. GF animals show defects in the structure and function of many intestinal immune components. This includes impaired development of gut associated lymphoid tissues (GALTs), reduced antibody production, fewer and defective Peyer's patches and reduced numbers of mesenteric lymph nodes (Falk *et al.* 1998, Macpherson and Uhr 2004). The rate of production of crypt cells is also reduced in the colon of rats bred in GF environments (Alam *et al.* 1994). Additionally, sensing of the commensal microbiota through TLR-MyD88 triggers several functions involved in intestinal homeostasis. Innate signals mediated by myeloid cells are required to enhance epithelial cell proliferation and induce repair of damaged intestinal epithelium via a

MyD88-dependent mechanism (Pull *et al.* 2005, Rakoff-Nahoum and Medzhitov 2007). MyD88- dependent bacteria signalling is also required for production of epithelial cell AMPs such as RegIIIγ (Vaishnava *et al.* 2011).
Commensal microbiota are also involved in the shaping of T-cell subsets. Luminal resident bacteria have been shown to upregulate the anti-inflammatory branch of the adaptive immune system by inducing differentiation of regulatory T-cells or interleukin-10 (IL-10) expression (Hooper *et al.* 2012). For example, polysaccharide A (PSA) of *Bacteriodes fragillis* was shown to induce IL-10 responses in T-cells that prevented expansion of Th17 cells and hence damage to the intestinal epithelial barrier. However, mutant *B. fragillis* that lacked PSA was shown to induce pro-inflammatory responses in the host (Round *et al.* 2011). Evidently, alterations in gut microbiota composition, termed dysbiosis, has the potential to significantly impact on host immune responses. Intestinal dysbiosis is linked to a diverse range of diseases that exhibit unique triggers, symptoms and localisations.

1.3 Dysbiosis of the gut microbiota in disease

The gut microbiota has been implicated in several distinct forms of chronic disease including disorders encompassing metabolic syndrome (obesity, type 2 diabetes), gastrointestinal disease (inflammatory bowel disease (IBD), colorectal cancer (CRC), irritable bowel syndrome (IBS)), allergic disease (allergic asthma) and neuronal disease (autism spectrum disorders (ASD)). Although these disorders exhibit distinct symptoms and localisations, most share the common characteristic of possessing a complex etiology that lacks a single causative agent. Recent evidence now indicates that specific alterations in individual microbiota profiles may provide the missing link (Table 1.1). Key examples of these will now be discussed.

Table 1-1: Dysbiosis of the gut microbiota in disease.

Disease	Evidence of dysbiosis	Microorganisms identified
Inflammatory bowel disease (IBD)	• IBD does not occur under germ-free conditions in animal model studies.(Kennedy *et al.* 2000, Madsen *et al.* 1999). • Colitis occurs in IL-2 deficient germ free mice with a non-pathogenic strain of *E. coli* but not with *Bacteroides vulgatus* (Waidmann *et al.* 2003). • Reduced diversity in luminal bacteria populations is consistently detected in human IBD patients compared to healthy controls (Manichanh *et al.* 2006, Ott *et al.* 2004). • Antibiotic treatment have shown beneficial effect on inflammatory symptoms in IBD patients (Khan *et al.* 2011).	• Decrease in Firmicutes and increased *Bacteroides* in IBD patients (Frank *et al.* 2007, Hedin *et al.* 2015). • Increase in Gammaproteobacteria in IBD patients (Li *et al.* 2012, Walters *et al*. 2014). • Presence of *E. coli*, specifically adherent invasive *E. coli* in IBD patients (A Darfeuille-Michaud *et al.* 2004). • Reduced *Faecalbacterium and Roseburia* (Hedin *et al.* 2015, Sokol *et al* 2009, Swidsinski and Weber 2005).
Colorectal cancer (CRC)	• Mice genetically susceptible for CRC develop less tumours when raised in germ free conditions (Uronis *et al.* 2009) • Antibiotic treatment reduces the development of tumours in the colon of mice with colitis-induced CRC (Chen *et al.* 2008, Klimesova *et al.* 2013).	• Increased abundance of *Fusobacterium* in colorectal biopsies (McCoy *et al.* 2013). • Increased Firmicutes and Fusobacteria; reduced Proteobacteria and Actinobacteria in CRC biopsies (Gao *et al.* 2015) • Distinct composition of mucosa-associated bacteria involving enriched *Fusobacterium* and *Peptostreptococcusten* (Gao *et al*. 2015). • Increased abundance of Enterotoxigenic *Bacteroides fragillis* (ETBF) (Toprak *et al.* 2006). • Increased mucosa-associated *E. coli* in colitis-associated

		CRC (Prorok-Hamon *et al.* 2014).
Allergic asthma	• Probiotic therapy improves allergic asthma symptoms (Ege *et al.* 2011, Isolauri *et al.* 2000, Murk *et al.* 2011) • Antibiotic exposure in first year of life increases risk of developing allergic asthma (Ege *et al.* 2011, Marra *et al.* 2009, Murk *et al.* 2011). • Reduced microbiota diversity in early infancy precedes asthma development at school age (Abrahamsson *et al.* 2014).	• Increased *Clostridium* genus (Atarashi *et al.* 2011). • Increased *Lactobacillus* spp, Reduced *Bacteroides* (Shannon L Russell *et al.* 2013).
Autism spectrum disorder	• Subset of patients show gastrointestinal symptoms (McElhanon *et al.* 2014). • Antibiotic treatment shown to temporarily alleviate ASD neuronal symptoms (Sandler *et al.* 2000). • GI symptoms in patients strongly correlated with severity of autism (Adams *et al.* 2011).	• Reduced *Bifidobacteria* spp and increased Lactobacillus spp (Adams *et al.* 2011). • *Bacteroides fragilis* treatment reduced neurological defects in mouse model of ASD (Hsiao *et al.* 2013).
Rheumatoid arthritis	• Animal models of spondyloarthropathy raised in a germ free environment do not develop peripheral joint disease. (J D Taurog *et al.* 1994) • Severe joint inflammation reported in adjuvant induced arthritis animal model when rats were raised in germ free conditions (Kohashi *et al.* 1979).	• Increased abundance of *Prevotella copri* in of patients with new-onset rheumatoid arthritis (NORA) (Scher *et al.* 2013).
Obesity	• Conventionally raised mice show 40% higher body fat	• Increased abundance of Actinobacteria in obese

	content compared to germ free mice, independent of diet (Bäckhed *et al.* 2007). • Germ-free mice fed high-sugar, high-fat Western diet do not develop obesity (Bäckhed *et al.* 2007). • Increased antibiotic exposure in childhood is associated with increased weight gain in adolescence (B S Schwartz *et al.* 2015). • Distinct microbiota composition detected in lean and obese human twins (Turnbaugh *et al.* 2009).	individuals compared to lean individuals in human twin study (Turnbaugh *et al.* 2009). • Reduced abundance of Bacteroideted in obese individuals compared to lean individuals in human twin study (Turnbaugh *et al.* 2009). • In overweight children, decreased beneficial bacteria such as *Bifidobacteria* that is associated with increased Gram-negative bacteria such as *Enterobacteriecea* (Kalliomäki *et al.* 2008).
Type 2 diabetes mellitus	• Antibiotic treatment reduced metabolic endotoxemia and cecal LPS in both high-fat-fed and ob/ob mice and caused improvement of insulin sensitivity (Cani *et al.* 2008). • Altered microbiota composition detected in diabetic subjects compared to healthy controls (Fredrik H Karlsson *et al.* 2013, Qin *et al.* 2012).	• TDM2 subjects show reduced number of Clostridiales bacteria (*Roseburia* species and *Faecalibacterium prausnitzii*), that produce the SCFA butyrate (Fredrik H Karlsson *et al.* 2013, Qin *et al.* 2012).
IBS	• Human IBS develops following recovery from enteric infections (Spiller and Garsed 2009). • Probiotic bacteria treatment alleviates IBS symptoms in animal models of post-infectious IBS (Lutgendorff *et al.* 2008)	• Increased Bacteroidetes and reduced Clostridials correlate with impaired barrier function in IBS patients (Jalanka-Tuovinen *et al.* 2014).

1.3.1 The role of the gut microbiota in the development of immune related disorders

1.3.1.1 Rheumatoid arthritis

A striking example of the role of the microbiota in immune related disorders is in the development of rheumatoid arthritis (RA). RA is a highly prevalent, systemic autoimmune disease with predilection for the joints (McInnes and Schett 2011). If left untreated, RA can lead to chronic joint deformity, disability, and increased mortality. The first report of a possible role for the gut microbiota in the pathology of arthritis was in the late seventies in GF rats. In an adjuvant induced arthritis model, severe joint inflammation and 100% penetrance was reported when rats were raised in germ free conditions (Kohashi *et al.* 1979). Furthermore, human leukocyte antigen (HLA) B27 transgenic rats (spontaneous model of spondyloarthropathy) raised in a GF environment do not develop peripheral joint disease or intestinal inflammation (J D Taurog *et al.* 1994). Recent work has shed light on a potential causative microbial agent for RA in at least a portion of patients. Littman and colleagues reported increased abundance of the bacterial species *Prevotella copri* in the faeces of patients with new-onset rheumatoid arthritis (NORA) compared with patients with established rheumatoid arthritis, patients with psoriasis or healthy controls (Scher *et al.* 2013). *P. copri* was detected in the microbiota of 75% of patients with NORA compared to only 21.4% of healthy controls. It remains to be determined whether expansion of *P. copri* is a causative factor in the development of rheumatoid arthritis in these patients (Table 1.1).

1.3.1.2 Allergic asthma

Intestinal dysbiosis is proposed to have wider implications on human health and is associated with allergic immune disorders. Allergic asthma affects approximately 100 million people worldwide and its incidence is increasing in developed countries (Mukherjee and Zhang 2011). Although controversial, the reported ability of probiotic therapy to improve allergic asthma symptoms and reduce inflammatory markers indicates a role for the gut microbiota in this disease (Isolauri *et al.* 2000). Epidemiological data has reported distinct gut microbiota composition between

asthmatic and non-asthmatic infants and early life, antibiotic exposure increases the risk of developing allergic asthma (Ege *et al.* 2011, Murk *et al.* 2011). Moreover, it was recently demonstrated that children developing asthma (n = 8) had a lower diversity of the total gut microbiota than non-asthmatic children at one week and one month of age (Abrahamsson *et al.* 2014). The mechanisms by which dysbiosis is able to induce allergic inflammation was proposed by Noverr and colleagues who showed that mice receiving antibiotic treatments followed by infection of *Candida albicans* exhibited an altered microbiota composition and altered immune regulation in the airways, leading to upregulation of Th2 responses to both mould spores and ovalbumin exposure in BALB/c mice (Noverr *et al.* 2005). Furthermore, these mice were more susceptible to developing allergic airways disease (Noverr *et al.* 2005). Evidently, these data show that functional input of the intestinal microbiota has far greater implications than maintaining intestinal homeostasis. Indeed, recent work carried out over the last decade also supports a role for gut microbiota in neuronal development.

1.3.1.3 Autism spectrum disorder

Autism spectrum disorders (ASD) describes a range of neurodevelopmental disorders including autism and Asperger's and has an incidence of approximately 1/1000 in the UK (Taylor *et al.* 2013). Autism is recognised as a multifactorial disorder and symptoms are associated with genetics, environmental triggers and also immune and neuronal dysregulation. Interestingly, a subset of ASD patients also display gastrointestinal symptoms indicating a role for the gut microbiota in ASD (McElhanon *et al.* 2014) (Table 1.1). Several independent studies have reported altered gut microbiota composition in ASD patients compared to healthy controls (Adams *et al.* 2011, Lv Wang *et al.* 2013). This has involved a diverse range of bacterial species including increased abundance of *Bifidobacterium* spp, *Sutterella* spp and increased mucolytic bacterium *Akkermansia mucinphila* that is also associated with IBD (Png and Lindén 2010, L Wang and Christophersen 2011, Lv Wang *et al.* 2013). Furthermore, it was demonstrated by Adams and colleagues that GI symptoms are strongly correlated with the severity of autism, indicating a causal or primary role for the gut microbiota in ASD symptom development (Adams *et al.* 2011). This is further supported by other work that

showed that vancomycin treatment temporarily alleviated autistic symptoms in 8 out of 10 patients (Sandler *et al.* 2000).

1.3.2 The role of the gut microbiota in the development of gastrointestinal disease

As described in this work, the gut microbiota has a strong functional input on the maintenance of intestinal homeostasis. As one may predict, intestinal dysbiosis is associated with a diverse range of gastrointestinal disorders. Key examples of these that show strong associations with dysregulated gut mucosa immune functions will now be discussed.

1.3.2.1 Colorectal cancer

Several types of cancer are associated with infectious agents and most of these cancers tend to occur in tissues that have a high level of exposure to microorganisms. A key example of this is colorectal cancer (CRC) that is the third most common cancer and the fourth leading cause of cancer deaths worldwide, accounting for approximately 1.2 million new cases and 600,000 deaths per year (Brenner *et al.* 2014). CRC is a heterogeneous disease, including at least three major forms: hereditary (attributed to less than 5% of cases), sporadic, and colitis-associated CRC (Danese *et al.* 2011, Jess *et al.* 2005). Much evidence now exists to support a role for the intestinal microbiota in the aetiology of CRC not only via the pro-carcinogenic activities of specific pathogens, but also via the immunoregulatory function of the resident microbial communities (Sobhani *et al.* 2013). Animal models have shown that mutant mice genetically susceptible to CRC develop significantly less tumours when maintained in a GF environment (Uronis *et al.* 2009). Additionally, depletion of the intestinal microbiota by antibiotic treatment reduces the development of tumours in the colon of mice affected by colitis-associated cancer induced by azoxymethane and dextran sodium sulphate (Klimesova *et al.*. 2013).

Pyrosequencing of sporadic CRC-associated microbiota isolated from colonic tumour tissue has revealed increased abundance of Firmicutes and Fusobacteria

and reduced numbers of Proteobacteria compared to healthy adjacent tissue (Gao et al 2015). Significant differences in the composition of mucosa-associated bacteria was also reported in CRC colonic samples that involved enriched *Fusobacterium* and *Peptostreptococcusten* (Gao *et al*. 2015). *Fusobacterium nucleatum* is recognised as a potential causative agent for CRC as it has the capacity to induce colonic neoplasia via the expression of the surface adhesion molecule FadA. FadA facilitates the attachment and invasion of *Fusobacterium nucleatum* into colonic epithelial cells (Han *et al.* 2005, Xu *et al.* 2007). FadA binds to E-cadherin, activates β-catenin signalling, and differentially regulates the inflammatory and oncogenic responses (Rubinstein *et al.* 2013). Increased abundance of *Fusobacterium* in rectal biopsies of CRC patients was recently demonstrated and this correlated with expression of tumour necrosis factor alpha (TNF-α) and IL-10 (McCoy *et al.* 2013). *16S* rRNA pyrosequencing of faecal samples from patients with sporadic CRC has also reported enriched *Bacteroides fragilis* operational taxonomic units (OTUs) compared with healthy controls (Tingting Wang *et al.* 2012). *B. fragillis,* that is shown to colonise humans asymptomatically, also secretes a toxin termed *B. fragillis* toxin (BFT). In the Min (multiple intestinal neoplasia) mouse model for colon cancer, enterotoxigenic *B. fragillis* (ETBF) promotes colonic tumorigenesis via the inflammatory Th17/IL-23 pathway (Wu *et al.* 2009). Higher levels of the *bft* gene were detected in mucosal samples of CRC patients, especially in late onset CRC compared to samples obtained from routinely screened healthy patients (Boleij *et al.* 2015).

Colitis-associated cancer, that develops in areas of active colonic inflammation, is listed as cause of death in 10%-15% of all IBD patients (Van Der Kraak *et al.* 2015). Gut dysbiosis detected in colitis-associated cancer is associated with specific microbiota alterations detected in IBD (Martin *et al.* 2004, Swidsinski *et al.* 1998). Colonic adenomas, carcinomas, and the gut mucosa of colitis-associated CRC patients are abnormally colonized by *E. coli* belonging to the B2 phylogroup that are also associated with CD (Bonnet *et al.* 2014, Martin *et al.* 2004, Swidsinski *et al.* 1998). These mucosa-associated *E. coli* strains that show adherent-invasive properties, have been detected in increased numbers of CRC and CD biopsies compared to healthy controls (Prorok-Hamon *et al.* 2014). A high prevalence of genotoxin producing *E. coli* has been reported in colitis-associated CRC (Buc *et al.* 2013, Raisch *et al.* 2015). Recent studies show that *E. coli* possessing the

polyketide synthase gene complex (*pks*) responsible for producing the genotoxin colibactin, induce inflammation-associated CRC in mice and are commonly mucosa associated in sporadic CRC (Prorok-Hamon *et al.* 2014). B2 CRC-associated *E. coli* are shown to invade and survive within macrophages that induces protumour responses including upregulated cyclooxygenase-2 (COX-2) expression (Raisch *et al.* 2015).

1.3.2.2 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is comprised of Crohn's disease (CD) and ulcerative colitis (UC) and is characterised by chronic and relapsing inflammation of the intestine. IBD is a disease of complex etiology and is proposed to occur in genetically susceptible hosts following periods of inflammation induced intestinal dysbiosis involving both the host mucosal immune system and the gut microbiota (Craven *et al.* 2012). Much evidence now exists to support an altered gut microbiota composition as a key characteristic of IBD. Under GF conditions, IL-10 $^{-/-}$ mice and HLA-B27 transgenic rats do not develop colitis (Kennedy *et al.* 2000). Furthermore, in spontaneous models of colitis intestinal inflammation is abolished if mice have a deficiency in individual TLRs or MyD88 (Hoshi *et al.* 2012). One hypothesis is that the onset of IBD is due to a general dysbiosis of gut microbiota composition involving an imbalance of resident commensal populations. Several independent studies have shown, by culture independent and dependent techniques, that intestinal inflammation is associated with reduced bacterial diversity and not bacterial numbers (Joossens *et al.* 2011). Metagenomic analysis of IBD patient microbiota has also reported approximately 25% fewer genes compared to healthy controls, indicating reduced diversity in microbiota species (Qin *et al*. 2012). IBD-associated dysbiosis involves lower abundance and complexity of the Bacteriodetes and Firmicutes phyla (Frank *et al.* 2007, Joossens *et al.* 2011, Manichanh *et al.* 2006). An increased abundance of Gammaproteobacteria has also been reported (Li *et al.* 2012, Walters *et al.* 2014). Several independent studies have reported reduced numbers of the anti-inflammatory commensal Firmicute, *Faecalibacterium prausnitzii* in IBD patients (Frank *et al.* 2007, Sokol *et al.* 2009, Swidsinski and Weber 2005). Interestingly, in other work, an increased abundance of *F. prausnitzii* was reported in paediatric

CD patients who had not yet been exposed to antibiotic therapies. Although highly controversial, this indicates a potential protective role for this bacterial species in IBD pathogenesis (Hansen *et al.* 2012). Among the Proteobacteria an overgrowth of *Enterobacteriaceae,* specifically *E. coli*, has been detected in IBD patients (Giaffer *et al.* 1992, Seksik *et al.* 2003, Sokol *et al.* 2006). More specific features of CD and UC associated dysbiosis also include reduced *Roseburia* and *Phascolarctobacterium,* that are involved in anti-inflammatory regulatory T-cell production and propionate production in the gut, respectively (Morgan *et al.* 2012). Other studies have reported decreased abundance of other short chain fatty acid (SCFA) producing bacteria including *Ruminococcaceae* (CD) *and Leuconostocaceae* (UC) (Morgan *et al.* 2012). The mechanisms by which the gut microbiota trigger intestinal inflammation in IBD are still unknown, however, it is proposed that individual immune defects determine the ability of intestinal bacteria to trigger colitis. This has been shown in GF mice deficient in IL-10 whereby colitis could be triggered by infection with *E. coli* or *Enterobacter faecalis* but not with *Bacteroides vulgatus* (Kim *et al.* 2005). Additionally, *Tlr-5*$^{-/-}$ mice develop spontaneous colitis in some housing facilities but not all, indicating microbiota composition affects disease susceptibility (Vijay-Kumar *et al.* 2007). Furthermore, deficiency in *Tlr-2, Tlr-4*, *Tlr-9* or *Myd88* in mice is associated with increased susceptibility to DSS-induced colitis (Lee *et al.* 2006, Saha *et al.* 2010).

A second hypothesis that has been gaining increased support is that IBD pathogenesis is associated with abnormal colonisation of the gut by a specific pathogenic agent. One example that has gained a great deal of attention over the last 2 decades is increased mucosal association of adherent-invasive *E. coli* (AIEC) in CD. AIEC is now proposed to be a likely cause of CD associated pathology.

1.4 Adherent-invasive *E. coli* infection associated with Crohn's disease

1.4.1 Crohn's disease

Crohn's disease was first recognised as a distinct entity of IBD approximately 80 years ago. Unlike UC, CD is characterised by discontinuous and granulomatous inflammation and may affect any region of the gastrointestinal tract from mouth to anus (Bandzar *et al.* 2013). In CD, TNF-α is present in extremely high quantities and is thought to be the major cause of excessive inflammation (Murray *et al.* 1997). Crohn's disease is debilitating and expensive to treat and affects approximately 150-200 /100,000 in the UK (Kalla *et al.* 2014). Once recognised as a disease of Western regions, the incidence of CD is increasing in both developing and developed countries (Benchimol *et al.* 2011, Molodecky *et al.* 2012). This trend is most pronounced in paediatric patients, confirmed recently by a study of paediatric Scottish patients (Henderson *et al.* 2012). CD is currently recognised as an incurable disorder and current therapies function to induce or sustain periods of remission. They include antibiotics, immune system suppressors, anti-inflammatory mediators and the last resort therapy of surgery.

1.4.2 Crohn's disease is a multifactorial disorder

CD is recognised as a multifactorial disorder of complex aetiology. It is proposed that disease development is a consequence of aberrant immune responses to intestinal bacteria following an environmental trigger, a process intimately linked to host genotype. The increased concordance for CD in monozygotic twins (~36%) and higher risk of developing IBD if a first degree relative has CD (relative risk 5-35) suggest a genetic determinant in disease pathogenesis (Van Limbergen *et al.* 2008, R K Russell and Satsangi 2004). Genome wide association studies (GWAS) have identified 71 (out of 163 IBD loci identified) distinct genetic susceptibility loci for CD (Jostins *et al.* 2012). However, CD associated polymorphisms are associated with only a 30-40 fold increase in risk of developing disease and expression of all polymorphisms in single individuals account for less than 20% of total risk (Van Limbergen *et al.* 2009). This prevents GWAS to predict disease

development and also emphasises that genetics alone cannot account for the tendency of an individual to develop CD. Many of the risk susceptibility genes identified have improved our understanding of the etiology of CD. The key genetic susceptibility loci contain genes that encode for various components that function in innate pattern recognition (NOD2/CARD15), differentiation of Th17 lymphocytes (IL-23R, JAK2, STAT3, CCR6, ICOSLG), autophagy (ATG16L1, IRGM, LRRK2) and intestinal epithelial barrier integrity (IBD5, DLG5, PTGER4, ITLN1, DMBT1, and XBP1) (Franke *et al.* 2010). The identified risk susceptibility genes indicate a strong microbial component in CD and a crucial role for bacterial recognition and clearance in disease pathogenesis.

Indeed, much evidence has reported an altered or dysbiotic intestinal microbiota composition in CD patients (Martin Baumgart *et al.* 2007, Fujimoto *et al.* 2013, Joossens *et al.* 2011, Kang *et al.* 2010, Manichanh *et al.* 2006). Gut dysbiosis in CD involves increased abundance of Proteobacteria, particularly *E. coli* and decreased bacteria of the Firmicutes phylum (Manichanh *et al.* 2006, Mukhopadhya *et al.* 2012). It is yet to be determined if gut dysbiosis is a primary factor in the development of CD, or is a secondary factor induced by environmental triggers or specific host factors.

A likely trigger for CD associated dysbiosis are variations in diet. Western society has shifted to a high-fat, high carbohydrate diet over the past-half century, and this has correlated with a significant increase in the incidence of disease in this region (Burisch *et al.* 2013). The mechanisms by which Western diet promotes dysbiosis and intestinal inflammation was demonstrated in mice whereby dietary fat, via changes in bile salt metabolism, promoted bacterial dysbiosis that involved intestinal blooms of *Bilophila wadsworthia* (Devkota *et al.* 2012). *B. wadsworthia* blooms were associated with colitis and increased production of pro-inflammatory cytokines in *Il-10*$^{-/-}$ mice, indicating a role for host genotype in dietary induced intestinal inflammation (Devkota *et al.* 2012). It was also shown that high-fat diet accelerated the development of intestinal inflammation in a murine Crohn's disease-like ileitis model, independent of weight gain (Gruber *et al.* 2013). In this case, accelerated pathogenesis was mediated by increased intestinal permeability via reduced occludin expression and altered immune responses involving increased Th17 responses (Gruber *et al.* 2013).

Furthermore, administration of emulsifiers to *Il-10*$^{-/-}$ mice was shown to induce perturbation of gut microbiota involving a significant reduction in microbial diversity, blooms of members of the Verrucomicrobia phyla and increased abundance of mucosa-associated pro-inflammatory Proteobacteria (Benoit Chassaing *et al.* 2015). Emulsifier exposure also increased faecal levels of bioactive LPS and increased the incidence of colitis in *Il-10*$^{-/-}$ mice (Benoit Chassaing *et al.* 2015).

Inflammation is also shown to be a key driver of intestinal dysbiosis, indicating that disease itself is a prerequisite for pathogenic shifts in the gut microbiota. It was shown that severe inflammation, induced by *Toxoplasma gondii* infection, caused significant gut dysbiosis in mice, involving a shift from >95% Firmicutes to >95% Proteobacteria. This also involved reduced bacterial diversity and increased mucosal invasion by *E. coli.* Administration of anti-TNF-α antibodies to mice alleviated gut dysbiosis and reduced bacterial mucosal invasion.

Intestinal dysbiosis in CD extends further than a perturbed microbiota composition and involves dysregulated mucosal immune responses and impaired intestinal barrier function (Figure 1.6). Increased intestinal permeability has been demonstrated in a larger proportion of CD patients than healthy subjects (Jenkins and Rooney 1987, Wyatt *et al.* 1997). Furthermore, patients with active CD demonstrate increased intestinal permeability that decreases during disease remission (Sanderson *et al.* 1987). Moreover, anti-inflammatory therapies such as infliximab, an anti-TNF-α blockade, also restores intestinal permeability in CD patients (Suenaert *et al.* 2002, Zeissig *et al.* 2004). The adaptive immune system is proposed to mediate and perpetuate intestinal inflammation in CD but is not recognised as the primary trigger for inflammatory symptoms. CD is characterised by an imbalance of inflammatory Th cells vs tolerance-maintaining Treg cells (Chao *et al.* 2014). Both human and murine studies have shown different T-cell subpopulations aberrantly activated in CD (Fuss *et al.* 1996). Initial studies showed that CD is predominately mediated by a T-helper Th1 immune response through activation of IL-12/STAT4 and interferon-gamma (IFN-γ)/STAT1 signalling pathways (Monteleone *et al.* 2005). However, there may be a partial Th1/Th2 polarisation in CD that involves other cytokines like TNF-α, IL-1β, and IL-17A, that primarily activate NF-κB, IL-6, and Il-10, that activate STAT3 (Zhan-Ju Liu *et al.* 2009, Sanchez-Munoz *et al.* 2008). Defects in processes crucial for microbial

sensing and eradication are also observed in CD. Impaired stimulation of the innate MyD88 pathway and also autophagy have been observed in CD monocytes (Homer *et al.* 2010, Kuballa *et al.* 2008, Lapaquette *et al.* 2010). Furthermore, reduced production of host AMPs including Paneth cell produced α-defensins has been observed (Wehkamp and Salzman 2005).

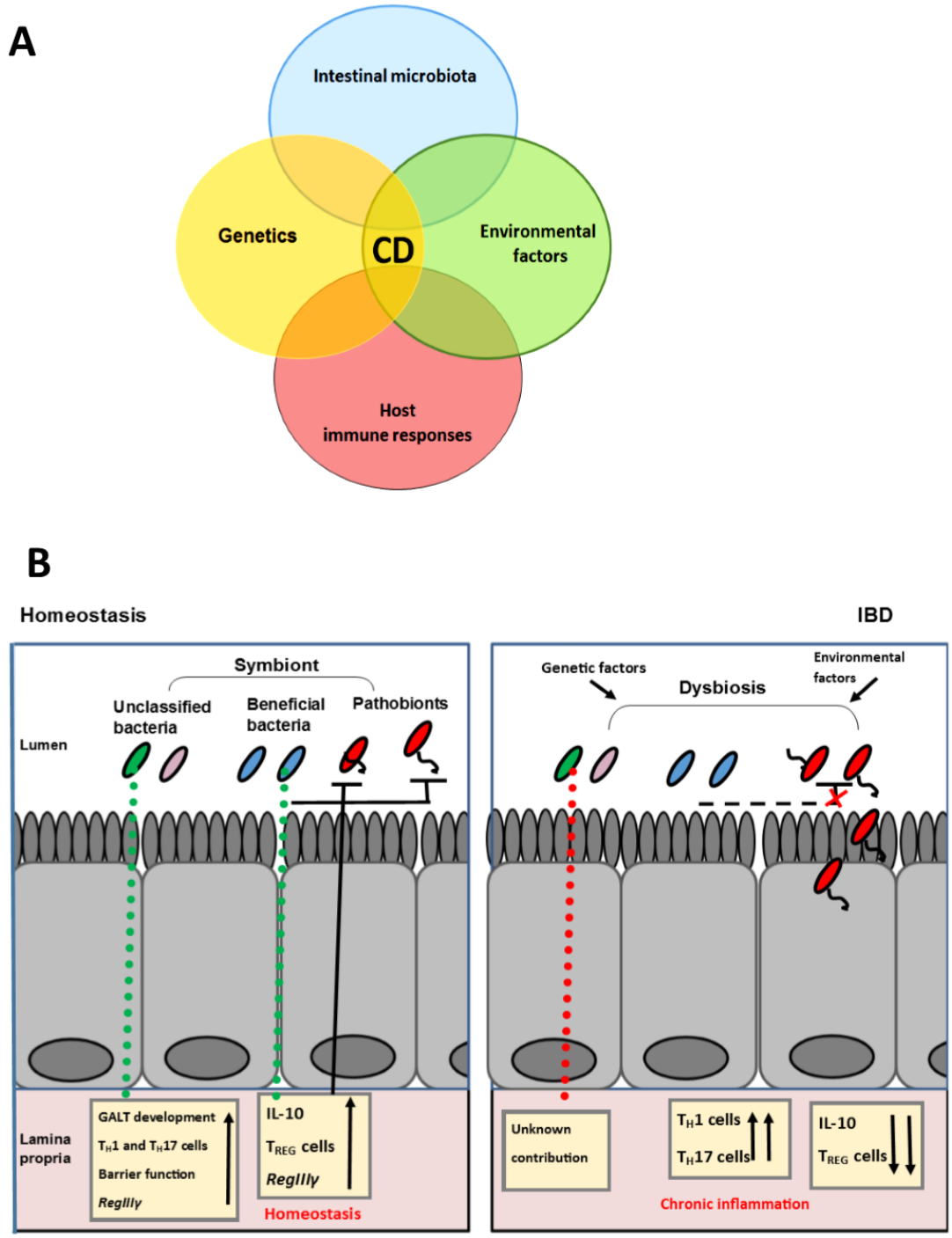


Figure 1-6: Crohn's disease (CD) is a multifactorial disorder.

A) CD possesses a complex etiology, associated with aberrant host immune responses to intestinal bacteria following an environment trigger in genetically susceptible hosts. B) The mechanisms involved in the pathogenesis of CD. A combination of genetic factors (incl. mutations in nucleotide-binding oligomerization domain 2 (*Nod2*), autophagy-related gene 16-like 1 (*Atg16l1*)) and environmental factors (such as infection, stress and diet) result in gut dysbiosis. Dysbiosis (infection or imbalance of beneficial bacteria) induces chronic inflammation involving hyperactivation of Th1 and Th17 cells. Adapted from (Kamada *et al.* 2013).

1.4.3 Increased abundance of *E. coli* in Crohn's disease

Intestinal overgrowth of *E. coli* in CD has been demonstrated by a wide range of culture based and molecular based studies (Martin Baumgart *et al.* 2007, Lopez-Siles and Martinez-Medina 2014, Neut *et al.* 2002, Willing and Halfvarson 2009). In the cohort of Martinez-Medina *et al. E. coli 16S* rRNA gene copies comprised 14% and 33% of total bacterial *16S* rRNA in healthy and ileal CD patients, respectively (Martinez-Medina, Aldeguer, et al 2009). Furthermore, increased levels of *E. coli* have been shown in active CD patients compared with those in remission (Martin Baumgart *et al.* 2007, Lopez-Siles and Martinez-Medina 2014, Schwiertz *et al.* 2010).

Additionally, in a previous study utilising fluorescent *in situ* hybridisation (FISH), increased *E. coli* numbers were detected in the epithelium and lamina propria of patients with active CD compared to inactive CD (Mylonaki and Rayment 2005). Increased *E. coli* numbers have also been shown to correlate with reduced time before disease relapse (Lopez-Siles and Martinez-Medina 2014). This correlates with work showing that high levels of antibodies against *E. coli* outer membrane protein C (OmpC) are associated with disease progression, longer duration and need for surgery in CD patients (Beaven and Abreu 2004, Landers *et al.* 2002, Mow *et al.* 2004). *E. coli* DNA is also more frequently detected in granulomas of CD patients (80%) compared with non-Crohn's control granulomas (10%) (Ryan *et al.* 2004). In addition*, E. coli* isolated from CD patients has been detected in the mucus layer, close to intestinal epithelial cells (Swidsinski and Weber 2005, Walmsley *et al.* 1998). Increased mucosa-associated *E. coli* are detected in CD patients relative to healthy controls and some genotypes appear to be associated with the disease. Further investigation of these mucosa-associated *E. coli* strains has demonstrated their strong adhesive and invasive properties . This phenotype has grouped these mucosa-associated strains as a novel *E. coli* pathovar associated with CD, designated AIEC for adherent-invasive *E. coli* (Arlette Darfeuille-Michaud 2002).

1.4.4 AIEC definition

The AIEC pathotype is defined as *E. coli* strains that (1) adhere and invade to intestinal epithelial cells; (2) involve host cell actin polymerization and microtubule recruitment in bacterial uptake; (3) survive and replicate within macrophages inducing pro-inflammatory cytokine production (Arlette Darfeuille-Michaud 2002). Since its definition, invasive determinants characteristic from ExPEC have been detected in diverse AIEC strains (Martinez-Medina, Mora, *et al.* 2009). These virulent phenotypes designate AIEC as a putative CD-associated pathogen that overlap significantly with other pathogenic *E. coli* (UPEC, APEC, DAEC, and APEC) (Nash *et al.* 2010).

1.4.5 Genetic classification of AIEC

AIEC strains that have been isolated from CD patients are clonally diverse and belong to different serogroups. No specific clone has been associated with disease pathogenesis (Martin Baumgart *et al.* 2007, Kleessen and Kroesen 2002, Sepehri *et al.* 2011). On the most part, AIEC belong to the B2 and D phylogroups as is the case for extra intestinal pathogenic *E .coli* (ExPEC) (Kotlowski *et al.* 2007). Furthermore, AIEC carry distinct sets of virulence genes that are characteristic of ExPEC (Elliott *et al.* 2013, Gombošová *et al.* 2011). Additionally virulence genes not associated with commensal *E. coli*, such as *afaC, pks, malX* or *lpf*, have been detected frequently in AIEC strains (Dreux *et al.* 2013, Rolhion *et al.* 2010). Moreover, analysis of sequenced AIEC genomes has failed to identify a unique genetic characteristic for this group (Miquel *et al.* 2010, Nash *et al.* 2010).

1.4.6 AIEC is associated with ileal and colonic Crohn's disease

AIEC can be described as a specialised pathogen in CD as it is also isolated from the gut mucosa of healthy individuals (Baumgart *et al*. 2007, Martinez-Medina *et al*. 2011). AIEC comprise approximately 3.58% and 0.9% of ileal and colonic *E. coli* in healthy subjects, however in this setting it is not shown to translocate across the intestinal mucosa barrier (Martinez-Medina, Mora, *et al.* 2009, Mylonaki and Rayment 2005).

The association of AIEC with CD was suggested over 2 decades ago in the first study by Darfeuille-Michaud (A Darfeuille-Michaud *et al*. 2004). In this early study,

it was shown by culture dependent techniques that AIEC could be more frequently isolated from patients with ileal CD (36%) compared to colonic CD (3.7%). This work was highly suggestive of a dominant role for AIEC in ileal disease. Indeed, many independent studies have reported a high prevalence of AIEC in ileal CD patients compared to healthy controls (Martin Baumgart *et al.* 2007, A Darfeuille-Michaud *et al.* 2004, Martinez-Medina, Aldeguer, *et al.* 2009, Sasaki *et al.* 2007). Additionally, recent work reported a prevalence of approximately 25% for AIEC in ileal CD patients compared to healthy controls (Dogan *et al.* 2013).

Other work has demonstrated a high prevalence of AIEC in colonic CD, suggesting a role for this *E. coli* pathotype in both colonic and ileal disease. A high number of mucosa-associated *E. coli* that displayed the AIEC phenotype were detected in mucosa preparations isolated from CD colonic biopsy samples (Martin *et al*. 2004). It was shown that mucosa-associated bacteria were isolated from 71% cases with ileal or ileocolonic Crohn's disease and from 86% of cases with colonic CD (Martin *et al.* 2004).

In other work, Baumgart and colleagues detected AIEC strains in the ileum of 38.5% of CD patients with ileal involvement but also in the colon of 37.5% of patients with colonic CD (Martin Baumgart *et al.* 2007). Furthermore, in other work that utilised a collection of 95 - 150 *E. coli* colonies per patient, AIEC strains were reported in the ileum of 54.5% of CD patients and in the colon of 50% of CD patients (Martinez-Medina, Aldeguer, *et al.* 2009). FISH analysis also reported the presence of mucosa-associated *E. coli* in 42% of colonic biopsy samples obtained from CD patients (Mylonaki and Rayment 2005).

The mechanisms by which expansion of AIEC in CD gut mucosa triggers intestinal inflammation have been described in both *in vitro* and *in vivo* studies of infection and will now be discussed.

1.4.7 AIEC and CD pathogenesis

1.4.7.1 Colonisation of intestinal mucosa

Much of the work that has aimed to identify the pathogenic mechanisms of AIEC in CD has utilised the AIEC reference strain LF82 (A Darfeuille-Michaud *et al.* 2004). AIEC LF82 was isolated from a CD chronic ileal lesion in 1999 and has been subsequently utilised by a number of groups as the model organism for AIEC associated CD infection (Boudeau *et al.* 1999). LF82 is an excellent biofilm forming bacteria and has been shown to form robust biofilms on the intestinal epithelial cell surface in several *in vitro* and *in vivo* studies (B Chassaing and Darfeuille-Michaud 2013). Biofilm formation is a common phenotype of AIEC strains. In one study, 17/27 AIEC strains were classified as biofilm producers compared with only 9/38 non-AIEC strains, with all being intestinal isolates (Weiss-Muszkat *et al.* 2010).

In ileal CD, LF82 colonises the intestinal mucosa by adhering to intestinal epithelial cells. LF82 adheres to intestinal epithelial cells via a common type I pili adhesin FimH variant that recognises and interacts with host intestinal receptor carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) in a mannose dependent manner (Barnich *et al.* 2003, 2007). Interestingly, CEACAM6 expression is upregulated in ileal CD patients and this may increase susceptibility to AIEC colonisation (Barnich and Darfeuille-Michaud 2007). This highlights an intimate link between genetic susceptibility, inflammation and intestinal dysbiosis in disease pathogenesis. Recent work has shown that AIEC express FimH variants that facilitate more efficient binding to intestinal epithelial cells (Dreux *et al.* 2013). AIEC flagella have also been shown to facilitate colonisation of the intestinal mucosa in a process related to active motility (Barnich *et al.* 2003). Flagella also play a role in the perpetuation of intestinal inflammation and induce secretion of pro-inflammatory cytokines from polarised intestinal epithelial cells causing recruitment of macrophages and dendritic cells (DCs) at the localised area (Eaves-Pyles *et al.* 2008, Subramanian, Rhodes, *et al.* 2008). Pro-inflammatory cytokines produced by these immune cells including TNF-α and IFN-y, induce expression of CEACAM6 receptors, hence promoting AIEC colonisation (Barnich and Darfeuille-Michaud 2007).

Colonisation of the colonic mucosa by AIEC is likely to occur via alternative adhesins to FimH namely the afimbrial adhesin Afa-1. Increased prevalence of afimbrial adhesion *afa-1* operon was detected in mucosal *E. coli* isolates from CD patients compared to healthy controls (Prorok-Hamon *et al.* 2014). Furthermore expression of Afa-1 correlated with the ability of *E. coli* to adhere and invade to intestinal epithelial cells (Prorok-Hamon *et al.* 2014).

1.4.7.2 Invasion of intestinal epithelial cells

AIEC are proposed to invade intestinal epithelial cells (IECs) via a macro-pinocytosis like process (Boudeau *et al*. 1999). Outer membrane vesicles (OMVs) also play a key role in AIEC invasion of intestinal epithelial cells (IECs). Rolhion and colleagues showed that outer membrane vesicles (OMVs) rich in OmpA fused to the membrane of host IECs via binding of LF82 OmpA to endoplasmic stress response chaperone glycoprotein 96 (Gp96) (Rolhion *et al.* 2010). This is predicted to facilitate release of bacterial effector proteins that induce actin polymerisation and microtubule rearrangement of the epithelium and hence bacterial translocation. Furthermore, Gp96 is also shown to be overexpressed in the ileum of CD patients that increases their susceptibility to AIEC infection and again demonstrates the multifactorial and complex etiology of this disease (Rolhion *et al.* 2010). It has been recently demonstrated for another AIEC prototype strain, NRG857c, that deletion of the gene encoding invasive protein IbeA, caused reduced invasion of, but not adhesion to intestinal epithelial cells *in vitro* (Cieza *et al.* 2015). Deletion of *ibeA* however did not affect AIEC persistence in the murine gastrointestinal tract, indicating that AIEC possess additional adhesins and invasins that facilitate interaction with the host.
Following bacterial invasion, LF82 are detected within intracellular compartments within intestinal epithelial cells including LC-3 positive autophagosomes (Lapaquette *et al.* 2010). It was recently demonstrated that LF82 reduces the level of autophagy in intestinal epithelial cells through activation of NF-κB that causes increased expression of microRNAs, *MIR30C* and *MIR130A*, and consequently reduced expression of key autophagy genes *ATG16L1* and *ATG5* (Nguyen *et al.* 2014). Ileal samples from patients with CD had increased levels of these microRNAs and reduced levels of *ATG5* and *ATG16L1* (Nguyen *et al.* 2014).

1.4.7.3 AIEC translocation of intestinal mucosa

Along with the ability to invade intestinal epithelial cells, other studies have also reported the ability of AIEC to translocate across the intestinal mucosa (Benoit Chassaing *et al*. 2011). AIEC translocate the gut mucosa via M cells of Peyer's patches (PPs). M cells, that cover approximately 10% of follicle-associated epithelia (FAE), are highly specialized cells for the phagocytosis and transcytosis of intestinal lumen macromolecules, antigens, and microorganisms. AIEC target M cells via type I pili and long polar fimbriae (LPF) (Benoit Chassaing *et al.* 2011). M cell specific cell surface protein, glycoprotein2 (Gp2) is reported to recognise the FimH component of bacterial type I pili that promotes bacterial uptake (Hase *et al.* 2009). The M cell specific receptor for AIEC LPF during bacterial translocation is currently unknown. Recent work has demonstrated a role for GipA factor in the colonisation and translocation of PP's by AIEC (Vazeille *et al.* 2016). Vazeille and colleagues reported that AIEC *gipA* deletion mutants showed impaired translocation of M cells in *ex vivo* murine models of infection (Vazeille *et al.* 2016). GipA was also reported to positively regulate expression of the *lpf* operon under bile-salt enriched conditions (Vazeille *et al.* 2016).
Furthermore, LF82 induce expression of pore-forming protein, claudin-2, and displace ZO-1 and E-cadherin from apical tight junctions, resulting in increased intestinal permeability (Denizot *et al.* 2012, Wine *et al.* 2009)

1.4.7.4 AIEC survival and persistence within host macrophages

Once AIEC has gained entry to the lamina propria it then invades and survives within macrophages inducing production of pro-inflammatory cytokines. (Bringer *et al.* 2006, Glasser *et al.* 2001). Extensive replication of LF82 within macrophages has been reported in several independent studies (Bringer *et al.*. (Mpofu *et al.* 2007, Subramanian, Roberts, *et al.* 2008); Martin *et al.* 2004). Within macrophages, intracellular LF82 survive within phagolysosome-like compartments indicating an ability to survive and persist in environments that contain acidic pH, oxidative stress and proteolytic enzymes (Bringer *et al.* 2006). Indeed, Bringer and colleagues showed that an acidic environment within the phagolysosome is

essential for AIEC intracellular replication (Bringer *et al.* 2006). The specific mechanisms involved in this process have yet to be determined. The stress protein HtrA and thiol-disulphide oxidoreductase DsbA have been reported to be important for survival and replication within macrophages (Bringer *et al.* 2005, 2007). LF82 *htrA* deletion mutants showed increased sensitivity to oxidative stress induced by hydrogen peroxide (H_2O_2) and demonstrated reduced growth in environments reproductive of the phagolysosome (Bringer *et al.* 2005). Additionally, Hfq, a RNA-binding protein that functions as a global posttranscriptional regulator of gene expression, is proposed to function in LF82 survival and replication within macrophage (Simonsen *et al.* 2011). Vazeille and colleagues have reported a crucial role for GipA in AIEC intramacrophagic persistence. It was shown that LF82 *gipA* mutants showed significantly reduced survival in macrophages at 7 h post infection compared to the wild-type strain. Reduced survival of isogenic mutant within macrophages was explained by the ability of GipA to increase tolerance of LF82 to oxidative stress and acidic pH (Vazeille *et al.* 2016). A role for *ibeA* in AIEC persistence within macrophages has also been demonstrated whereby mutant AIEC that lack *ibeA* show significantly reduced intramacrophagic survival up to 24 h post-infection (Cieza *et al.* 2015). AIEC persistence in macrophages causes increased secretion of pro-inflammatory cytokines including IL-6 and TNF-α, without inducing host cell death (Glasser *et al.* 2001). This has been linked to granuloma formation, the hallmark of CD associated inflammation, and has been demonstrated *in vitro* (Meconi *et al.* 2007). Prolonged survival of AIEC infected macrophages has been explained by the direct role of LF82 in delaying apoptosis. It was recently demonstrated that LF82 infection of macrophages increased proteosomal degradation of caspsase-3, a key regulator of apoptosis, hence promoting LF82 intracellular persistence (Dunne *et al.* 2013).

These data obtained from a multitude of *in vitro* studies show AIEC expansion in CD is a multifactorial process involving host genetic determinants, environmental triggers and also the presence of specific *E. coli* with enhanced capacity to interact with the host mucosa. Animal models for AIEC intestinal infection, that have been recently established, have given greater insights into the mechanisms involved in AIEC induced gut inflammation.

1.4.8 Insights from AIEC animal models

The current animal models for AIEC colonisation further highlight the multifactorial and complex mechanisms involved in the role of this *E. coli* pathotype in CD pathogenesis.

In the first model of LF82 colonisation of the murine intestine, Carvalho and colleagues demonstrated that intestinal persistence of AIEC was dependent on expression of human CEACAMs (Frédéric a Carvalho *et al.* 2009). In CEABAC10 mice (Chan *et al.* 2007), that express human CEA, CEACAM3, 6 and 7, LF82 persistence induced severe intestinal colitis involving bloody diarrhoea, weight loss, epithelial damage and immune cell infiltration of colonic mucosa. Wild-type mice that lacked CEACAM receptors, infected with LF82, did not exhibit any clinical or histological symptoms of colitis (Frédéric a Carvalho *et al.* 2009). It was recently demonstrated by Small and colleagues that persistent colonisation of the intestine of conventional mice with AIEC strain NRG 875c required depletion of gut microbiota. Chronic infection with AIEC was established following streptomycin treatment of mice and infection induced intestinal inflammation involving heightened Th17 responses, increased secretion of pro-inflammatory cytokines and transmural fibrosis and inflammation (Small *et al.* 2013). In a different model, where intestinal inflammation was induced in mice via *Toxoplasma gondii* infection or administration of indomethacin, inflammation was shown to drive severe dysbiosis that primarily involved proliferation of AIEC and increased bacterial invasion of intestinal mucosa (Craven *et al.* 2012). Furthermore, administration of anti-TNF-α antibodies was shown to limit inflammation induced dysbiosis and also bacterial invasion. Compelling work by Chassaing and colleagues has given great insight into the triggers and mechanisms involved in AIEC colonisation. Mice engineered to lack the flagellin receptor,TLR5, are prone to developing spontaneous intestinal inflammation(B M Carvalho *et al.* 2012, Vijay-Kumar *et al.* 2007). The work of Chassaing and colleagues demonstrated that mice lacking TLR5 (T5K0), raised in a GF environment, were more susceptible to LF82 infection (Frederic A Carvalho *et al.* 2012). Furthermore, LF82 infection of T5KO mice induced chronic colitis that was not detected in wild-type mice (Frederic A Carvalho *et al.* 2012).

Interestingly, LF82 induced colitis was shown to be greater in T5KO mice that were exposed to LF82 and then placed in specific pathogen-free (SPF) housing

after LF82 bacterial load was cleared from intestine (Benoit Chassaing, Koren, *et al.* 2014). This indicates that exposure of T5KO mice to AIEC alters the intestinal immune responses to other microbiota, highlighting its potential as a pro-inflammatory/ immunoregulatory bacterium. Moreover, LF82 induced colitis in T5KO mice was linked to dramatic shifts in microbiota composition, involving reduced species diversity and also increased gut LPS and flagellin, activating TLR4 and NLRC4 dependent pathways. Remarkably, altered microbiota components and increased levels of bioactive LPS and flagellin persisted beyond clearance of LF82(Benoit Chassaing, Koren, *et al.* 2014). Wild-type mice that possessed TLR-5, colonised by AIEC did not exhibit altered inflammatory markers, bacterial loads, microbiota composition or pro-inflammatory immune responses to intestinal microbiota.

This evidence further highlights the requirement for multiple triggers for the onset of CD associated pathology. This factor may in part explain the poor efficacy of current therapies for CD that frequently target one or few components of disease pathogenesis. Key examples of CD therapies will now be discussed.

1.5 CD therapies currently used in clinical practice

1.5.1 Anti-inflammatory therapies

CD is currently recognised as an incurable disease and displays a range of therapeutic challenges. Therapies aim to induce or sustain periods of remission and those currently in clinical use include broad spectrum antibiotics, immune system suppressors, anti-inflammatory agents and also resection surgery. The most common treatments for CD are the use of synthetic antibodies against TNF-α or use of an alpha-4 integrin subunit. Alpha-4 integrin is expressed on leukocytes and controls cell migration to inflammation site. Key examples of anti-TNF-α antibodies include infliximab (REMICADE) and adalimumab (HUMIRA) (Bandzar *et al* 2013). The currently used alpha-4 integrin inhibitor in clinical practice is natalizumab (Bandzar *et al.* 2013). This class of CD therapies effectively control symptoms in some patients but fail to alleviate growth impairment shown in paediatric patients or induce complete remission. Moreover, recipients often become resistant to these therapies and use of anti-TNF-α antibodies is

associated with a range of adverse side effects including opportunistic infections, malignancy, hematologic disorders and formation of autoantibodies (Antoni and Braun). Corticosteroids (CS) are a well-established treatment for active CD and have been used widely in clinical practice for decades. Common systemic CS include hydrocortisone, budesonide and prednisolone (Yang and Lichtenstein 2002). Corticosteroids down regulate production of inflammatory cytokines such as IL-1, IL-6, and TNF-α (Ho *et al.* 2006). If used correctly this class of therapy is cheap, safe and effective in a proportion of patients. However CS use is linked to serious toxic side effects and approximately 50% of recipients fail to respond to CS or become steroid resistant after only one year (Tung *et al.* 2006, Yang and Lichtenstein 2002).

1.5.2 Antimicrobial therapies

A recent addition to the group of CD therapies are broad spectrum antibiotics. The use of antibiotics for the treatment of this complex disorder however, is highly controversial as their role is not clearly defined. However, recent meta-analysis data has indicated use of broad spectrum antibiotics as a potential primary treatment for CD (Khan *et al.* 2011). In the large cohort study (n=402) of Prantera *et al.* 62% of CD patients who received rifaximin (61 of 98) were in remission, compared with 43% of patients who received placebo (43 of 101). However, in this work disease remission required prolonged courses and high doses (800 mg for 12 weeks) of antibiotic treatment that is associated with a range of adverse effects including secondary *Clostridium difficile* infections that is associated with disease relapse in a portion of patients (Huebner and Surawicz 2007). Furthermore, important work carried out over the last decade, now demonstrates a strong association between CD development and exposure to broad spectrum antibiotics in early childhood (Hildebrand *et al.* 2008, Ungaro *et al.* 2014). Indeed, increased antibiotic exposure in childhood has been shown to increase the risk of being newly diagnosed with CD(Hildebrand *et al.* 2008). Furthermore, additional work that demonstrates both short-term and long-term intestinal dysbiosis induced by antibiotic treatment highlights a potential limitation for this class of therapy in patients that exhibit altered microbiota composition (Dethlefsen and Relman 2011, Jernberg *et al.* 2007).

Recent studies also suggest a potential role for therapies able to specifically manipulate the microbiota in CD patients. These include faecal transplant therapy, probiotics and prebiotics (Fujimori *et al.* 2007, Gordon and Harbord 2014). However, due to the lack of long-term and large cohort studies, it is difficult to assess the *in vivo* efficacy of these types of therapies. Indeed, a therapy that employed such a technique would be highly desirable for the treatment of disorders such as CD, in which targeting of a specific species of the microbiota is required.

1.6 Bacteriocins

1.6.1 Bacteriocins as alternative antimicrobials

The production of antimicrobial peptides is not confined to multicellular organisms. Bacteriocins are ribosomally synthesised antimicrobial peptides produced by one bacterium that are active against other bacteria either of the same species (narrow spectrum) or across genera (broad spectrum) (Figure 1.7). It has been suggested that 30-99% of bacteria and archaea make at least one bacteriocin, however, advances in genome analysis will provide a more definite figure (Margaret a Riley and Wertz 2002). Bacteriocins show potential as novel antibiotics to specifically target a narrow spectrum of the bacterial population. This specificity combined with their unmatched potency may enable their use to treat conditions where currently available antibiotics are inadequate. Furthermore, genomic data can be used to identify large numbers of bacteriocin encoding genes in the human microbiota (Zheng *et al.* 2015). Limitations associated with the use of bacteriocins as antimicrobials include instability of a protein therapeutic, antigenic stimulation of the host immune response and also generation of resistance in target species. Despite this, a number of studies have demonstrated good efficacy of bacteriocins *in vivo* for the treatment of a range of bacterial infections. Bacteriocin treatments have been utilised as both bacteriocin producing probiotics and also as purified peptides. Key examples of both will now be discussed.

Figure 1-7: Bacteriocin production by *E. coli* human faecal bacterial isolate.

Faecal *E. coli* isolate was spotted on eosin methylene blue (EMB) agar containing mitomycin C and tested against AIEC LF82 indicator strain. Zones of inhibition are shown as clearing around faecal *E. coli* spots where growth of LF82 has been inhibited.

1.6.1.1 Bacteriocin producing probiotic strains

A number of studies have demonstrated bacteriocins as important colonisation peptides that provide a competitive advantage in high cell density environments such as the human gut (Figure 1.9). Important work of Hillman and colleagues (Hillman 1987) showed a strong correlation between the ability of *Streptococcus mutans* to colonise the human oral cavity and the production of a bacteriocin, mutacin 1140. It was demonstrated that a mutacin producing strain was stably maintained in a human subject, persisting for 14 years following a single administration (Hillman 1987, 2002, Hillman *et al.* 1998). In other work, Gillor and colleagues reported persistence of commensal colicin-producing *E. coli* in the mouse gastrointestinal tract (Osnat Gillor *et al.* 2009). Colicin producing *E. coli* were shown to produce sufficient levels of colicin to outcompete the isogenic non colicin-producing *E. coli* strain and higher numbers of colicin- producing *E. coli* were detected in the mouse GI tract at day 120 (~10^5 CFUs/g faeces) compared to the non-producing isogenic strain (<10^1 CFUs/g faeces) (Osnat Gillor *et al.* 2009).

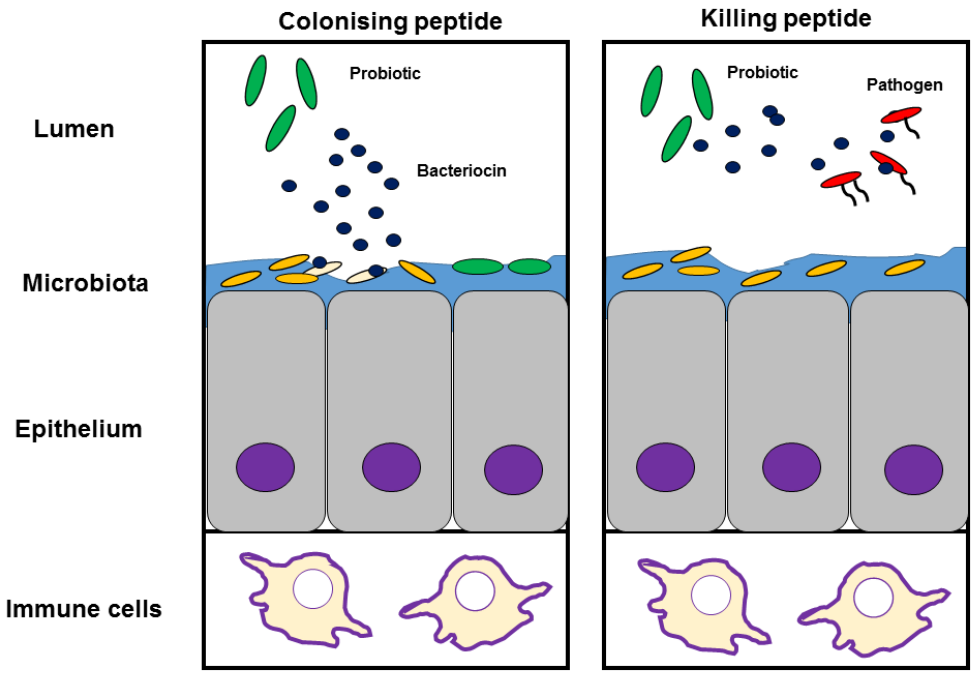


Figure 1-8: The ecological role of bacteriocins in highly populated microbial communities. Figure shows the role of bacteriocins as colonising peptides (left) that are produced by probiotic bacteria for killing of competing microbiota to facilitate colonisation of probiotic producing bacteria. Right panel shows the role of bacteriocins as killing peptides; when produced by probiotic bacteria elicit killing against pathogenic bacteria. Adapted from Dobson *et al.*(Dobson *et al.* 2012).

Other work also demonstrated the anti-infective ability of probiotic bacteriocin producing *Lactobacillus salivarius* strain UCC118 against the food-borne pathogen *Listeria monocytogenes* in the murine GI tract (Corr *et al.* 2007). In this work, mice were infected with *L. monocytogenes* and then orally treated with probiotic *L. salivarius* that produces bacteriocin Abp118. Oral administration of the bacteriocin producing probiotic significantly reduced infection by *L. monocytogenes* that was represented by reduced counts detected in the liver and spleen (Corr *et al.* 2007). This protective effect was abolished when mice received a stable mutant of *L. salivarius* UCC118 that was unable to produce bacteriocin. In a later study, oral administration of *L. salivarius* UCC118 to mice had no significant effect on the major phyla with comprised the microbiota, but increased numbers of Firmicutes were detected (Riboulet-Bisson *et al.* 2012). Other work reported the ability of colicin-producing commensal *E. coli* to inhibit growth of UPEC biofilms on an abiotic catheter surface (Trautner *et al*. 2005). The presence of colicin-producing

E. coli K-12 on the catheter surface prevented catheter colonization by colicin-susceptible *E. coli* but not by colicin resistant *E. coli*. One possible method for the treatment of bacterial infections in humans, is use of probiotic species isolated from the healthy gut environment that produce bacteriocins active against pathogenic bacteria. The ability to engineer specific colicin-producing probiotic bacteria was recently assessed (Trivedi *et al.* 2014). Colicin E2 isolated from a bacteriocin producing vaginal *Lactobacilli* strain was overexpressed in the probiotic strain *Lactobacillus brevis* DT24. This strain showed superior activity against UPEC *E. coli in vitro* compared to wild-type producer strain in overlay spot plate assays (Trivedi *et al.* 2014).

1.6.1.2 Activity of isolated bacteriocins

The ability of bacteriocin producing strains to inhibit pathogenic strains *in vitro* has been extensively investigated. However, there are limited studies demonstrating a correlation between *in vitro* activity and *in vivo* efficacy. This represents a key challenge in the development of bacteriocins as an antimicrobial therapy. However, good activity of a purified lantibiotic bacteriocin, mersacidin, was demonstrated against methicillin-resistant *Staphylcoccus aureus* (MRSA) strain 99308, colonising the mouse nasal epithelium (Kruszewska *et al.* 2004). Intranasal administration of mersacidin reduced *S. aureus* 99308 bacterial counts below the detection limit and also reduced production of pro-inflammatory cytokines (Kruszewska *et al.* 2004). Additionally, bacteriocins isolated from *Paenibacillus polymyxa* and *Lactobacillus salvairius* showed good efficacy against *Campylobacter jejuni* infection in chickens when microencapsulated and incorporated into chicken feed (N J Stern *et al.* 2006, Norman J Stern *et al.* 2005). In this case, the use of the equivalent bacteriocin producing strains was unsuccessful with no detectable reduction in bacterial counts (N J Stern *et al.* 2006, Norman J Stern *et al.* 2005).

The treatment of antibiotic resistant bacterial infections represents a great clinical challenge and is one that current antibiotics frequently fail to overcome. One example of this is ‘superbug’ infection or hospital-acquired *Clostridium difficile* infection (CDI). CDI frequently displays resistance to multiple antibiotics, is very difficult to treat and hence is associated with relapse and increased risk of

mortality. Interestingly, a novel narrow spectrum bacteriocin, thuricin CD, isolated from *Bacillus thuringiensis* showed good activity against CDI in an *ex vivo* human distal colon model (Rea *et al.* 2011). Thuricin CD showed superior killing activity against CDI compared to the commonly used broad spectrum antibiotics vancomycin and metronidazole. Furthermore, unlike both of these broad spectrum antibiotics, thuricin CD did not significantly alter the composition of the wider microbiota (Rea *et al.* 2011). Additionally, work by Smith and colleagues reported activity of purified pyocins against *Pseudomonas aeruginosa* (Smith *et al.* 2012). *P. aeruginosa* forms biofilms that are highly tolerant to antibiotics and is associated with high mortality rates in cystic fibrosis and burns patients. In this work, pyocin S2 displayed superior killing activity against *P. aeruginosa* biofilms compared to the commonly used antibiotics tobramycin and azetronam (Smith *et al.* 2012). Additionally, using the waxmoth larvae model of infection, pyocin S2 also showed good efficacy against *P. aeruginosa in vivo* (Smith *et al.* 2012).

The main conclusions derived from these data are 1) bacteriocins specifically target pathogens and may not significantly alter other populations present in the environment (Rea *et al.* 2014) ; 2) bacteriocin producing strains produce sufficient levels of bacteriocin *in vivo* to eradicate susceptible target strains (Osnat Gillor *et al.* 2009); 3) the presence of bacteriocin producers in the environment can induce desirable alterations in microbial communities (Trautner et al 2005); and 4) in some cases bacteriocins show superior killing activity against bacterial infections compared to broad spectrum antibiotics currently used in clinical practice (Rea *et al.* 2011, Smith *et al.* 2012). Together these properties demonstrate bacteriocins as a putative novel, targeted therapy with highly potent activity.

1.6.2 Bacteriocins of Gram-negative bacteria

The first description of bacteriocin mediated inhibition was reported approximately 90 years ago and showed antagonism between *E. coli* strains (Gratia 1925). Since this original discovery, bacteriocin production has been reported in many bacterial species and is associated with both clinical and environmental isolates. Within a species, tens and even hundreds of bacteriocins are often produced (Margaret a Riley and Wertz 2002). The bacteriocin family includes a vast diversity of proteins in terms of size, targets, modes of action and immunity mechanisms. In general, the Gram-negative bacteriocins are named after the bacterial genus (klebicins of *Klebsiella pneumonia*) or after species (colicins of *E. coli*; cloacins of *Enterobacter cloacae*; marcescins of *Serratia marcesens*) (Cascales *et al.* 2007, Margaret a Riley and Wertz 2002). The bacteriocins produced by *Pseudomonas aeruginosa* are generally termed pyocins (Michel-Briand and Baysse 2002). The diversity of Gram-negative bacteriocins is divided into three groups: 1) large colicin-like bacteriocins (25-80 kDa); 2) smaller sized microcins (<10 kDa); 3) phage tail-like bacteriocins that are large, multimeric peptide assemblies (Margaret a Riley and Wertz 2002). The remainder of this section will focus on the colicin-like bacteriocins, specifically the colicins and pyocins that are the best characterised.

1.6.3 Colicin-like bacteriocins

Colicins that are produced by *E. coli* are the best characterised type of the colicin-like bacteriocins (CLBs). They are described as high molecular weight (30-80 kDa) bactericidal proteins and are produced by a wide range of *E. coli.* Colicins are produced by *E. coli* strains that harbour a colicinogenic plasmid (pCol) (Cascales *et al*. 2007). These strains, termed colcinogenic strains, are widely distributed in nature and are detected in the human gut (Micenková *et al.* 2014). Over 30 bacteriocins from *E. coli* have now been identified and are shown to employ diverse killing activities. Colicins target cells by 1) pore formation that depolarises the inner membrane (e.g colicin A, B, K, N, E1); 2) an endonuclease activity that non-specifically degrades chromosomal DNA (e.g colicin E2, E7, E8, E9); a ribonuclease activity that specifically cleaves *16S* rRNA (e.g colicins E3, E4, E6) or specific tRNAs (e.g colicin D and E5) and 4) inhibition of peptidoglycan synthesis (colicin M) through cleavage of lipid II (El Ghachi *et al.* 2006). Pyocin

genes are chromosomally encoded and are ubiquitous among *Pseudomonas* spp. Three types of pyocins have been classified; F-type, R-type and S-type. In a previous study, R- and F-type pyocins were produced in over 90% of *Pseudomonas aeruginosa* strains and S-type, that are colicin-like bacteriocins, in approximately 70% of tested strains (Michel-Briand and Baysse 2002). S-type pyocins have been shown to kill by DNase activity (pyocin S1, S2, S3, AP41), tRNase activity (S4) or pore-forming activity (S5) (Michel-Briand and Baysse 2002, Nakayama *et al.* 2000, Sano *et al.* 1993). Other examples of colicin-like bacteriocins such as klebicins (*Klebsiella*), alveicins (*Hafnia alvei*) and marcesens (*Serratia marcesens*) are not as well characterised.

1.6.3.1 Colicin-like bacteriocin gene organisation

Colicin-encoding operons are comprised of three tightly linked genes (Cascales *et al.* 2007). The genetic organisation of many known colicin operons has been reviewed elsewhere (MA Riley 1993). Colicin gene operons are comprised of: i) a colicin gene that encodes the toxin; ii) an immunity gene that encodes a protein conferring specific immunity to the producer cell by inactivating the toxin protein and iii) the lysis gene that encodes a protein (known as the lysis protein or bacteriocin release protein) involved in release of colicin i.e lysis of producing cell (Figure 1.9). Nuclease-type colicins are released as heterodimers with their cognate immunity protein and all other colicins are released as monomers (Cascales *et al.* 2007). In nuclease-type colicins, expression of the immunity gene is regulated by the promoter that controls colicin expression and also by its own constitutive promoter that ensures a constant level of immunity protein production that prevents death of the producing cell. This promoter is located within the structural gene for the nuclease-type colicin (Figure 1.9). In pore forming colicins, the immunity protein is located on the opposite DNA strand of the intergenic space between colicin and lysis genes and is transcribed from its own constitutive promoter. Some nuclease colicins (e.g E3) have additional genes within the cluster that provide immunity to other colicins (e.g E8) (Toba *et al.* 1988).
Gene clusters of other pore forming and nuclease colicin-like bacteriocins show a similar organisation to pore forming and nuclease colicin gene clusters (M A Riley *et al.* 2001, Wertz and Riley 2004). For S-type pyocins, genes are also organised

on a single operon, however, only two open reading frames (ORFs) are present. The first encodes the bactericidal or toxin protein and the second encodes the immunity protein. Immunity genes and toxin genes of S-type pyocins are expressed from the same strand similar to the nuclease-type colicins. However, for pyocin S5 a pore forming pyocin, the immunity gene is encoded on the opposite strand to the pore forming colicins. Interestingly, no lysis gene has been identified in pyocin gene clusters (Margaret a Riley and Wertz 2002).

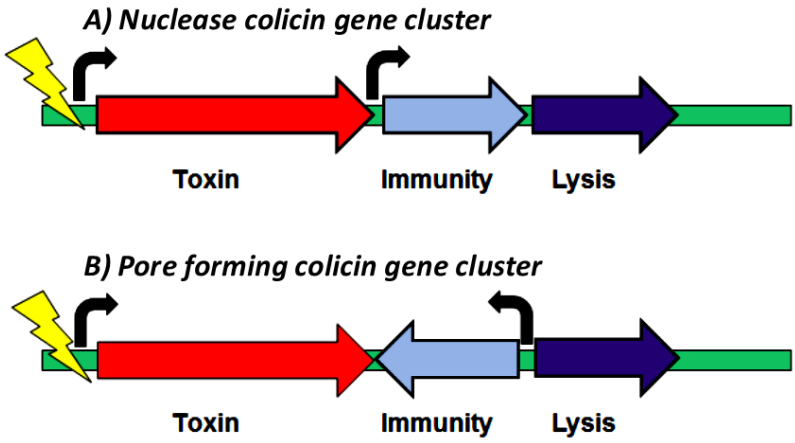


Figure 1-9: Genetic organization of i) nuclease and ii) pore-forming colicins.
The orientation of genes encoding for killing activity, immunity and lysis proteins is depicted with large coloured arrows. The transcriptional starts are indicated by curved arrows. The LexA-binding region is indicated by bolt symbol.

1.6.3.2 Colicin-like bacteriocin expression

Colicin expression is regulated by the SOS induction system mediated by the LexA repressor that binds to the inverted repeat sequence between the promoter and the ribosome binding site (Varley and Boulnois 1984). Briefly, upon DNA damage, the nucleoprotein filament formed by RecA binding to single stranded DNA, stimulates autocleavage of LexA and derepression of LexA regulatory genes (i.e on accessory plasmids such as colcinogenic plasmids) (Kreuzer 2013). The SOS response in *Pseudomonas aeruginosa* is more complex and is coordinated by LexA and two related regulators, PrtR and PtrN. Pyocin operons do not possess a LexA binding box (Margaret a Riley and Wertz 2002). Pyocin production is initiated during the SOS response when autocleavage of PrtR derepresses PrtN.

PrtN then binds to the P-box in the promoter region of pyocin genes to activate their expression (Nakayama *et al.* 2000, Sano *et al.* 1993). The SOS response is triggered in cells during times of stress, more specifically bacteriocin gene expression is swiftly induced leading to very high levels of protein being produced upon DNA damage. Colicin production at the population level was investigated in the early study of Ozeki (1959) (Ozeki *et al*. 1959). This study reported that approximately 0.1% of colcinogenic cells produced colicin under normal conditions and this increased to 50% of the total population after induction by DNA damaging agents (Ozeki *et al*. 1959). A later study showed that 3% of cells in a population produced colicin when induced by nutrient starvation (Mulec *et al.* 2003).

1.6.3.3 Functional domains of colicin-like bacteriocins

Colicins are composed of three domains with each encoding a separate function. These are 1) the receptor binding (R) domain that is involved in binding to a specific surface receptor on the target cell; 2) the translocation (T) domain that mediates transport across the outer membrane by binding to either the Tol or Ton protein complexes); 3) the cytotoxic (C) domain that possesses a specific killing function (Figure 1.10). The S-type pyocins also possess 3 functional domains, however they show different organisation and include an additional domain of unknown function. Organisation of domains for the majority of S-type pyocins from N to C- terminus is; receptor-binding domain, domain of unknown function, translocation domain and the cytotoxic domain (Figure 1.10). Pyocin S1 lacks the second domain of unknown function indicating that it is not essential for the cytotoxic function for this pyocin.

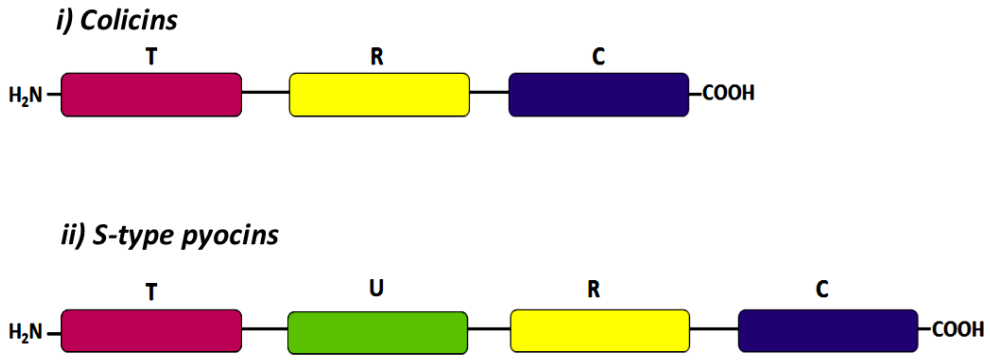


Figure 1-10: Colicin-like bacteriocins show similar organisation of functional domains. Colicin like bacteriocin (CLB) protein domain organization for A) colicins and B) S-type pyocins. Functional domains are translocation domain (*T*), receptor-binding domain (*R*), cytotoxic domain (*C*) and pyocin-specific domain (*U*), of as yet unknown function.

1.6.4 Receptor binding and translocation of colicin-like bacteriocins

1.6.4.1 Colicins translocate the outer membrane via the Tol- or TonB system

The N-terminal translocation and central receptor binding domains of nuclease-type colicins are highly conserved. This reflects the existence of a common delivery apparatus for structurally diverse cytotoxic domains. Colicin N-terminal domains parasitize two distinct and conserved complexes that are essential for the development of the bacterial cell; Tol and TonB systems. Group A colicins (e.g colicin A, E1 to E9, K, N, S4, U and Y) are translocated via the Tol-system and Group B colicins (colicin B, D, H, Ia, Ib, M, 5 and 10) are translocated via the TonB system (JK Davies and Reeves 1975). The Tol and TonB systems are coupled to the proton motive force across the inner membrane and act as energy transducers for outer membrane (OM) stability and active transport, respectively. Colicin translocation is initiated by binding of the colicin to a specific outer membrane receptor on the cell surface.

1.6.4.2 Receptor binding

Group A and group B colicins target *E. coli* cells by interacting with specific outer membrane proteins that are frequently nutrient acquisition proteins. Group A colicins, colicin A and E1- E9 target the TonB-dependent vitamin B_{12} transporter, BtuB (Cascales *et al.* 2007). Colicin K binds the nucleoside transporter Tsx and colicin U binds OmpA. For translocation of these colicins a second OM protein is required (i.e OmpF or TolC for colicin E1). For pore-forming colicins from group A, Cavard showed that target cells lacking BtuB could be killed if incubated in low osmotic strength buffers (Cavard 1976). In this case, OmpF functioned as the sole receptor for pore forming colicins. For group A colicins it is thought that the primary receptor does not function in the process of colicin translocation across the OM. Group B colicins also target TonB dependent transporters in the OM. These include ferric enterobactin receptor, FepA, (colicin B and D) and also ferrichrome receptor FhuA (colicin M). Additionally, colicin Ia and Ib recognise the colicin I receptor, Cir. In these systems only one OM protein is required for both reception and translocation (Jakes and Finkelstein 2010).

1.6.4.3 Tol-dependent colicin translocation

For group A colicins, all but colicin N require two OM proteins for colicin translocation. These proteins are the TonB dependent cell surface receptor as described and either OmpF or TolC. The Tol-system is comprised of 5 proteins that form a multiprotein complex in the cell envelope of most Gram-negative bacteria. The five tol genes are *tolQ, tolR, tolA, tolB* and *pal.* Different group A colicins utilise distinct subsets of Tol proteins for translocation. Colicin A, E2-E9 and K require TolABQR and colicin E1 requires the TolAQ proteins. Structural data for colicin E2 and E3 bound to BtuB suggest a mechanism for colicin translocation across the OM (Kurisu *et al.* 2003, Sharma *et al.* 2007) (Figure 1.11). These data indicate that these colicins do not utilise the central channel of BtuB for outer membrane translocation(Kurisu *et al.* 2003, Sharma *et al.* 2007). Instead, the E-type colicins are proposed to form a complex known as the translocon that is comprised of the outer membrane receptor, BtuB, and OmpF and TolB (Housden *et al.* 2005, Jakes and Finkelstein 2010, Zakharov *et al.* 2008). Translocon

assembly is facilitated by the unstructured region of the translocation domain, termed the intrinsically unstructured translocation domain (IUTD). The IUTD recruits the OM translocator OmpF and delivers an epitope to the periplasm in order to interact with TolB. Group A colicins recruit Tol proteins to the translocon through one or more Tol binding sites in the IUTD (Cascales *et al.* 2007). Tol proteins that are commonly required for group A colicin killing are the IM components of the Tol system; TolQRA. Indeed, the main target is TolA that makes contact with IUTD directly or indirectly via TolB (Bonsor *et al.* 2009).

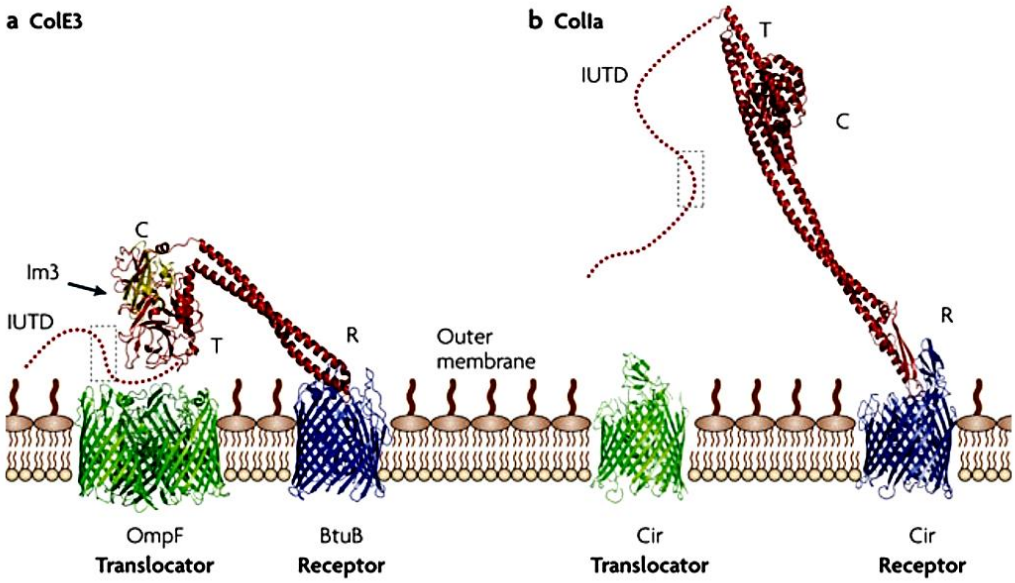


Figure 1-11: Colicin E3 and Ia binding to outer membrane receptor proteins on target cells. The receptor-bound colicins ColE3 (part a) and ColIa (part b) use their amino-terminal intrinsically unstructured translocation domains (IUTDs) to recruit the outer-membrane translocators outer-membrane protein F (OmpF) and colicin I receptor (Cir) respectively. The signalling epitope in ColE3, that is shown bound to its immunity protein Im3, is the TolB-binding epitope, whereas for ColIa the signalling epitope is the TonB box. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology, (Colin Kleanthous 2010), Copyright 2010.

1.6.4.4 TonB dependent colicin translocation.

OM translocation of group B colicins is less defined with the mechanism still unknown (Figure 1.11). The TonB system is comprised of an OM transporter and 3 known inner membrane (IM) proteins; TonB, ExbB, ExbD (Lazdunski *et al.* 1998). The three IM proteins show amino acid and functional similarity to the Tol system; TolA, Q and R. *E. coli* strains that lack functional TonB show tolerance to all group

B colicins. In this system colicins are able to bind OM transporters but are unable to translocate into the periplasm (JK Davies and Reeves 1975). Structural data for colicin IA bound to its receptor Cir, suggests a mechanism for group B colicin translocation (Buchanan *et al.* 2007). Following receptor binding colicin Ia recruits another copy of its receptor to capture TonB. For translocation, a short sequence located in the nutrient transporter termed the TonB box is also detected in the IUTC domain. Under normal conditions, binding of the TonB box by the specific nutrient leads to TonB recruitment from the periplasm and therefore opening of central pore in transporter. The presence of the TonB box in both the transporter (Cir) and colicin IA IUTD is required for translocation of colicin Ia (Buchanan *et al*. 2007, Jakes and Finkelstein 2010).

1.6.5 Immunity proteins of colicin-like bacteriocins

Colicin-like bacteriocins are targeted against species closely related to the producing strain. Due to this, both the target cells and producing cells possess the import apparatus required for binding and translocation of the bacteriocin. Therefore, in order to prevent killing of the producing cell, an effective mode of immunity is utilised to prevent cell suicide. This mode of immunity occurs in the form of a small (~10 kDa) protein, termed the immunity protein (Espesset *et al.* 1994). The immunity protein is highly specific for each type of colicin (Cascales *et al.* 2007). Two distinct types of immunity proteins are detected for nuclease and pore-forming colicins that function by unique mechanisms. This section will briefly summarise the function of immunity proteins for both nuclease and pore forming colicins.

1.6.5.1 Nuclease colicins

The target of nuclease colicins is located in the cytoplasm of target cells. In this case, the immunity protein forms an high affinity complex with the cognate colicin in the producing cell that neutralises its catalytic activity (Cascales *et al.* 2007). The affinity of colicin E9 binding to its immunity protein, Im9, has been found to be in the femtomolar range. This is recognised as one of the strongest associations observed for a complex of two proteins (Wallis *et al.* 1995). Immunity proteins of nuclease colicins bind at the cytotoxic domain of colicin but at a site distinct from

the active site (C Kleanthous and Walker 2001). The immunity protein binding region of the cytotoxic domain is the most sequence variable region of this functional domain that is thought to permit the evolution of novel binding specificities in conjunction with the maintenance of the catalytic activity of the toxin (C Kleanthous and Walker 2001). The interaction of enzymatic colicin E3 with its cognate immunity protein was revealed in previous work that proposed that bipartite recognition of the immunity protein by colicin facilitates disassociation of immunity protein from the toxin on binding to a target cell (Walker *et al.* 2003). It was shown that binding of colE3 to BtuB, via the receptor binding domain triggered translocation of the T-domain into the periplasm where it forms a complex with TolB. The affinity of the toxin for the immunity protein is now reduced allowing it to dissociate from the toxin. This triggers translocation of the cytotoxic domain into the periplasm and ultimately the cytoplasm (Walker *et al.* 2003). The colicin E9 immunity protein, Im9, binds with very high affinity and specificity to E9 DNase domain (K_d - $2.4x10^{-14}$ M). Energy input in the form of the cytoplasmic membrane proton motive force has been shown to promote Im9 release from the colicin E9/Im9 complex and initiate OM translocation of DNase domain (Bonsor *et al.* 2009). Recent work has indicated that this cellular energy is used for unfolding and structural rearrangement required for membrane translocation (Vankemmelbeke *et al.* 2013).

1.6.5.2 Pore-forming colicins

The immunity proteins of pore forming colicins are integral membrane proteins with several transmembrane spanning regions (Mankovich *et al.* 1986). The cytotoxic function of pore-forming colicins is inhibited through direct interaction in the inner membrane. Little is known about the immunity protein residues involved in recognition of the lethal domains of pore-forming colicins (Cascales *et al.* 2007, YL Zhang and Cramer 1993). Immunity proteins of pore forming colicins are classified into two distinct groups respective of their sequence homologies: type A) immunity proteins for colicins A, B, N, and U; type E1) (immunity proteins for colicins E1, 5, K, 10, Ia, and Ib (Cascales *et al.* 2007, Espesset *et al.* 1994, Song *et al.* 1991). Data obtained in several independent studies over the last two decades has suggested that prior to formation of pore, the A-type immunity

proteins diffuse laterally in the membrane and interact with their cognate hydrophobic helical hairpins (Espesset *et al.* 1994, Pilsl and Braun 1995, Pilsl *et al.* 1998). For E1-type immunity proteins, a similar mechanism is proposed but they are thought to interact with the voltage responsive segment of the E1-type colicin instead of the hydrophobic helical hairpin (Lindeberg and Cramer 2001, Pilsl and Braun 1995).

1.7 Aims and Hypothesis

1.7.1 Hypothesis

The hypothesis for this work is that colicins would show good activity against AIEC strains associated with CD. We hypothesised that these potent and narrow-spectrum antibiotics would display good efficacy against AIEC infection *in vitro* and *in vivo* and hence may represent a potential antimicrobial therapy for CD patients that display gut dysbiosis involving enriched mucosa-associated *E. coli*.

1.7.2 Aims

The aim of this work is to demonstrate the potential of colicins as novel antimicrobial therapeutics for the treatment of CD-associated AIEC infection. This aim will be achieved by:

1) Assessing the efficacy of colicins against AIEC in vitro.

- AIEC adhere to and invade intestinal epithelial cells and survive within macrophages, inducing production of pro-inflammatory cytokines. Investigation of colicin activity against AIEC associated with mammalian cells will provide good insight into the efficacy of colicins against this distinct *E. coli* pathotype.

2) Assessing the efficacy of colicins against AIEC in vivo.

- AIEC abnormally colonise the gut mucosa of a portion of CD patients and this is associated with disease pathogenesis. Investigating colicin activity against AIEC growing in the murine gastrointestinal tract will demonstrate the efficacy of colicins as a future antimicrobial therapy against intestinal infection.

3) Assess the prevalence of AIEC-associated phenotypes in E. coli isolated from HLA-B27 (+) juvenile idiopathic arthritis (JIA) patients

- JIA is a juvenile spondyloarthropathy (SpA). SpAs, associated with HLA-B27 genotype, demonstrate clinical overlap with CD. Gut dysbiosis involving increased *E. coli* is associated with the HLA-B27 genotype. To ascertain the association of AIEC with HLA-B27 genotype, the prevalence of AIEC in HLA-B27 (+) patients will be investigated. This work will demonstrate the wider applicability of colicins against AIEC overgrowth in other inflammatory gut-associated disorders.

2 Materials and methods

2.1 Chemicals, growth media and strains

2.1.1 Chemicals

All chemicals used in this study were of analytical grade and were purchased from Sigma-Aldrich, Thermo Scientific, Invitrogen or Melford unless otherwise stated.

2.1.2 Strains

The bacterial strains used in this study are described in Table 2.1

Table 2-1: Strains used in this study

Strain	Genotype or description	Reference or source
LF82	AIEC reference strain. Isolated from ileal biopsy of CD patient.	Kindly donated by Arlette Darfeuille-Michaud (A Darfeuille-Michaud *et al.* 2004)
LF82-GFP	WT LF82 strain harbouring pDM15 plasmid that encodes *gfpmut2* gene	Constructed by Karen Smith, Walker lab
LF82::Strp^R	Streptomycin resistant WT LF82 strain that harbours a K42T mutation in *rpsL* that confers streptomycin resistance. Strain also harbours chromosomal integration vector containing a synthetic lux operon at *16S* rRNA region that confers erythromycin resistance.	Kindly donated by Marjenberg and Douce (University of Glasgow)
HM95	Colonic mucosally-associated AIEC strain isolated from ileal CD patient	University of Liverpool (Martin *et al.* 2004)
HM154	Colonic mucosally-associated AIEC strain isolated from colonic CD patient.	University of Liverpool (Martin *et al.* 2004)
HM419	Colonic mucosally-associated AIEC strain isolated from ileo-colonic CD patient.	University of Liverpool (Martin *et al.* 2004)

HM580	Colonic mucosally-associated AIEC strain isolated from colonic CD patient	University of Liverpool (Martin *et al.* 2004)
HM605	Colonic mucosally-associated AIEC strain isolated from colonic CD patient	University of Liverpool (Martin *et al.* 2004)
HM615	Colonic mucosally-associated AIEC strain isolated from colonic CD patient	University of Liverpool (Martin *et al.* 2004)
DH5α	*F^-, φ80dlacZΔM15, Δ (lacZYA-argF) U169, deoR, recA1, endA1, hsdR17(rk-, mk+), phoA, supE44, λ thi-1, gyrA96, relA1*	Invitrogen
BL21(DE3)pLysS	F^- *ompT hsdS*B (rB^-mB^-) *gal dcm* (DE3) pLysS (Cam^R)	Invitrogen
K-12 W3110	F- lambda- IN(*rrnD-rrnE*)1 *rph-1*	(Bachmann 1972)
F18	Commensal *E. coli* strain isolated from human intestine	Kindly donated by Donal Wall (University of Glasgow) (Wall *et al.* 2007)
BZB2101	*E. coli* K12 W3110 (Wild type) *gyrA,* pColA-CA31	(Pugsley 1985)
BZB2102	*E. coli* K12 W3110, pColB-K260	(Pugsley 1985)
BZB2104	*E.coli* K12 W3110, pColE1-K53, Tra^- Mob^+	(Pugsley 1985)
BZB2108	*E. coli* K12 W3110, pColE5-099 Tra^-, Mob^+	(Pugsley 1985)
BZB2103	*E. coli* K12 W3110, pColD-CA23	(Pugsley 1985)
BZB2123	*E. c*oli K12 W3110 *gyrA,* pColN-284, Tra^- Mob^+	(Pugsley 1985)
BZB2114	*E. coli* K12 W3110 *gyrA,* pColIa-CA53, Tra	(Pugsley 1985)
BZB2115	*E. coli* K12 W3110 *gyr,* pCollb-P9drd	(Pugsley 1985)
BZB2125	*E. coli* K12 W3110 *gyrA,* pColE2-P9	(Pugsley 1985)

2.1.3 Growth media

Lysogeny broth (LB) was prepared in dH_2O using the recipe shown and was adjusted to pH 7.5 and sterilised by autoclaving (Table 2.2). MacConkey agar and EMB agar were purchased and were prepared as per manufacturer's instructions (Sigma-Aldrich).

Table 2-2: Growth media used for cultivation of bacterial strains and mammalian cell lines in this study.

Media	Composition / Source	Sterilisation
Lysogeny broth (LB)	10 g Tryptone, 10 g NaCl, 5 g yeast extract, 1 L dH_2O	Autoclave at 121ºC for 15 min
LB agar	10 g Tryptone, 10 g NaCl, 5 g yeast extract, 15 g nutrient agar, 1 L dH_2O	Autoclave at 121ºC for 15 min
Eosin methylene blue agar	Sigma	Autoclave at 121ºC for 15 min
MacConkey agar	Sigma	Autoclave at 121ºC for 15 min

2.2 Bacteria cultivation techniques

2.2.1 Storage of bacteria

One millilitre aliquots of frozen bacterial stocks were made by adding 500 µl of glycerol to 500 µl of an overnight LB culture and storing in cryo tubes at -80°C.

2.2.2 Growth of bacteria

All strains were inoculated into LB or onto agar plates with the appropriate antibiotics. When required, ampicillin was added at 100 µg ml^{-1}, kanamycin was added at 50 µg ml^{-1}, streptomycin added at 100 µg ml^{-1} and erythromycin at 500 µg ml^{-1}. Cultures were propagated from a single colony and grown at 37°C with shaking.

2.3 Molecular techniques

2.3.1 Extraction of genomic DNA

Bacterial cells were pelleted from 1 ml of overnight LB cultures grown at 37°C. Genomic DNA (gDNA) was extracted using the Qiagen QIAamp DNA Mini kit (Qiagen, UK), as per the manufacturer's instructions. DNA was resuspended in 200 µl of distilled water and stored at -20°C until required. DNA was quantified using a Nanodrop spectrophotometer

2.3.2 Preparation of plasmid DNA

Bacterial cells were pelleted from 5 ml of overnight LB cultures grown at 37°C. Plasmid DNA was extracted using the QIAprep Spin Miniprep kit (Qiagen, UK), as per the manufacturers instructions. DNA was resuspended in 30 µl of elution buffer (10 mM Tris-HCl, pH 8.5) and stored at -20°C until required.

2.3.3 Polymerase chain reaction

A typical PCR used 1 µl Pfu turbo (Agilent, UK) with 0.2 µM forward and reverse primers, 100 ng genomic DNA and 200 µM dNTP mix in a final volume of 50 µl.

reaction. The standard PCR protocol used in this study is shown in Table 2.3. If initial conditions did not yield the desired product the reaction was optimised by the inclusion of 5 - 10% dimethylsulfoxide (DMSO) and variation of the annealing temperature.

For colony PCR a single bacterial colony was resuspended in 25 µl of distilled water, and incubated at 96°C for 5 min. The lysate was centrifuged and 5 µl of the supernatant was added to 95 µl of water and 1 µl was used as DNA template in the PCR reaction described above.

Table 2-3: Standard PCR protocol

Step	Temp (°C)	Time (s)	Cycles
Initial denaturation	95	120	1
Denaturation	95	30	
Annealing	58	30	18-30*
Extension	72	60 / kb	
Final extension	72	600	1
Hold	4	-	

*30 cycles used for standard PCR and 18 cycles used for PCR mutagenesis.

2.3.4 Primers

All primers used in this study were synthesised by Eurofins MWG Operon. Primers listed in Table 2.4 were used to amplify DNA. Appendix A shows DNA sequence for colicin E9H575A mutant.

Table 2-4: Primers used in this study

Name	Restriction enzyme site	Sequence 5' to 3'
Colicin E9H575AF (mutagenesis)	*NcoI*	ACT ACA CCT AAG CGA GCT ATC GAT ATT CAC CGA GGT AAG
Colicin E9H575AR (mutagenesis)	*XhoI*	CTT ACC TCG GTG AAT ATC GAT AGC TCG CTT AGG TGT AGT
Colicin E9-RFPF	*NcoI*	GTT GAC GTC CAT GGC CTC CAC CGA GGA CGT CAT CAC CGA)
Colicin E9-RFP	*XhoI*	CCA CTG TGC TCG AGC AGG AAC AGG TGG TGG CGG CCC TCG
88F	-	AYTGGGYDTAAAGNG
91R	-	CCCGTCAATTYYTTTRAGTTT

Y, C/T; N, A/T; R, A/G

2.3.5 PCR mutagenesis

Constructs to express the colicin E9 mutant H575A were created using the QuikChange Site Directed Mutagenesis kit (Stratagene) as per manufacturer's instructions, utilising pCS4 as template and E9H575AF and E9H575AR mutagenesis primers (See Appendix A for peptide sequence) (Garinot-Schneider *et al.* 1996).

2.3.6 *Agarose gel electrophoresis*

For a 0.8% (w/v) agarose gel, 0.8 g of agarose was added to 100 ml 100mM Tris-Hydrochloride, pH 8.2 / 7.7 mM Borate/ 2.5 mM ethylenediaminetetraacetic acid (EDTA) (TBE) buffer. This was heated until the agarose dissolved and allowed to cool to approximately 42ºC. GelRED gel stain (Cambridge Biosciences, UK) was added 1:10,000 and the gel poured into the gel tray to set. The appropriate volume of 6x loading buffer (10 mM Tris-HCl pH 7.6 0.03% bromophenol blue, 60% glycerol, 60 mM EDTA was added to DNA samples prior to loading and the gel was run at 200 mA for 45 min. The 100bp or 1 kb^+ size marker (New England Biolabs Inc (NEB), UK) was used to determine the size of sample bands and gels were visualised using an UVIpro gold Uvitec transilluminator (Uvitec, UK).

2.3.7 Restriction enzyme digest

A typical digest reaction consisted of 1 µg of prepared DNA, restriction enzyme digestion buffer, 10 U restriction enzymes and distilled water to final volume of 25 µl. The reaction was mixed and incubated at 37ºC for 1 hr. The digest products were subjected to agarose gel electrophoresis to visualise and purify fragments.

2.3.8 DNA gel purification

Bands of interest were excised from 0.8% agarose gels and were purified using the Qiagen Gel Extraction Kit (Qiagen, UK), as per the manufacturer's instructions. The purified DNA was eluted into 30 µl of elution buffer (10 mM Tris-HCl, pH 8.5) and stored at -20°C.

2.3.9 DNA sequencing and identification of bacterial strains

DNA for sequencing was prepared using Qiagen Gel Extraction Kit (Qiagen, UK) as described. Sequencing reactions required 50-200 ng of DNA and were performed by Source Bioscience Sequencing (Glasgow; UK). For sequencing of *16S* rRNA and identification strains, nucleotide sequences were compared with other strains by using NCBI BLAST analysis.

2.3.10 Ligation reaction

A typical ligation reaction consisted of 50 ng digested plasmid, 10x DNA ligase buffer and 5 U T4 DNA ligase (NEB; UK). Insert was added at a 1:1 and 1:3 molar ratio (plasmid: insert). The reaction was incubated at room temperature overnight and then used in transformation reactions.

2.3.11 Transformation of *E. coli*

Five microlitres of ligation reaction or 1 µl of plasmid was added to 50 µl of chemically competent *E. coli* BL21 (DE3) pLysS, Top 10 or DH5α cells and incubated on ice for 30 min. Cells were then heat shocked at 42°C for 45 s or 30 s (Top 10) and then placed on ice for 5 min. Five hundred microlitres of LB broth was added to the cells and incubated shaking at 37°C for 1 h. The cells were then plated onto LB agar plates containing appropriate antibiotic and incubated overnight at 37°C.

2.3.12 Plasmids

The plasmids used in this study are described in Table 2.5.

Table 2-5: Plasmids used in this study.

Plasmids	Description	Reference or source
PCs4	Colicin E9-Im9 expression plasmid, Amp^R, IPTG-inducible T7 promoter	(Garinot-Schneider *et al.* 1996)
pDM15	Kan^R expression vector, Cp25 promoter, encodes *gfpmut2* gene	Kindly donated by John Butcher, University of Glasgow
pAR5	Encodes RFP-tagged colicin constructed by amplification of the RFP gene using primers RFPF and RFPR	Kindly donated by Angela Rinaldi, Walker lab, Univeristy of Glasgow

2.4 Microbiology

2.4.1 Overlay spot plate method

Soft agar overlay spot plates were performed using the method of Fyfe *et al.* (Fyfe *et al.* 1984). One hundred and fifty microlitres of log phase test strain culture (OD_{600}= 0.6) was added to 6 ml of 0.8% soft agar and overlayed on LB agar plate. Five microlitres of purified colicin, at varying concentrations, was spotted onto overlay plates and incubated for 18 h at 37°C. Clear zones indicate killing. Colicin production of *E. coli* strains was assessed via adapted overlay spot plate method. Five µl of log phase test strain culture (OD_{600} = 0.6) was spotted onto an LB agar plate containing mitomycin C (0.75 µM) and incubated overnight at 37°C. The cells were lysed using chloroform and the chloroform allowed to evaporate before 50 µl of the required indicator strain culture (adjusted to OD_{600} = 0.6) was added to 5 ml of soft agar (0.8% agar in distilled water) and poured over the LB agar plate (containing the lysed cells). Plates were incubated overnight at 37°C and diameter of inhibition zones was measured.

2.4.2 Minimum inhibitory concentration assay

MIC assay with serial broth microdilution was used to determine antimicrobial susceptibility, in sterile 96-well plates (Corning). In the first column, a starting concentration of test compound was prepared in LB (180 µl) in triplicate. Serial 2-fold dilutions were performed for each test compound in subsequent columns. Overnight cultures were diluted in sterile PBS to OD_{600}= 0.6. The bacterial cultures were then diluted 1000-fold in LB and 20 µl of the final solution was added to each well of the 96-well plate within 30 min of inoculum preparation. The microtitre plates were then wrapped in parafilm and incubated at 37°C for 18 – 24h. The lowest concentration at which there was no visible growth (the first clear well), was taken as the MIC of the test compound.

2.5 SDS-PAGE and silver staining

Samples were mixed 4:1 in sample buffer (62.5 mM Tris-HCl pH 6.8, 2.5% sodium dodecyl sulphate (SDS), 0.002% bromophenol blue, 0.7135 M (5%) β-mercaptoethanol, 10% glycerol), boiled at 95°C for 10 min and 15 µl loaded onto a sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) of the appropriate size (typically 12% or 18% gels). Broad range protein markers (2 - 212 kDa, NEB, UK) or broad range ColourPlus Prestained protein markers (10 - 230 kDa, NEB, UK) were used throughout. SDS-PAGE gels were stained using Coomassie stain (500 ml dH_2O, 400 ml methanol, 100 ml acetic acid, 0.5 g Coomassie blue R250) and destained with destain solution (500 ml dH_2O, 400 ml methanol, 100 ml acetic acid).

2.6 Western blotting

For preparation of protein samples, log phase cultures (OD_{600} = 0.6) were centrifuged at 1700 g for 10 min and cell pellets were resuspended in PBS and heated at 65°C to liberate cell surface Ag43α domain. Following SDS PAGE (10% polyacrylamide) proteins were transferred from the gel onto nitrocellulose membranes using an ECL semi dry transfer unit at 30 mA for 1 h. After the transfer the membrane was blocked in blocking buffer consisting of 20 mM Tris HCl, 200

mM NaCl, 0.01% (v/v) Tween, 5% (w/v) skimmed milk (Marvel; UK) for 2 hours at room temperature. A polyclonal rabbit antiserum raised against the α-domain of Ag43 (1:2000) was applied to the nitrocellulose membrane for 1 h at room temperature and agitated. Excess unbound antibody was washed from membrane using 20 mM Tris HCl, 200 mM NaCl, 0.01% (v/v) Tween. The membrane was then incubated with a goat anti rabbit secondary antibody (1:10,000) coupled with horseradish peroxidase (HRP) for 1 h shaking at room temperature and washed as described above. The blot was developed using enzyme-linked chemiluminescence (ECL) with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, UK).

2.7 Protein purification

2.7.1 Overexpression and purification of recombinant colicin protein E9

Tandom overexpression of colicin E9 in complex with it's His_6-tagged immunity protein (Im9) was performed as previously described (Garinot-Schneider *et al.* 1996). Colicin E9-Im9His_6 was overexpressed from *E.coli* BL21 (DE3) cells carrying the plasmid pCS4. 5 L of LB broth was inoculated (1:100) and overnight culture and cells were grown at 37°C in shaking incubator to an OD_{600} =0.6. Protein production was induced by addition of 1 mM IPTG and cells were grown for further 4 h and harvested by centrifugation. The cell pellet was re-suspended in 200 mM Tris-HCl and 500 mM NaCl containing 10 mM imidazole pH 7.5. Cells were lysed using MSE Soniprep 150 (Wolf laboratories, UK) and cell debris separated by centrifugation. The cell-free lysate was applied to a 5 ml His trap™ HP column (GE healthcare, USA) and the protein eluted over a 0-350 mM imidazole gradient. E9-Im9 (His6) containing fractions were dialysed overnight into 50 mM Tris-HCl, 200 mM NaCl and the protein was further purified by gel filtration chromatography on a Superdex S75 26/60 column (GE Healthcare) equilibrated in 50 mM Tris-HCl, 200 mM NaCl pH 7.5. The protein was stored at -20°C in this buffer until required. E9H575-Im9 (His6) was purified as described above. Purification of E9-RFP was performed by Angela Rinaldi (Walker lab; Appendix B).

2.7.2 Purification of colicin E1

Purification of colicin E1 was performed as previously described (SA Schwartz and Helinski 1971). *E. coli* BZB2104 (pColE1-K53) was sub-cultured overnight in LB broth at 37˚C. The culture was used to inoculate 6 x 625 mL LB culture that was grown to log-phase (OD_{600}=0.6) and then induced with mitomycin C (0.4 µM) and incubated at 37˚C for 18 h. The cultures were collected by centrifugation at 6360 g for 30 min and supernatant was retained. The supernatant was re-suspended in ammonium sulphate (404 g/L) for 45 min at 4°C and centrifuged as described previously. The pellet was re-suspended in 50 mM Tris 200 mM NaCl buffer pH 7.5. The sample was collected in a porous membrane and dialysed for 18 h in 5 L of 50 mM Tris pH 8.0 at 4ºC. The next morning the sample was dialyzed in fresh buffer for 3h and cell debris was removed by centrifugation for 20 min at 6360 g and 4˚C. The protein was purified using anion exchange column constructed following manufacturers guidelines (DE52 preswollen microgranular DEAE cellulose, Whatman) and eluted on a 0-1 M NaCl gradient using an Akta-purifier chromatography system (GE Healthcare, USA) The protein was detected in elution flow through that was concentrated by centrifugation using Vivaspin® centrifugal concentrator (GE Healthcare Life Sciences) and further purified by gel filtration chromatography on a Superdex S75 26/60 column (GE Healthcare) equilibrated in 50 mM Tris-HCl, 200 mM NaCl pH 7.5. The protein was stored at -20°C in this buffer until required. The total protein was evaluated by SDS-PAGE (12% polyacrylamide) (Appendix C).

2.7.3 Lipopolysaccharide removal from purified proteins

Removal of lipopolysaccharide (LPS) from colicin protein was performed using polymyxin B-agarose columns (Detoxi Gel, Pierce) according to manufacturer's recommendation. One ml of purified colicin E9 (3 mg ml^{-1}) was loaded onto each 0.5 ml Detoxi column and incubated at room temperature for 60 min. The protein was then eluted with endotoxin free water in 1 ml fractions. Fractions were pooled and stored at -20°C.

2.8 Tissue culture

2.8.1 T84 cells

The T84 colon carcinogen cell line obtained from the American Type Culture Collection (ATCC #CCL-248) was maintained in a 50:50 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Hams F-12 Medium (both Sigma) supplemented with 10% FBS, 2 mM L-glutamine, 15 mM HEPES buffer (pH 7.5), 14 mM sodium bicarbonate and 2% penicillin (200 U/ml) /streptomycin (200 µg ml^{-1}) (Sigma-Aldrich). Media was changed every 2 days and cells were grown for 6-9 days before being used in assays. Confluent T84 monolayers were subcultured by 0.25% trypsin + EDTA (Invitrogen) treatment. Experiments that required infection of T84 cells were performed using antibiotic-free medium. T84 cells were passaged after reaching 70% confluency.

2.8.2 RAW 264.7 cells

RAW 264.7 macrophages obtained from the European Collection of Authenticated Cell Cultures (ECACC # 91062702) were cultured in RPMI-1640 media (Sigma) supplemented with 10% FBS, 2 mM L-glutamine and 2% penicillin (200 U/ml) /streptomycin (200 µg ml^{-1}) (all Sigma). RAW 264.7 cells were maintained at 37°C at 5% CO_2. The media was replaced daily. When cells reached 90% confluency, old media was replaced and cells were split using cell scrapers (Invitrogen) that were used to dislodge cells from the bottom of the flask. The cells were counted using a haemocytometer and then seeded at the appropriate density into a new flask. The cell line was used between 3-30 passages.

2.8.3 J774.1 cells

J774A.1 mouse BALB/c macrophages (ECACC # 91051511) were grown in RPMI-1640 media supplemented with 10% FBS, 2% penicillin (200 U/ml) /streptomycin (200 µg ml^{-1}) and 2 mM L-glutamine (all Sigma). Cells were kept in a humidified incubator at 37°C with 5% CO_2. Media was replaced daily. When cells reached 90% confluency, old media was replaced and cells were split using cell scrapers (Invitrogen) that were used to dislodge cells from the bottom of the flask. The cells

were counted using a haemocytometer and then seeded at the appropriate density into a new flask. The cell line was used between 3-30 passages.

2.9 Colicin treatment of mammalian cells

2.9.1 Intracellular colicin killing assay

Investigation of colicin activity against intramacrophagic LF82 was measured by the gentamicin protection assay (Falkow *et al.* 1987). RAW 264.7 macrophages (2 x 10^5 /well) were seeded on to 24-well plates (Corning) and were grown for 18 h to confluency. Prior to infection of cells, bacteria were washed and re-suspended in antibiotic-free RPMI-1640 media and added to cells at a multiplicity of infection (MOI) of 50. After 2 h infected macrophages were washed with PBS and exposed to RPMI media containing gentamicin (100 µg ml^{-1}) to kill extracellular bacteria that had failed to invade macrophages. After 1 h, the media was removed and macrophages were then treated with culture media containing antimicrobial treatments in the presence of gentamicin (20 µg ml^{-1}) at 37°C, 5% CO_2. For LF82 growth curves, media was free from antimicrobial treatments and was replaced every 24 h. Antimicrobial treatments used were colicin E9, colicin E1 and the antibiotic ciprofloxacin (100 µg ml^{-1}). After incubation with antibiotic for desired time point, RAW264.7 cells were washed three times with sterile PBS. If colicin was added, macrophages were also treated with trypsin (0.25%) in EDTA for 5 min at 37°C. This was to ensure all macrophage bound colicin protein was inactivated. The macrophages were then scraped from the surface of the plate and lysed with 2% Triton X-100 for 5 min. Recovered intracellular bacteria were quantified by plating serial dilutions on LB agar containing ampicillin (50 µg ml^{-1}). The plates were incubated overnight at 37°C and CFU counts were performed. Bacterial survival in RAW 264.7 cells (% survival) were calculated as a % of viable internalised bacteria relative to the number recovered from untreated cells infected with LF82, considered as 100%.

2.9.2 Colicin killing of cell-associated *E. coli*

To assess efficacy of colicin against LF82 associated with T84 monolayers, adhesion assays were performed. T84 cells were grown in 24 well plates for 3-5

days at 37°C in 5% CO_2 until an epithelial monolayer was formed. Prior to adhesion assays T84 cell monolayers were washed with sterile PBS. LF82 were grown to OD_{600} = 0.6 in LB broth and resuspended in DMEM culture and added to cells at a multiplicity of infection (MOI) of 10 for 3 h at 37°C, 5% CO_2. To assess antimicrobial killing of adherent bacteria, colicin E9 and the antibiotic ciprofloxacin were diluted in DMEM culture media to concentrations of 100 µg ml^{-1} and added to separate wells at 15, 30, 45 and 60 min post infection. After 3 h, monolayers were washed three times with sterile PBS to remove non-adherent bacteria and treated with trypsin (0.25%) in EDTA for 10 min at 37°C. The cell suspension was removed by aspiration and homogenised by passing through a 22-gauge needle. Cells were lysed by sonication and cell-associated LF82 were serially diluted and plated on LB agar plates containing ampicillin (50 µg ml^{-1}). Plates were incubated overnight at 37°C and CFU counts were performed. All adhesion assays were repeated in triplicate.

To assess inhibition of LF82 adhesion by colicin producing commensal *E. coli*, co-culture adhesion assays were performed using LF82 and *E. coli* BZB2104 (pColE1-K53). For co-culture assays, bacteria were added in equal volumes at a MOI of 10 for 3 h and number of cell-associated LF82 were determined as stated before.

2.9.3 Photomicroscopical analysis of T84 cell morphology

For photomicroscopy T84 cell monolayers were grown on glass coverslips in 6 well plates (Corning) for 5-7 days. Cells were infected with LF82 (MOI of 10) and when required were treated with colicin E9 (100 µg ml^{-1}) 15 min post infection. After 3 h monolayers were washed three times with sterile PBS and fixed with 10% methanol in PBS for 10 min. Cells were washed again in sterile PBS as above and then Giemsa stained (20% (v/v) Giemsa in dH_2O) for 20 min at room temperature. Cover slips were washed and mounted on slides using clear nail varnish solution. Images were captured using Zeiss Axioskop attached to QImaging Micropublisher 3.3 RTB camera at 40 x magnification.

2.9.4 Confocal microscopy

In order to visualise colicin and bacteria associated with cells, RAW 264.7 macrophages were seeded (2 x 10^5/well) on glass cover slips in 6 well plates

(Corning) and grown to confluency for 18 h at 37°C. Culture media used for infection contained 50 µg ml^{-1} kanamycin to select for growth of the GFP-expressing LF82 strain. Where required, macrophages were infected with LF82-GFP (MOI of 50) for 2 h and exposed to culture medium (RPMI-1640, FBS, L-glutamine and kanamycin) containing gentamicin (100 µg ml^{-1}) for 1 h to ensure killing of extracellular bacteria. Cells were treated with culture media containing colicin E9-RFP (100 µg ml^{-1}) and gentamicin (20 µg ml^{-1}) for the required time. Infected cells were washed three times in sterile PBS and treated with 0.25% trypsin in EDTA for 5 min at 37°C to inactive extracellular colicin E9-RFP. RAW 264.7 macrophages were washed as above to remove macrophage bound protein and fixed with 2% paraformaldehyde (PFA) in PBS and 0.5 M sucrose for 10 min at room temperature. Fixed cells were permeabilised with 0.5% Triton X-100 for 3-5 min at room temperature. Actin cytoskeleton was stained using Alexa-647 labelled phalloidin (Life Technologies) and nuclear DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies). Cover slips were air dried and mounted on slides with Dako Mounting Medium (Dako UK). Slides were examined using a Zeiss LSM410 Laserscan Microscope equipped with periphera argon-UV laser. Image processing was performed using the LSM Image Browser software (Zeiss).

2.9.5 Drug treatment of macrophages

The following drugs were purchased from Sigma Aldrich and used as described previously (Marina-García *et al.* 2009). Cytochalasin B, that blocks phagocytosis, was used at 10 µg ml^{-1}; polyinosinic acid, that blocks scavenger receptor mediated endocytosis was used at 50 µg ml^{-1}; dynasore that blocks clathrin mediated endocytosis was used at 80 µM; dimethylamyloride (DMA) that blocks pinocytosis was used at 500 µM and mannan that blocks mannose receptors was used at 1 mg ml^{-1}. RAW 264.7 cells were treated with specific drug for 30 mins and then treated with colicin E9-RFP for 4 h and visualised by confocal microscopy as stated.

2.9.6 Enzyme linked immunosorbent assay (ELISA) for detection of tumour necrosis factor-α

RAW 264.7 macrophages were infected and treated as described. At various time points, post-infection supernatants were collected and assayed for TNF-α by cytokine ELISA kits (BioLegend). Optical density was determined at wavelength 450nm and cytokine concentration was determined as by manufacturer's instructions.

2.9.7 Lactate dehydrogenase hydrogenase cytotoxicity assay

For RAW 264.7 macrophages cytotoxicity was determined by LDH release. Lactate dehydrogenase (LDH) activity as a measure of cytoxicity was measured in cell supernatants according the manufacturer's protocol (LDH Cytotoxicity Assay Kit, Abcam). LDH release was measured colormetrically according to manufacturer's protocol. % Cytotoxicity = (Compound treated LDH activity – Spontaneous LDH activity) / (Maximum LDH activity – Spontaneous LDH activity) x 100.

2.10 Isolation of clinical *E. coli* isolates

2.10.1 Ethics statement

Stored stool samples were provided from ongoing and past studies exploring the role of gut microbiota in health and disease. All participants and their legal carers provided written informed consent for their stored samples to be used in future studies that explore the role of gut microbiota in health and disease. The studies were approved by the West of Scotland Research Ethics committee and the local health board Research and Development office and participants were recruited according to the Good Clinical Practice standards for research. R&D reference number: GN11KH060.

2.10.2 Stool samples

Patients were recruited in a pilot study of Dr Konstantinos Gerasimidis investigating gut microbiota composition of juvenile idiopathic arthritis (JIA) patients (Yorkhill Children's foundation; University of Glasgow). Patient recruitment and stool collection was performed between April 2011 and October 2012. The age range of patients was 3 -14 years old. Stool samples from 25 HLA-B27 negative JIA patients and 10 HLA-B27 positive JIA patients were kindly donated (Table 2.6). Stool samples of 35 healthy patients were also also kindly donated. All samples were age and sex matched and samples were labelled blind for all assays. Stool samples were stored at -80ºC.

Table 2-6: Table showing JIA and healthy patient stool samples used in this work.

Healthy control samples			JIA patient samples		
Patient	HLA-B27	Study sample no	Patient	HLA-B27	Study sample no
PWS029	Negative	1	JIA017	Negative	21
PWS035	Negative	2	JIA024	Negative	22
PWS070	Negative	3	JIA029	Negative	23
PWS082	Negative	4	JIA034	Positive	24
PWS116	Negative	5	JIA042	Positive	25
PWS118	Negative	6	JIA049	Negative	26
PWS126	Negative	7	JIA006	Positive	29
PWS139	Negative	8	JIA021	Negative	30
PWS073	Negative	10	JIA031	Negative	31
PWS030	Negative	11	JIA036	Positive	32
PWS032	Negative	12	JIA041	Negative	33
CHIC449	Negative	13	JIA046	Negative	34
CHIC443	Negative	14	JIA052	Negative	35
CHIC439	Negative	15	JIA058	Negative	36
CHIC441	Negative	16	JIA008	Positive	37
CHIC428	Negative	17	JIA023	Negative	38
CHIC419	Negative	18	JIA032	Positive	39
PWS085	Negative	19	JIA038	Positive	40
PWS092	Negative	20	JIA039	Negative	61
CHIC451	Negative	41	JIA045	Negative	62
CHIC459	Negative	42	JIA051	Positive	63
CHIC462	Negative	43	JIA062	Negative	64

PWS105	Negative	44
CHIC429	Negative	45
CHIC461	Negative	47
CHIC458	Negative	48
PWS142	Negative	49
CHIC446	Negative	50
CHIC467	Negative	51
CHIC427	Negative	52
CHIC457	Negative	53
CHIC463	Negative	54
CHIC435	Negative	57
CHIC465	Negative	59

JIA009	Negative	65
JIA022	Negative	66
JIA027	Negative	67
JIA035	Negative	68
JIA040	Negative	69
JIA050	Negative	70
JIA059	Negative	72
JIA019	Positive	73
JIA026	Negative	74
JIA030	Negative	75
JIA033	Negative	76
JIA043	Negative	77
JIA053	Negative	78
JIA048	Negative	80
JIA061	Negative	81
JIA063	Negative	82
JIA064	Negative	83
JIA065	Negative	84

2.10.3 Isolation of faecal *E. coli*

0.1 g of stool sample was mixed in one hundred microliters of sterile PBS and vortexed thoroughly until a faecal slurry was produced. Stool slurry was then serially diluted in PBS and 10^{1}, 10^{-1}, 10^{-2} and 10^{-3} dilutions were plated on to triplicate plates of Gram-negative selective MacConkey agar (Sigma, UK). These plates were incubated for 24-48 hr at 37 °C. Colonies that showed *E. coli* phenotype (pink / purple colouration) were counted and an average CFU count was determined for each patient stool sample. 3 - 5 *E. coli* colonies were picked from each stool sample and restreaked on LB agar plates and eosin methylene blue (EMB) agar plates for 24 h at 37°C to screen for *E. coli* species. Colonies that showed metallic green phenotype on EMB agar were stored as previously described.

2.11 Determination of AIEC phenotype

2.11.1 Invasion of intestinal epithelial cells

T84 cells were grown in 24 well plates (Corning) for 3-5 days at 37°C in 5% CO_2 until an epithelial monolayer was formed. *E. coli* strains were grown shaking in overnight culture at 37°C and then subcultured in fresh media to OD_{600} = 0.6 *E. coli* were diluted in antibiotic-free DMEM media and added to T84 monolayers at a MOI of 10 for 2 h at 37°C, 5% CO_2. Infected T84 cells were then treated with gentamicin or kanamycin (100 µg ml^{-1}) for 1 h to kill extracellular bacteria that had not invaded T84 epithelial monolayer. Cells were then washed and were removed from wells by treatment with trypsin (0.25%) in EDTA for 5 min. Cell suspension was removed by aspiration and homogenised by passing through a 22-gauge needle. Cells were lysed by sonication and were serially diluted and plated on LB agar plates. Plates were incubated overnight at 37°C and CFU counts were performed. All invasion assays were repeated in triplicate. LF82 was used in every assay as a positive control for invasion. Commensal strain F18 was used as a negative control. Invasion of T84 cells was expressed as an invasion index (I_INV) calculated by: (number of intracellular bacteria / number of bacteria in original inoculum) * 100. Assays were performed in triplicate for all *E. coli* isolates.

2.11.2 Intramacrophagic proliferation

The ability of *E. coli* strains to proliferate within macrophages was determined by the gentamicin protection assay as previously described (Falkow *et al.* 1987). J774.1 macrophages were seeded (2 x 10^5 cells/ well) onto 24 well plates and were infected with *E. coli* strains at a MOI of 50 for 2 h. Cells were then treated with gentamicin or kanamycin (100 µg ml^{-1}) for 1 h and then fresh media containing antibiotic was added for 3 h, 24 h or 48 h. For time points exceeding 24 h, media was removed and replaced. Macrophages were then washed extensively with PBS and were then scraped from the surface of the plate and lysed with 2% Triton X-100 for 5 min. Recovered intracellular bacteria were quantified by plating serial dilutions of cell suspension on LB agar. The plates were incubated overnight at 37°C and CFU counts were performed. LF82 was used in all assays as a positive control for intramacrophagic replication and F18 was used as a negative

control. Replication within J774 cells was expressed as a replication index (I_REP) that was calculated by: (number of intracellular bacteria at 4 h / number of intracellular bacteria at 1 h) * 100. Persistence within macrophages was expressed as a persistence index (I_PERS) that was calculated by: (number of intracellular bacteria at 48 h / no of intracellular bacteria at 1 h)* 100. Assays were performed in triplicate for all *E. coli* isolates.

2.11.3 Detection of AIEC-associated virulence genes

The presence of potential virulence determinants was confirmed by PCR amplification of two AIEC associated virulence genes, *lpfA* that encodes long polar fimbriae and *malX*, pathogenicity island marker. The PCR reaction conditions were the same as described previously and 1.0% agarose gel was run for qualitative analysis. The primers used for amplification of virulence determinants genes are listed in Table 2.7. Multiplex PCR for detection of 10 virulence factor gene regions associated with extraintestinal *E. coli* was performed at the Gastrointestinal Bacteria Reference Unit, Public Health, Colindale. Investigated genes were *pap (*pyelonephritis associated pili), *fimH* (type 1 fimbriae), *ibeA* (invasion protein), *fyuA* (yersiniabactin siderophore), *iutA* (aerobactin siderophore), *ompT* (unknown virulence function), *traT* (associated with serum resistance), *k1* (capsule synthesis) and *uidA*, a molecular marker for *E. coli* (Johnson and Stell 2000).

Table 2-7: Primers used for detection of AIEC-associated virulence genes, *lpfA* and *malX*.

Target gene	Primer name	Sequence (5' to 3')	Product size (bp)	Genbank Accession number
lpfA	lpfAF	GAAGCGTAATATTATAGGCGG	570bp	CU651637.1
	lpfAR	TTCGACAGTAAATTGTGAATC		
malX	malXF	GGACATCCTGTTACAGCGCGCA	1593bp	U00096
	malXR	TCGCCRCCAATCACAGCCGAAC		

2.11.4 Serotyping of faecal *E.coli* isolates

Serotyping was performed elsewhere by agglutination of somatic antigens by the use of anti-*E. coli* polyclonal antiserum (Gastrointestinal Bacteria Reference Unit, Public Health, Colindale).

2.12 *In vivo* colicin treatment

2.12.1 Ethics statement

All animal experiments were performed in accordance with the UK Animals Scientific Procedures Act, authorised under a UK Home Office License and approved by the animal ethical review committee of the University of Glasgow. Animal studies were not randomised and blinding was not used in this study. Project licence number: 60/4128

2.12.2 Mice

For all experiments six week-old, female, murine specific pathogen-free CF-1 mice were used (Harlan, UK). All mice received food and water *ad libitum* and were housed in groups (n =5) during the experiments.

2.12.3 Pectin Zein beads preparation

Pectin zein complex hydrogel beads were prepared as described previously (LinShu Liu, Fishman, Hicks, *et al.* 2006). Pectin solution prepared from pectin (poly-D-galacturonic acid methyl ester; ≥74% esterfied) from citrus peel was utilised (Sigma). Beads were prepared by dropping pectin solution, 6% (w/v) into an ethanol solution (85%) containing zein, 1% (w/v) and calcium chloride 0.5% (w/v) at room temperature using a 30 gauge needle. The droplets (~50 µl/drop) were left to cure in reception phase for 15 min and were then washed with distilled water and then dried in a drying oven at ambient temperature and then stored at 4ºC until required. To prepare colicin loaded hydrogel beads, colicin E1 protein was predissolved in dH_2O (10 mg ml^{-1}) and then mixed with 6% pectin solution prior to droplet formation. To determine colicin content in hydrogel beads, air dried beads were dissolved in 85% ethanol containing 0.5% NaOH and 20% Tween20 and diluted in SDS running buffer and run on SDS-PAGE (12% polyacrylamide). Beads were run on SDS-PAGE with standard concentrations of recombinant E1 and band intensities were used to approximate amount of loaded colicin in hydrogel beads. Encapsulation efficiency was calculated by: (amount E1 encapsulated /total amount E1 added) * 100.

2.12.4 Murine model of LF82 infection

Female CF-1 mice received streptomycin (100 mg ml^{-1}) supplemented drinking water 72 h prior to infection and 0.1 M sodium bicarbonate by oral gavage 30 min prior to infection with 1 x 10^9 CFU of LF82::Str^R in 0.1 ml PBS also by oral gavage. Following infection, the number of bacteria was monitored in faecal output. Faecal pellets were collected every 18 h and were homogenised in sterile PBS. Serial dilutions of faecal homogenates were plated on LB agar containing 100 µg ml^{-1} streptomycin and 500 µg ml^{-1} erythromycin and incubated at 37°C overnight to determine bacterial load. Bacterial load was expressed as CFUs/g of faecal material. Streptomycin (100 mg ml^{-1}) supplemented drinking water was used throughout experiment.

2.12.5 Colicin treatment of LF82 intestinal infection

Female CF-1 mice (n=5) were streptomycin treated and infected with LF82::Str^R as described. 18 h post infection, mice received selected colicin bead treatment in 100 µl distilled water by oral gavage. After 18 h faecal pellets were collected and plated to monitor bacterial load as described. Where required mice received colicin bead treatments daily for 7 days and faecal pellets were collected every 18 h. Control group received colicin empty pectin zein beads in dH_2O (Table 2.7).

For infections that involved treatment by colicin E1 solution, 18 h post infection, mice were treated with 0.1 sodium bicarbonate and then received purified colicin diluted in 100 µl dH2O (Table 2.7). Mice received colicin E1 solution every 22 h for 8 days. Control group for this colicin treatment received dH_2O.

Table 2-8: Delivery and dosing for colicin treatment of LF82 murine intestinal infection.

Experimental Attributes	Method		
	1	2	3
Colicin Treatment	Colicin E1 pre-loaded pectin zein beads	Colicin E1 pre-loaded pectin zein beads	Recombinant colicin E1 protein
Dosage	5 beads (~5µg)	10 beads (10µg)	10mg ml^{-1}
Adminstration schedule	18 h post infection	18 h post infection and daily for 8 days	18 h post infection and daily for 8 days

2.12.6 Colicin stability assay

To assess colicin stability and activity in the murine intestine, CF-1 mice that had not been exposed to LF82 received colicin E1 (10 mg ml^{-1}) diluted in 100 µl dH_2O by oral gavage for 30, 60 or 120 min. Mice were then culled by cervical dislocation and intestinal organs (stomach; small intestine; caecum; colon) were aseptically extracted and placed in sterile PBS. Organs and liquid content were then homogenised for 5 - 10 min using Stomacher Lab blender 80 (Steward). To test colicin activity, one hundred and fifty microliters of LF82::StrpR culture at OD_{600} = 0.6 was added to 6 ml of 0.8% soft agar and poured over an LB agar plate before 10 µl of intestinal organ homogenate was spotted onto the plates and incubated for 24 h at 37°C. Clear zones indicate cell death.

2.13 Statistical analysis

Non-parametric analyses were selected when a small sample size was present in data. For macrophage assays one way ANOVA followed by post-hoc analysis with Bonferroni correction was used to compare bacterial numbers. For *in vitro* adhesion assays unpaired student's t-test was used to compare bacterial numbers. Bacteria counts in co-culture assays were compared using non-parametric Mann-Whitney U test. One way ANOVA followed by post-hoc analysis

with Bonferroni correction was used to compare amount of cytokines and LDH present in cell supernatants. For all tests $P < 0.05$ was regarded as statistically significant. For faecal bacterial count data, comparison of CFUs were performed using the nonparametric Mann–Whitney U test. For assays investigating AIEC phenotypes, comparison of invasion, replication and persistence index values between groups were performed using the nonparametric Mann–Whitney U test. Differences were considered statistically significant at $P < 0.05$. For murine infection assays, non-parametric Mann–Whitney U-test was carried out at all time points to compare bacteria counts. All mice, including outliers were included in the statistical analysis. For all tests $p < 0.05$ was regarded as statistically significant.

3 Colicins show activity against Crohn's disease associated adherent-invasive *E. coli*

3.1 Introduction

3.1.1 Colicin antibiotics; Fighting bugs with their own weaponry

Antibiotic treatments are currently used to kill or inhibit the growth of pathogenic or unwanted bacterial species and are utilised for this purpose in numerous sectors including health care, food production and veterinary medicine. The wide spread application of antibiotics is thought to be a major contributing factor to antibiotic resistance. This is in part due to the broad spectrum nature of antibiotics used for these purposes, that results in a build-up of resistant determinants in the wider bacterial population (Julian Davies and Davies 2010).

However, inhibition of bacteria by highly targeted, narrow spectrum antibiotics is also observed widely in bacterial communities in the form of bacteriocins; protein antibiotics produced by bacteria during intraspecies competition (O Gillor *et al.* 2008). Although the specific ecological role of bacteriocins is still unknown, it is proposed that they function as anti-competitors, by enabling the invasion of a producer strain into an established microbial community or by limiting the advance of neighbouring cells in an occupied niche (Margaret a Riley and Wertz 2002). Overall, the detection of bacteriocins in all surveyed prokaryotic lineages, highlights the significance of these products for bacterial survival and competition, making them ideal candidates for further investigation as therapeutics (Cascales *et al.* 2007).

In order to function as an antimicrobial therapy in human health, bacteriocins must show good efficacy against target strains that demonstrate abnormal colonisation of the host (i.e are present in increased numbers and interact with host cells); be active against a range of isolates; be non-cytotoxic to host cells and also be successfully delivered to the site of infection. In this work, the efficacy of colicins was tested against AIEC infection associated with Crohn's disease.

3.1.2 CD associated adherent-invasive *E. coli* as a target for colicin treatment

AIEC bacteria are more frequently isolated from ileal and colorectal biopsies of Crohn's disease patients compared to healthy controls (Conte *et al.* 2014, Martin *et al.* 2004, Mylonaki and Rayment 2005). It is currently unknown if abnormal colonisation of AIEC detected in CD patients is a primary cause of inflammatory symptoms or is a secondary event facilitated by host genetic factors, impaired epithelial barrier function and dysregulated mucosal immune responses associated with disease. CD patients demonstrate intestinal dysbiosis relative to healthy individuals and in a proportion of patients, AIEC expansion can be recognised as a component of this dysbiosis. In this case, it would be preferential to use targeted antibiotics to prevent further dysbiosis of resident microbial communities. This represents a complex challenge for broad spectrum antimicrobial therapies as they are unable to target specific members of the intestinal microbiota. Colicins are species-specific antibiotics that show activity against *E. coli* and closely related species and as such may make a useful potential treatment for AIEC infection associated with CD.

3.2 Aims

The aim of the work described in this chapter was to test the efficacy of colicins against CD associated AIEC in *in vitro* models (Figure 3.1). To achieve this, colicin killing was investigated against:

1) A range of colonic and ileal AIEC clinical isolates growing in planktonic culture;
2) AIEC associated with T84 intestinal epithelial cells;
3) AIEC growing within RAW 264.7 macrophages.

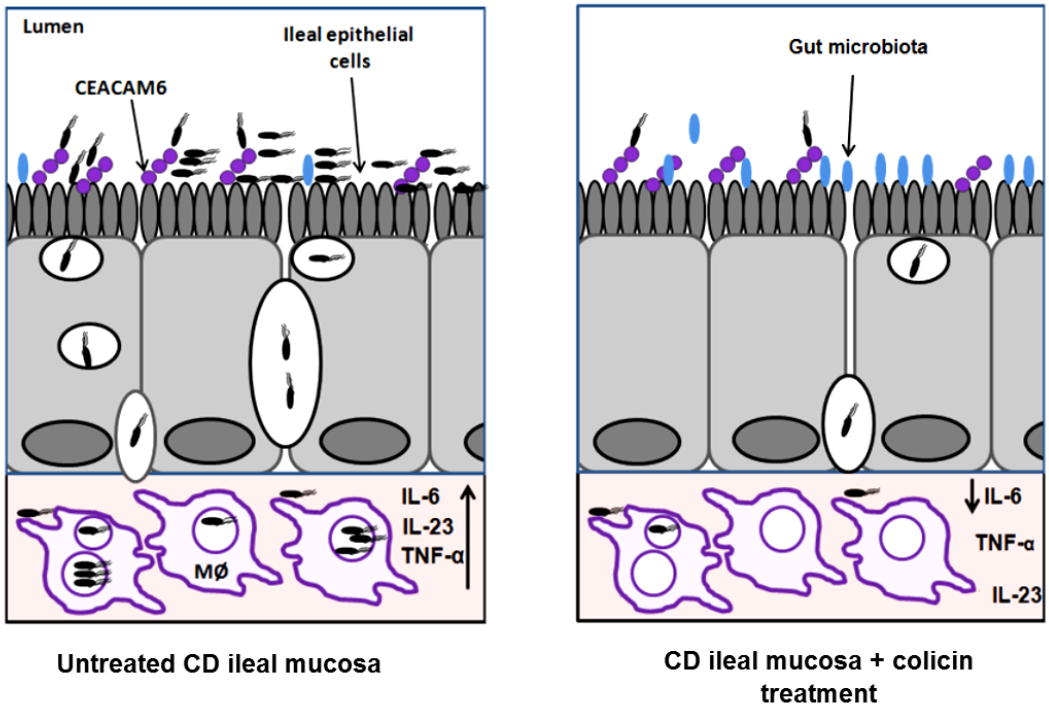


Figure 3-1: Hypothetical target sites for colicin treatment of AIEC.

Figure shows model for AIEC infection in CD (left) and the investigated sites for colicin treatment of AIEC (right). In this work, we hypothesise that targeted killing of AIEC by colicins would preserve gut microbiota populations, reduce the number of AIEC interacting with intestinal epithelial cells at gut mucosa and therefore decrease the number of AIEC gaining entry to lamina propria and host macrophages. Reduced AIEC infection of macrophages may result in decreased production of pro-inflammatory cytokines and therefore inflammatory symptoms associated with CD.

3.3 Results

3.3.1 Colicins show activity against planktonic AIEC

In order to investigate the activity of colicins against AIEC, overlay assays were performed. A range of indicator AIEC strains were tested. This included the AIEC reference strain LF82 (ileal CD) that was kindly gifted by Darfeuille-Michaud (Clermont-Ferrand, France) and colonic mucosally associated AIEC clinical isolates HM95 (ileal CD), HM419 (ileo-colonic CD) and HM154, HM580, HM605 and HM615 (colonic CD) that were kindly gifted by Barry Campbell (University of Liverpool). *E.coli* W3110 strains that possessed colicinogenic plasmids encoding colicin A, B, N, E1, Ia, Ib (pore-formers); E2, E9 (DNase) and D (tRNase) were used. These data showed that AIEC reference strain LF82 was susceptible to all tested colicins (Figure 3.2). Similarly, AIEC clinical isolates HM419 and HM95 showed high level of susceptibility to all tested colicins tested when grown in planktonic culture (Table 3.1). AIEC clinical isolate HM615 showed sensitivity to colicins E1- E9, A, D and N but was tolerant to colicins B, Ia and Ib. By this method, AIEC clinical isolates HM154, HM580 and HM605 were tolerant to all tested colicins (Table 3.1).

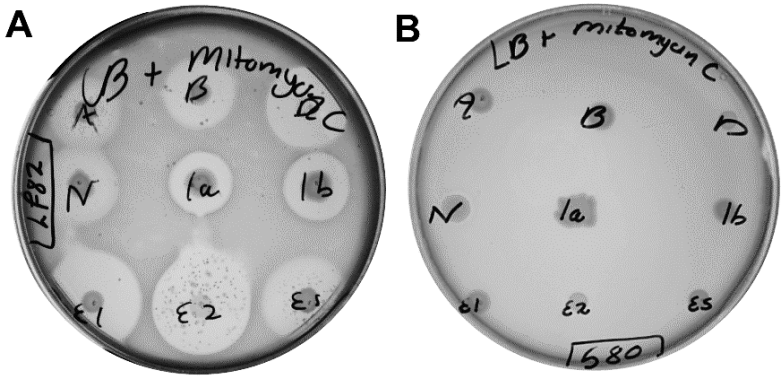


Figure 3-2: AIEC isolates show different susceptibility profiles to colicins.

A) AIEC reference strain LF82 is susceptible to a wide range of colicins. Colcinogenic *E. coli* W3110 strains were spotted on LB agar containing mitomycin C for induction of colicin production and an AIEC soft agar lawn was grown on top. Colicin killing is shown by a zone of clearing around colicin producer strains. B) AIEC clinical isolate HM580 is tolerant to all colicins investigated in overlay assay. Image shown is a representative example of three independent overlay spot plate assays.

Table 3-1: Killing spectrum of colicins against AIEC reference strain LF82 and AIEC clinical isolates HM95-HM615.

Indicator strain	Colicin producing strains									
	A	B	D	N	IA	IB	E2	E5	E1	E9
LF82	+	+	+	+	+	+	+	+	+	+
HM95	+	+	+	+	+	+	+	+	+	+
HM154	-	-	-	-	-	-	-	-	~	~
HM419	+	+	+	+	+	+	+	+	+	+
HM580	-	-	-	~	-	-	-	-	~	~
HM605	-	-	-	-	-	-	-	-	-	-
HM615	+	~	+	+	-	-	+	+	+	+

Colicin producing strains were grown on LB agar containing mitomycin C, chloroform killed and then overlain with soft agar suspension of AIEC indicator strain. For results; + denotes killing and zone of inhibition, ~ denotes small diffuse zone and - denotes no zone of inhibition detected.

3.3.2 Potent activity of colicins against AIEC

To produce purified colicin E9, the plasmid pCS4, encoding full length colicin E9 and its cognate immunity protein Im9, was transformed into *E. coli* BL21 (DE3) cells. Colicin E9 was overexpressed as previously described (Garinot-Schneider *et al.* 1996). Colicin E1 was produced in mitomycin C induced *E. coli* BZB2104 (pColE1-K53) that carries the colicin E1 encoding plasmid. Purification of colicin E1 was performed by ammonium sulphate fractionation and ion exchange chromatography as previously described (SA Schwartz and Helinski 1971). Chromatograms and SDS-PAGE gels showing the final purified products for colicin E1 and E9 are shown in Appendices B and C (Figure B-1, C-1). Following purification colicins were filter sterilised and stored at -20°C until required. To test the activity of purified colicins E1 and E9, 5-fold serial dilutions (1 mg ml^{-1} – 0.32 µg ml^{-1}) were spotted on soft agar overlays of AIEC indicator strains. Plates were incubated overnight at 37°C and colicin killing was detected by zones of

clearance. Colicins E1 and E9 showed potent killing activity against sensitive AIEC strains, killing at a concentration of 0.32 µg ml^{-1} for AIEC strains HM95, HM419 and HM615 and AIEC reference strain LF82 (Figure 3.3).

These data showed that colicin E9 poorly inhibited HM154 and HM605 at 1 mg ml^{-1} that was shown by a small and diffuse zone of inhibition (Figure 3.3). HM605 was resistant to colicin E9 killing at a concentration of 1 mg ml^{-1}. Colicin E9 showed activity against HM580 at a concentration of 0.25 mg ml^{-1} and against HM154 at a concentration of 1 mg ml^{-1}. HM605 was resistant to colicin E1 killing at 1 mg ml^{-1}. The MICs of colicins E1 and E9 against AIEC growing in liquid broth are shown in Appendix D.

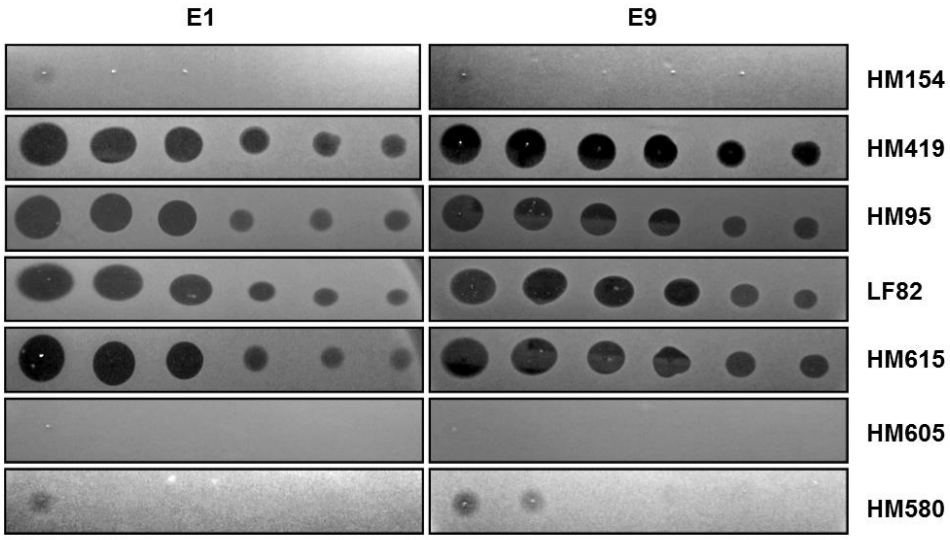


Figure 3-3: Colicins show highly potent activity against some AIEC isolates.

A) AIEC reference strain LF82 (ileal CD) and colonic AIEC clinical isolates HM95 (ileal CD) HM419 (ileo-colonic CD) and HM164, HM580, HM605 and HM615 (colonic CD) were grown in planktonic culture and purified colicin E9 and colicin E1 protein was serially diluted 5-fold (1 mg ml^{-1} - 0.32 µg ml^{-1}) and spotted on molten agar overlay. Zones of clearing show killing of AIEC bacteria by each colicin and dilution. Data shown is a representative example of three independent overlay assays.

3.3.3 Colicins show activity against AIEC LF82 cells adhered to T84 intestinal epithelial cells

It has been proposed that one of the initial stages of AIEC infection in CD may involve receptor mediated bacterial adhesion to intestinal epithelial cells (IECs) (Barnich and Darfeuille-Michaud 2007). To determine if colicins are active against

AIEC that are adhered to IECs, monolayers of the T84 human intestinal epithelial cell line were infected with LF82 and colicin E9 (100 µg ml^{-1}) was added 15, 30, 45 and 60 min post-infection. T84 cells are known to express CEACAM6 receptors and therefore represent a suitable *in vitro* model for ileal AIEC adherence to IECs (Barnich and Darfeuille-Michaud 2007). On addition of colicin E9 at 15, 30 and 45 min post infection, live and adhered LF82 could no longer be detected by CFU counts (Figure 3.4). Colicin treatment at 1 h post-infection caused a 2-log unit reduction in CFU counts of cell associated LF82 compared to untreated controls (Figure 3.4). These data show that colicin E9 can effectively kill AIEC bacteria adherent to human IECs that express CEACAM6 receptors.

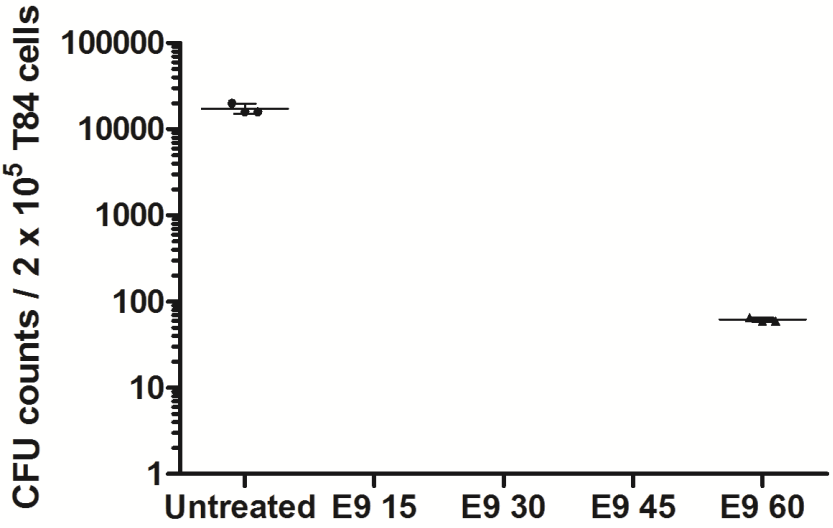


Figure 3-4: Colicins show activity against ileal AIEC strain LF82 associated with intestinal epithelial cells.

T84 intestinal epithelial cell monolayers were infected with AIEC LF82 (MOI of 10 for 3 h) and when required colicin E9 (100 µg ml^{-1}) was added in presence of AIEC bacteria at 15, 30, 45 and 60 min post infection. Cell associated LF82 numbers were determined by CFU counts. Black circle is mean value for each experiment. Black line represents mean value. Error bars shown are SE of three independent experiments. No CFU counts were detected for E9 at 15; 30; 45 min by this method. Comparison of CFUs for untreated and colicin E9 treated showed a significant reduction on colicin treatment at 60 min. ($P< 0.05$, unpaired Student's t test).

3.3.4 Colicin killing of cell associated AIEC preserves healthy cell morphology of intestinal epithelial cells

A defining feature of the AIEC pathovar is the ability to invade IECs that is proposed to be mediated by actin induced alterations of the host cell cytoskeleton. To determine if changes in morphology of IECs were induced by AIEC infection and subsequent colicin treatment, T84 cells were grown on glass coverslips, infected with LF82 and treated at 15 min post infection with colicin E9 (100 µg ml^{-1}) where required. At 3 h post infection T84 cells were washed, Giemsa stained and visualised by light microscopy. Light micrographs showed that LF82 infection of T84 cells caused alterations in cell morphology (Figure 3.5). LF82 infected cells displayed high levels of cell detachment and increased production of abnormal cell protrusions and lamellipodia. (Figure 3.5b). LF82 adhered to T84 cells were also observed (Figure 3.5b). Colicin E9 treatment of infected T84 cells significantly reduced the production of cell protrusions and a smoother cell monolayer was observed (Figure 3.5c). LF82 infected T84 cells treated with colicin E9 also showed reduced cell detachment and reduced levels of debris in the apical supernatant. Following colicin E9 treatment, very few cell-associated LF82 could be visualised. Addition of colicin E9 to uninfected T84 cells did not induce changes in cell morphology (Figure 3.5d).

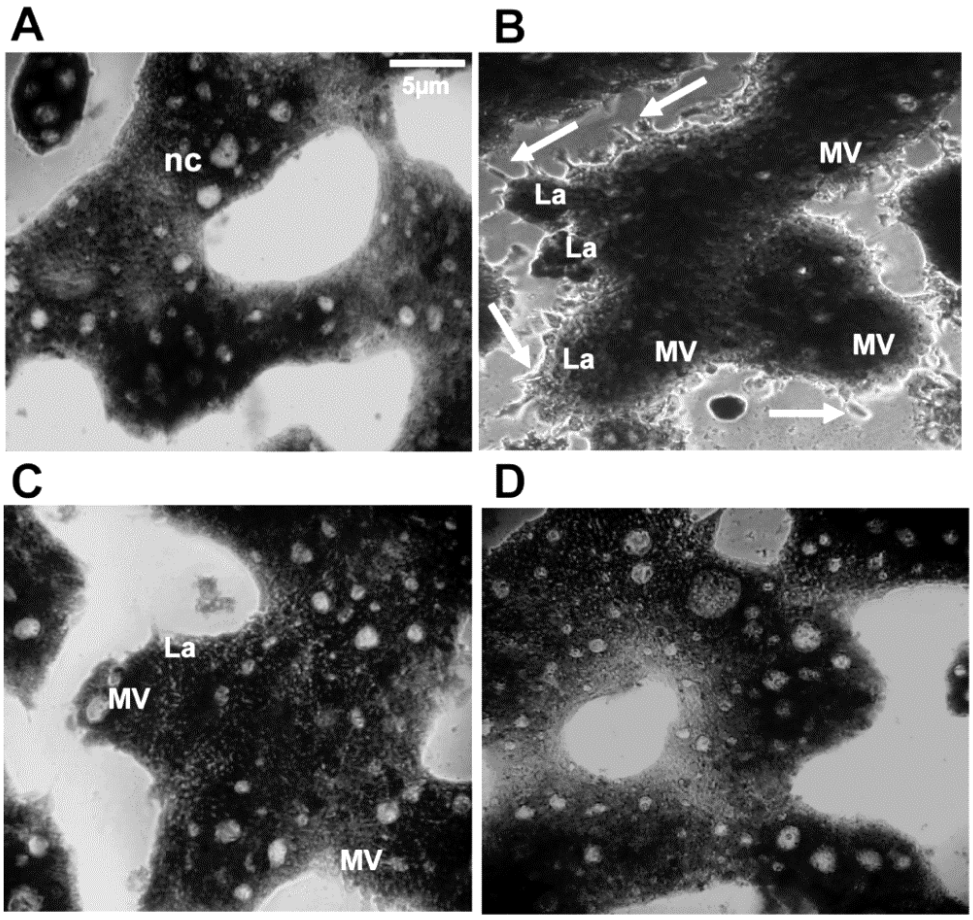


Figure 3-5: Colicin killing of IEC associated AIEC reduced appearance of abnormal cell morphology induced by infection.

T84 cells were grown on glass cover slips in 6 well plates and infected with AIEC LF82 (MOI of 10 for 3 h). For colicin treated samples, colicin E9 (100 µg ml^{-1}) was added 15 min post-infection. Cells were Giemsa stained and visualised with Zeiss Axioskop (40 x magnification). Light micrographs show: A) Untreated control B) LF82 infected T84 cells, C) LF82 infected T84 cells treated with colicin E9 (100 µg ml^{-1} for 3 h) D) Uninfected T84 monolayer treated with colicin E9 (100 µg ml^{-1} for 3 h). Scale bar shown is 5 µm. Image labelling is as follows; nc; nuclei; MV; membrane vesiculation, white arrow; adhered bacteria, La; lamellipodia. Image is representative of two independent experiments.

3.3.5 Co-culture with colicin producing commensal *E. coli* reduces adherence of LF82 to intestinal epithelial cells

For disorders such as CD, that are associated with intestinal dysbiosis, the use of probiotic bacteria that can manipulate the microbiota composition is highly desirable. In order to investigate the ability of colicin-producing commensal *E. coli* strains to inhibit AIEC adherence to IECs, co-culture assays were performed. For this, the colicin producing commensal *E. coli* strain BZB2014 (W3110 pColE1-K53)

was co-cultured with LF82 in T84 cell adhesion assays. Equal volumes of LF82 and BZB2014 (MOI=10) were added to T84 monolayers and incubated for 3 h at 37°C, 5% CO_2. *E. coli* W3110 that did not carry the ColE1 plasmid was also used in co-culture assays with LF82.

Co-culture of LF82 with colicin-producing commensal *E. coli* significantly reduced CFU counts of cell associated LF82 compared to LF82 mono-culture (P= 0.0256). No significant difference in CFU counts of cell associated LF82 was detected following co-culture of LF82 with the non colicin-producing W3110 strain (P=0.136) (Figure 3.6). These data show that concentrations of colicin produced by colcinogenic *E. coli* strains *in vitro* is sufficient to inhibit cell- associated AIEC.

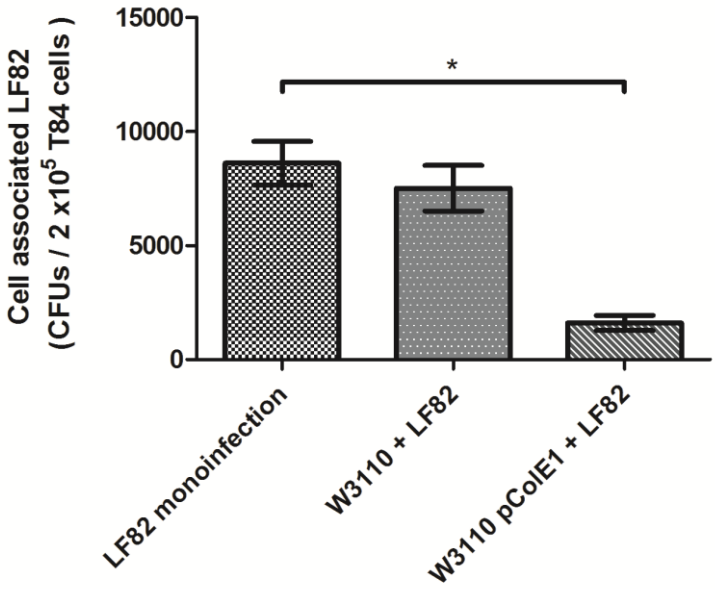


Figure 3-6: Co-culture of LF82 with colicin producing commensal *E. coli* strain reduced adherence of AIEC to T84 IECs.

T84 intestinal epithelial cell monolayers grown in 24 well plates (2 x 10^5 cells / well) were infected with *E. coli* (MOI of 10) for 3 h. AIEC LF82 was added as a monoculture, in co-culture with colicin producing *E. coli* BZB2014 (W3110 pColE1-K53) and in co-culture with non colicin-producing isogenic BZB2014 strain. Number of cell-associated LF82 were determined by CFU counts on LB agar containing ampicillin (50 µg ml^{-1}). Data shown represents mean and SD (error bars) of two independent experiments. Comparison of LF82 CFUs following co-culture with colicin producing strain and non colicin producing strain showed a significant reduction on colicin treatment (P=0.0256). Comparison of mean CFUs were performed using the nonparametric Mann–Whitney U test. * denotes significant difference between groups.

3.3.6 Colicins show activity against AIEC bacteria residing within macrophages

3.3.6.1 LF82 is an intramacrophagic persister

Although previous studies have reported extensive replication of AIEC strain LF82 within macrophages *in vitro*, this result was not reproduced in this work (Bringer *et al.* 2006, Subramanian, Roberts, *et al.* 2008). Bacterial survival in RAW 264.7 macrophages was determined using the gentamicin protection assay (Falkow *et al.* 1987). RAW 264.7 macrophages were infected with LF82 at an MOI of 50 for 2 h and treated with gentamicin until the required time point. Viable bacteria were determined by CFU counts following plating of lysed cells on LB agar. LF82 was shown to survive within macrophages and persist intracellularly for extended periods with viable bacteria being detected after 48 h (Figure 3.7a). Approximately 2 x 10^4 CFUs of LF82 were detected in macrophages at 1 h. This decreased to approximately 1 x 10^4 CFUs at 4 h, 1 x 10^3 CFUs at 24 h with approximately 40 CFUs of LF82 detected at 48 h.

To visualise intramacrophagic LF82 bacteria, LF82 strain expressing GFP was constructed. RAW macrophages were grown to confluency on glass coverslips in 6 well plates and LF82-GFP was used in gentamicin protection assays. At 24 h, cells were stained with DAPI and Alexa-647-phalloidin and visualised using confocal microscopy. Consistent with CFU counts LF82 bacteria were only detected in a small proportion of macrophages in the population. LF82 bacteria were detected in 0.5% of macrophage cells and formed cell aggregates within macrophages (Figure 3.7b).

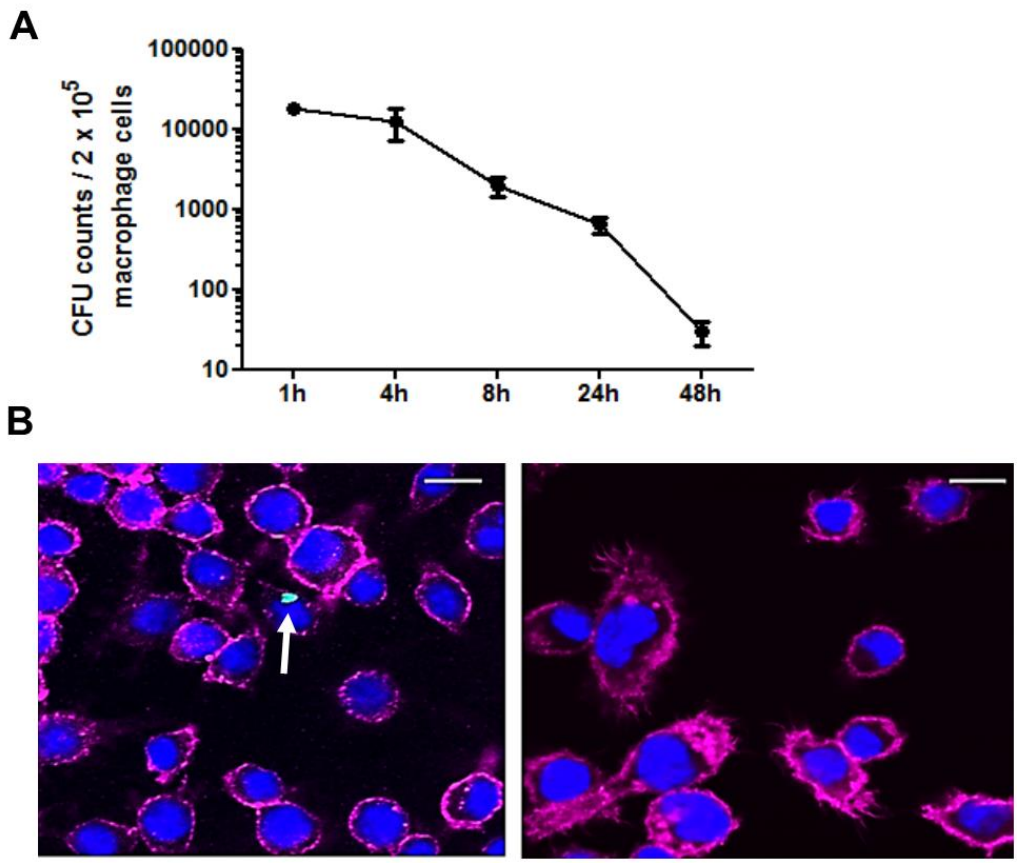


Figure 3-7: AIEC LF82 shows persistence within macrophages.

A) LF82 persists in macrophages at low numbers at 48 h. RAW 264.7 macrophages (2 x 10^5 cells) were infected with LF82 (MOI of 50; 2 h) and treated with gentamicin (100 µg ml^{-1} for 1 h) to kill extracellular bacteria. Fresh media containing gentamicin was added and incubated for 4, 8, 24 and 48 h. Macrophages were lysed and viable intracellular bacteria determined by CFU counts. Data represents mean and SE of three independent experiments. B) LF82 persists in few macrophages in population at 24 h. Figure shows confocal micrographs of RAW 264.7 macrophages infected with green LF82-GFP (left) and mock-infected control (right). Actin is stained with phalloidin-Alexa 647 conjugate (purple) and nuclear DNA is stained with DAPI (blue). Arrow shows intracellular aggregate of LF82 bacteria in single macrophage. Scale bars shown are 10 µm. Confocal micrographs represent two independent experiments.

3.3.6.2 Colicins kill LF82 residing within macrophages

To investigate the ability of colicins to inhibit LF82 persistence within RAW macrophages gentamicin protection assays were performed that included treatment of intracellular bacteria with a range of antibiotics. Antimicrobial treatments were colicin E1, colicin E9, colicin E9-H575A, a variant protein that lacks antibacterial activity due to a mutation in the active site of the cytotoxic

DNase domain (Appendix A), or ciprofloxacin (100 µg ml^{-1}), that has previously been shown to be highly active against intramacrophagic AIEC (Subramanian, Roberts, *et al.* 2008). For antibiotic free controls, the number of bacteria present in RAW 264.7 macrophages at 4 h and 24 h was approximately 1.6 x 10^4 and 800 CFUs / well, respectively. Both colicin E9 and colicin E1 treated macrophages showed reduced intracellular survival of LF82 at 4 h and 24 h but to a lesser extent than ciprofloxacin (Figure 3.8). In contrast, treatment with inactive colicin E9H575A following gentamicin treatment did not reduce survival of intramacrophagic LF82 that indicated that the cytotoxic function of the protein was directly responsible for bacterial killing (Figure 3.8).

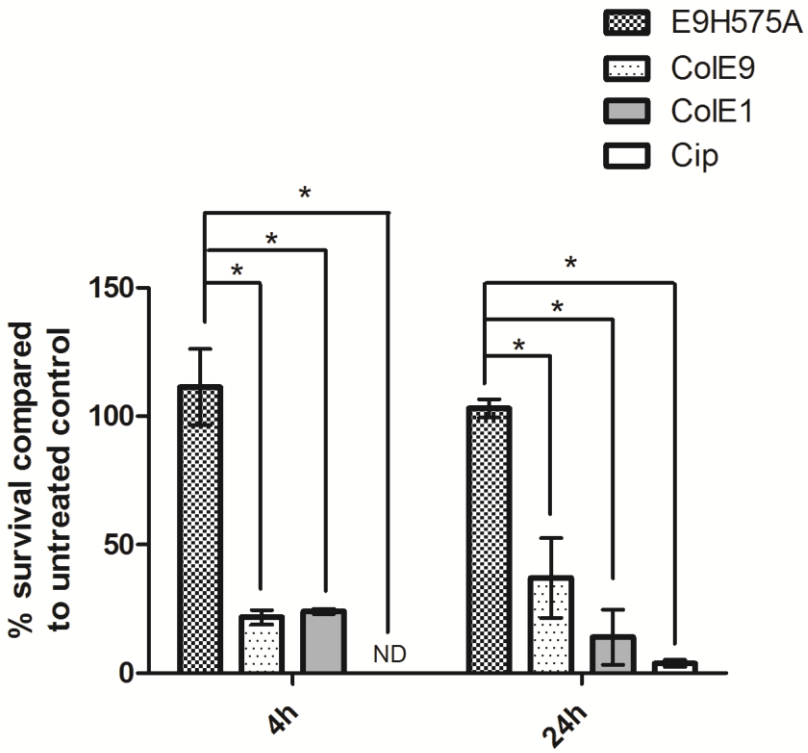


Figure 3-8: Colicins kill intramacrophagic AIEC.

A) Colicins show activity against LF82 residing within RAW 264.7 macrophages. RAW 264.7 macrophages were infected with LF82 (MOI of 50; 2 h) and treated with gentamicin (100 µg ml^{-1} for 1 h) to kill extracellular bacteria. Antimicrobial treatments were performed for 24 h at 100 µg ml^{-1} for all tested. At 4 h and 24 h macrophages were lysed and viable intracellular bacteria determined by CFU counts. E9H575A is an active site mutant protein of colicin E9. ND; None detected. Data shown represents mean and SE of three independent experiments. One way ANOVA followed by post-hoc analysis with Bonferroni correction showed significant differences between the inactive colicin H575A group and colicin E9, colicin E1 and ciprofloxacin treated groups at 4 and 24 hours ($P < 0.05$). *, $P < 0.05$.

3.3.7 Colicins enter AIEC containing compartments within infected RAW264.7 macrophages

In order to demonstrate colicin entry into RAW 264.7 macrophages, RFP-tagged fluorescent colicin E9 (colicin E9-RFP), in which the DNase domain is replaced with RFP leading to a cytotoxically inactive protein, was constructed (Walker lab). RAW 264.7 macrophages grown on glass coverslips in 6 well plates were infected with LF82-GFP (green) at a MOI of 50, treated with gentamicin as described and treated with colicin E9-RFP (red) and incubated for 24 h. In order to selectively visualise intracellular colicin, macrophages were treated with trypsin to inactivate extracellular and cell associated colicin E9-RFP. Using confocal microscopy the presence of both colicin and LF82 within infected macrophages was observed (Figure 3.9a). Confocal micrographs showed that colicin E9-RFP was present within infected RAW macrophages and in some cases co-localised together with LF82-GFP within specific compartments (Figure 3.9a). Although co-localisation of colicin with LF82 was detected, colicin-RFP that was not co-localised with bacteria was also detected within macrophages at 4 h (Figure 3.9b).

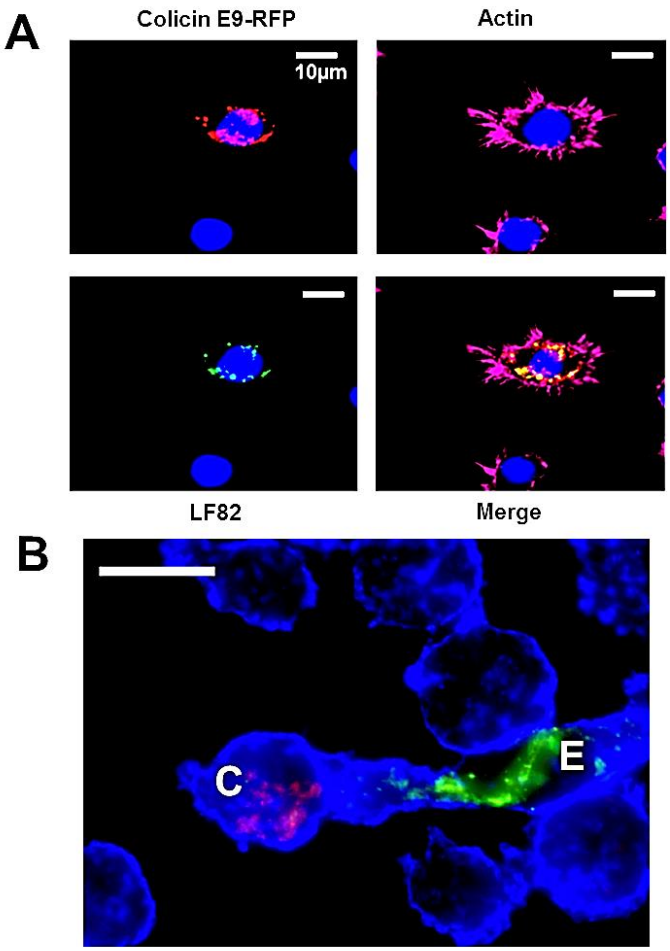


Figure 3-9: Colicin adheres to macrophages, enters AIEC containing compartments and localises with LF82.

A) Colicin enters AIEC containing compartment in RAW 264.7 macrophages. Figure shows confocal micrographs of RAW 264.7 macrophages infected with GFP expressing LF82 bacteria (green) and treated with colicin E9-RFP (red) for 24 h. Trypsinised macrophage cells were fixed and permeabilised and actin stained with phalloidin-Alexa 647 conjugate (purple) and nuclear DNA stained with DAPI (blue). Scale bars shown are 10µm. A total number of 50-200 macrophages were assessed per experiment. Confocal micrographs represent three independent experiments. B) Colicin E9-RFP detected in macrophages and not co-localised with AIEC. Fluorescent micrographs of RAW264.7 macrophages infected with GFP expressing LF82 bacteria (green) and treated with colicin E9-RFP (red) for 4 h. Trypsinised macrophage cells were fixed and permeabilised and actin stained with phalloidin-alexa 647 conjugate (blue). Labels are C: Colicin-RFP; E: *E. coli* LF82. Scale bar shown is 10 µm.

3.3.8 Colicin uptake in macrophages involves actin mediated endocytosis

AIEC are proposed to enter macrophages by phagocytosis due to their residence within compartments highly representative of the phagolysosome (Bringer *et al.* 2006). Protein uptake by phagocytosis in macrophages is not an established entry mechanism, therefore it was postulated that colicin utilised distinct endocytic pathways to gain entry to macrophage cells. To further investigate colicin uptake, RAW macrophages treated with colicin E9-RFP that were not exposed to trypsin were visualised by fluorescent microscopy (Figure 3.10). Interestingly, in the absence of trypsin treatment, high levels of colicin adherence at the macrophage cell surface were observed (Figure 3.10). This indicated binding of colicin to cell surface macrophage receptors.

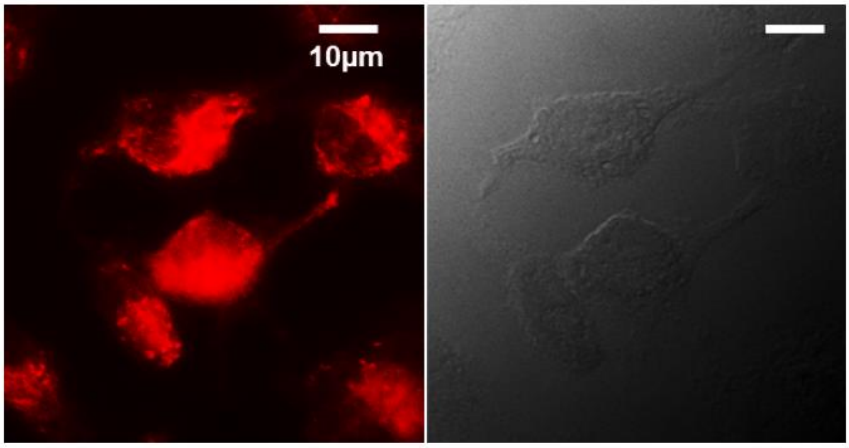


Figure 3-10: Colicin adheres to RAW 264.7 macrophages.
Figure shows fluorescent micrograph of RAW264.7 macrophages treated with colicin E9-RFP for 4 h that were not trypsinised prior to staining and fixing. Second panel shows DIC image. Scale bar shown is 10 µm.

In order to determine the mechanism of colicin uptake into cells, macrophages were pre-treated with inhibitors of multiple endocytic pathways and then treated with colicin E9-RFP for 4 h and trypsin treated and washed as described. Confocal micrographs showed that pre-treatment of macrophage cells with cytochalasin B, an inhibitor of actin polymerisation significantly reduced the uptake of colicin E9-RFP (Figure 3.11). The processes of clathrin-mediated endocytosis, pinocytosis, mannose receptor-mediated endocytosis and scavenger receptor-endocytosis inhibited by dynasore, DMA, mannan and polyinosinic acid respectively, were not shown to influence colicin uptake (Figure 3.11). These data indicate that an actin regulated endocytic pathway regulates colicin uptake into AIEC-infected macrophage compartments.

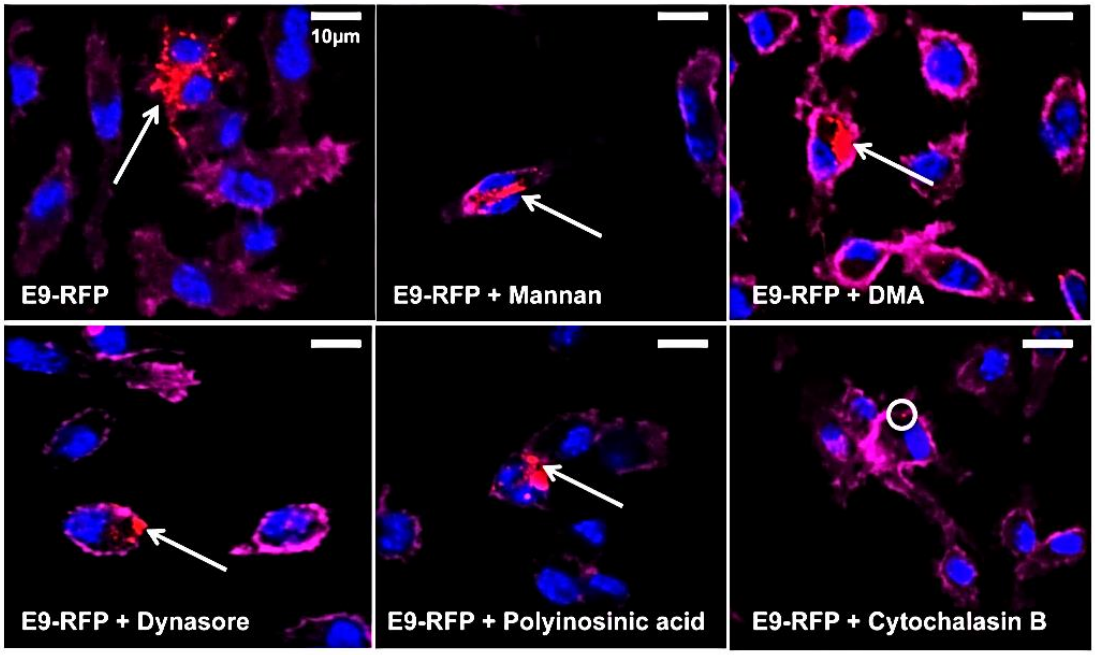


Figure 3-11: Pre-treatment of RAW 264.7 macrophages with chemical inhibitor of actin polymerisation significantly reduced uptake of colicin E9-RFP.
Confocal micrographs of RAW 264.7 macrophages pre-treated with inhibitors of endocytosis (30 min) and then treated with colicin E9-RFP (red) for 4 h. Inhibitors used were mannan (mannose receptor-mediated endocytosis), dynasore (clathrin mediated-endocytosis), dimethylamiloride (pinocytosis), polyinosinic acid (scavenger receptor-mediated endocytosis) and cytochalasin B (actin polymerisation). Actin was stained with phalloidin-Alexa 647 conjugate (purple) and nuclear DNA stained with DAPI (blue). Arrows and circle highlight colicin E9-RFP inside macrophage cells. Scale bars shown are 10 µm. Confocal micrographs represent two independent experiments.

3.3.9 Effect of colicin treatment on macrophage TNF-α production

In previous *in vitro* studies AIEC infected macrophages have been shown to produce increased amounts of the pro-inflammatory cytokine, TNF-α (Glasser *et al.* 2001). We therefore sought to investigate if colicin killing of intramacrophagic AIEC would cause a decrease in the production of pro-inflammatory cytokines. To achieve this, RAW 264.7 macrophages were infected with LF82 and treated with colicin E9 (100 µg ml^{-1}). For this assay, colicin was further purified using endotoxin removal columns to ensure LPS was extracted from protein fractions. LPS (1 µg ml^{-1}; Sigma) was used as a positive control for cytokine release. For cytokine detection, macrophage cell supernatants were removed at 24, 30 and 40 h post-treatment and ELISAs (BioLegend) were performed for TNF-α. Although colicin E9 did not reduce production of TNF-α by LF82 infected macrophages, increased production of TNF-α was not detected following colicin treatment (Figure 3.12a). A similar result was obtained for ciprofloxacin treated LF82 infected macrophages indicating that at least in this assay, AIEC killing is not associated with reduced levels of TNF-α. Colicin E9 treatment of uninfected macrophages was shown to produce similar levels of TNF-α as the untreated mock controls at all-time points tested (Figure 3.12a). Additionally, lactate dehydrogenase (LDH) assays showed that colicin E9 treatment was not cytotoxic to macrophages (Figure 3.12b). Untreated macrophages were shown to produce similar amounts of LDH compared to colicin treated, uninfected cells at 24 h (Figure 3.12c).

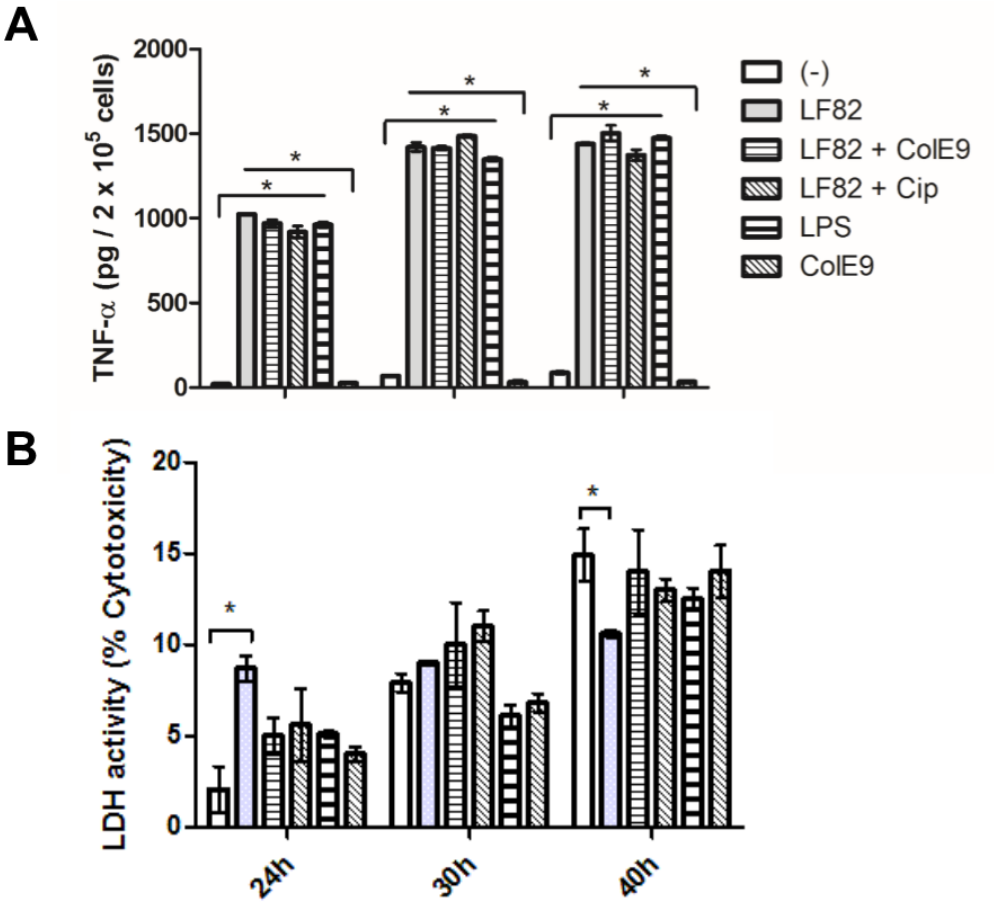


Figure 3-12: Colicin is non-cytotoxic to macrophage cells and fails to increase production of TNF-α.

TNF-α (A) and LDH (B) production by RAW 264.7 macrophages infected with LF82 (MOI of 50; 3 h) and treated with E9; ciprofloxacin (100 µg ml^{-1}) for 24 h, 30 h and 40 h following gentamicin treatment. (-) represents mock infected negative control. LPS (1 µg ml^{-1}) stimulation of macrophages was control for cytokine production. % Cytotoxicity was determined by measurement of LDH release relative to uninfected controls. Data are expressed as mean +/- SE of two independent experiments. In (A) ANOVA followed by post-hoc analysis with Bonferroni correction indicated differences ($P < 0.05$) for both mock infected and ColE9 treated vs LF82, LF82 + ColE9, LF82 + Cip and LPS treated cells at 24, 30 and 48 h and no difference ($P > 0.05$) between mock infected and ColE9 treated cells. In (B) ANOVA followed by post-hoc analysis with Bonferroni correction indicated differences ($P < 0.05$) at 24h and 40 h for mock infected vs LF82 but no other significant differences between groups on pairwise comparison. *, $P < 0.05$.

3.4 Discussion

A range of chronic immune related diseases such as IBD, diabetes and rheumatoid arthritis are associated with intestinal dysbiosis and in a subset of patients associations with specific bacterial species have been determined. For the most part, the use of broad spectrum antibiotics for the treatment of chronic immune related disorders, such as CD generally show poor efficacy (Selby *et al.* 2007). This itself can lead to the development of severe secondary infections resulting in extended hospital care and requirement for further treatments (Hickson *et al.* 2007, Meropol *et al.* 2008).

A potential alternative treatment for the correction of dysbiosis is the use of narrow- spectrum antibiotics such as the colicin-like bacteriocins. As stated in section 3.1.1, to be considered as a potential therapeutic, colicins must show activity against target strains that demonstrate abnormal colonisation of host, be active against a range of isolates and also be non-cytotoxic towards mammalian cells. These data demonstrate clearly that colicins fulfil the majority of these requirements and also exhibit several unexpected but useful attributes (Figure 3.13).

In this work colicins were shown to kill a range of clinical AIEC isolates that highlights their potential as a therapeutic against diverse infections. Colicins were also shown to kill AIEC-associated with intestinal epithelial cells and killed AIEC bacteria growing intracellularly within macrophages. For the latter, colicins entered AIEC containing compartments within RAW 264.7 macrophages by exploiting actin-mediated endocytosis. The unexpected ability of colicins to inhibit intramacrophagic AIEC highlights their potential as a therapy for CD as AIEC persistence within host macrophages has been associated with inflammation and also granuloma formation (Meconi *et al.* 2007). Indeed, good efficacy of a wide range of broad spectrum antibiotics has been demonstrated against intramacrophagic AIEC HM605 in previous work, that also reported complete killing with ciprofloxacin (Subramanian, Roberts, *et al.* 2008). However, the ability of a narrow spectrum and potent antimicrobial to penetrate eukaryotic cells sets colicins apart from traditional antibiotics.

When considering the treatment of AIEC infections in CD patients, we envisage that colicin delivery would take the form of a colicin producing probiotic. Indeed in

this work, we demonstrated the ability of a colicin producing commensal *E. coli* strain to kill AIEC associated with intestinal epithelial cells that express CEACAM6 receptors. This result indicates that colicin producing bacteria, that can be readily isolated from the healthy gut microbiome, may be effective in the treatment of AIEC bacteria colonising the gut mucosa of CD patients (Cursino *et al.* 2006). Additionally, this work has provided further support for the utilisation of colicin treatments as it showed that colicin treatment of eukaryotic cells did not induce cytotoxicity (Figure 3.13). Although yet to be confirmed *in vivo*, this highlights the potential applicability of colicins as antimicrobial therapies in humans.

Due to the targeted killing mechanism shown by colicins, they represent a novel class of 'personalised medicine' i.e therapies that can be 'matched' to patient gut profiles. As a major feature of CD is intestinal dysbiosis, colicins in the form of personalised medicine could be administered to juvenile patients who show genetic susceptibility of developing CD. Indeed, a multitude of data now demonstrates an association between increased antibiotic exposure in early childhood and increased risk of developing disorders such as asthma, IBD and rheumatoid arthritis (Arvonen *et al.* 2015, Shannon L Russell *et al.* 2012, Ungaro *et al.* 2014). By similar concepts, colicins could function in a reverse manner to broad spectrum antibiotics and shift microbiota populations to a 'non – risk' composition in patients who are genetically susceptible to these disorders. Long term colicin treatment assays in murine models will give insights into the ability of colicin-like bacteriocins to permanently alter the gut microbiota composition even after treatment has ceased. Animal models will also contribute to overcoming the current limitations of colicins. These include instability of colicins *in vivo* and stimulation of host immune response that may involve the production of anti-colicin antibodies. Furthermore, for treatment of intestinal infections, a key limitation is the method of delivery. Alternative methods of delivery aside from probiotic producing strains may include methods of encapsulation that protect colicin protein from the protease enriched intestine (see Chapter 4).

Good efficacy of colicin-like bacteriocins will rely on extensive knowledge on the target strain involved. In coherence with current attempts to improve detection methods for pathogenic agents (Longitude prize), colicin-like bacteriocins represent an ideal personal medicine that will partner this strategy. Colicin-like

bacteriocins represent an innovative and clinically relevant form of antibiotics and deserve increased opportunity within the clinical setting.

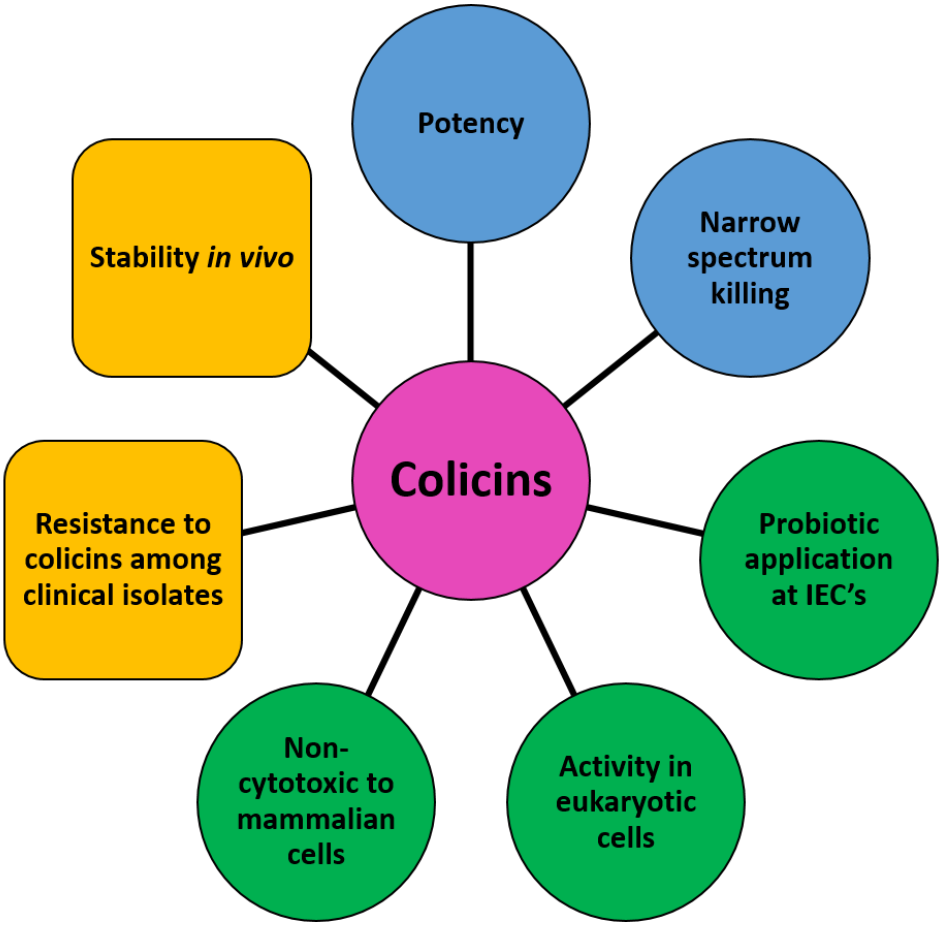


Figure 3-13: Colicins as an alternative to traditional antibiotics.

Figure shows attractive properties of colicins for their role as antibiotics demonstrated previously (blue), in this work (green) and that have yet to be extensively demonstrated (yellow).

4 Encapsulation of purified colicin for the treatment of persistent LF82 intestinal infection in mice

Foreword

Probiotic colicin producing bacteria represent an ideal approach for the treatment of AIEC infection that is associated with CD and hence intestinal dysbiosis. However, limitations of this method include variations in the composition of patients' gut microbiota, establishing long term colonisation of the probiotic strain and also the potentially low concentration of colicin produced. The use of purified protein is an alternative strategy for colicin treatment of AIEC infection. This method would facilitate the use of increased, but also known concentrations of colicin and would allow use of combinations of different proteins or 'colicin cocktails'. The work described in this chapter investigates the use of purified colicin for treatment of intestinal LF82 infection in mice following its encapsulation into colon targeting pectin zein hydrogel beads.

4.1 Introduction

4.1.1 Colonic microbiota digestible biopolymers as carriers for colon specific drugs

Protein therapeutics have increased dramatically in number and frequency of use since the introduction of the first recombinant protein therapeutic, human insulin, 25 years ago (Clark *et al.* 1982). At present, more than 130 different proteins or peptides are approved for clinical use by the FDA, and many more are in development (Leader *et al.* 2008). However, oral administration of protein therapeutics is very difficult due to their low bioavailability. Protein degradation occurs at high levels in the stomach and duodenum but less so in the ileum and colon (Sinha and Kumria 2003). As such, various delivery systems have been developed to target drug absorption from the colon and ileum. An effective delivery system must protect the encapsulated drug en route to colon and minimise exposure to digestive enzymes. The approaches commonly used in

formulating oral protein delivery systems include use of specific excipients (absorption enhancers, enzyme inhibitors and mucoadhesive polymers) or protective formulations (Park *et al.* 2011). A key example of the latter are biodegradable polymers that are specifically digested by enzymes produced by colonic bacteria (Sinha and Kumria 2003).The ability to utilise complex dietary and host glycans is essential for the survival of prominent members of the gut microbiota (Bourquin *et al.* 1993, Macfarlane and Macfarlane 2012). Fruits, vegetables and cereals are major components of human diet and provide carbohydrates, digested by human intestinal enzymes and also fibrous material that is not readily digested in the small intestine. Members of the microbiota extract energy from these undigested dietary products by fermentation (El Kaoutari *et al.* 2013, Miller and Wolin 1979, Sinha and Kumria 2003). For this purpose, members of the colonic microbiota produce a wide range of enzymes that include glucosidases, xylosidases, araninosidases, galactosidases and deaminases. These are referred to as carbohydrate–active enzymes (CAZymes) (El Kaoutari *et al.* 2013) The main saccharolytic species in the colon are *Bacteroides* and *Bifidobacterium* (Sinha and Kumria 2003). As these bacterial enzymes are produced exclusively in the colon, the use of microbial biodegradable polymers for drug delivery represents a site specific approach and hence highly suitable for treatment of colonic disorders.

4.1.2 Pectin / zein hydrogel beads for colon specific colicin delivery

Pectin is a natural heteropolysaccharide distributed abundantly in fruits and vegetables (LinShu Liu, Fishman and Hicks 2006). It is comprised of 1, 4 linked alpha-D-galactosyluronic acid residues with a range of neutral sugars including rhamnose, galactose, and arabinose (Figure 4.1).

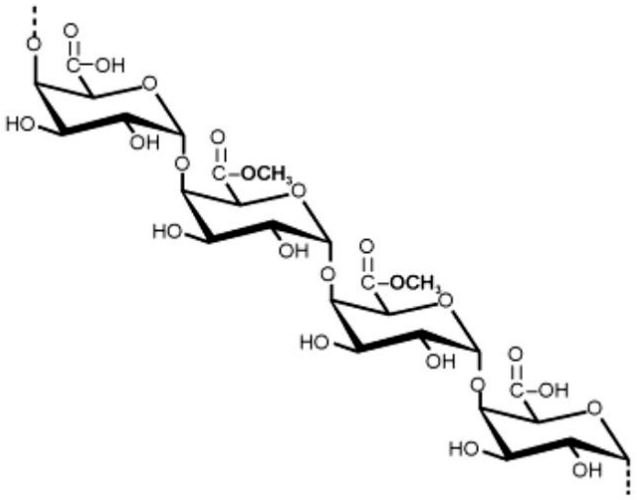


Figure 4-1: Chemical structure of pectin.

Repeating segment of pectin molecule and functional groups. Image adapted from (Thakur *et al*. 1997).

Pectin possesses several properties that make it an ideal colon specific drug delivery vehicle. Pectin is gastro-resistant and is selectively digested by microflora in the colon as a soluble dietary fibre. Pectin has also been shown to increase gastric transit time that would be useful when swift drug delivery is required (Wong *et al.* 2011). Additionally, pectin undergoes 100% fermentation in the colon that generates the production of short chain fatty acids that perform several beneficial roles for bowel health. The functional model for pectin as a colon targeted delivery vehicle involves: 1) adsorption of colonic microbiota and enzymes upon pectin matrix surface; 2) degradation of matrix by bacterial pectinases and 3) drug release at desired site. Pectin gels have been extensively tested for applications in oral drug administration. This has included amidated pectin hydrogel beads prepared for colon specific delivery of indomethacin and sulfamethoxazole (Munjeri *et al.* 2015). One limitation associated with the use of pectin gels for colon-specific drug delivery is the pectin based matrix is prone to swelling as well as erosion in aqueous medium leading to premature drug release at the upper GI tract. Numerous approaches have been investigated to prevent pectin based matrix undergoing premature drug release. For example, crosslinking pectin with calcium ions or zinc ions and complexation with polymers (chitosan and Eudragit TM) has been shown to reduce solubility, swelling and erosion of the

polymer, whilst increasing stability (LinShu Liu, Fishman and Hicks 2006, Turkoglu and Ugurlu 2002).

Recent work has investigated complexation of pectin with zein, a major storage protein of corn kernels (LinShu Liu, Fishman, Hicks, *et al.* 2006). Zein has been patented for the production of protein microspheres, that are able to protect encapsulated drugs from stomach acid and release them in the small intestines on protease digestion (Mathiowitz *et al.* 1993). It was shown previously that inclusion of a small portion of zein into the pectin matrix suppressed the swelling behaviour of pectin and stabilised the pectin matrix (LinShu Liu, Fishman, Hicks, *et al.* 2006). In a later study, pectin / zein hydrogel beads were used to successfully deliver a probiotic protein, p40, to the mouse colon where it activated its target protein, EGFR, in colonic epithelial cells (Yan *et al.* 2011). These data highlight the potential of pectin / zein hydrogel beads as a novel type of colon specific drug delivery vehicle. Along with the premenitoned prebiotic properties of pectin, this system represents a potential method for delivery of colicin proteins to the CD colon, which in a portion of patients is abnormally colonised by AIEC.

4.1.3 Model of LF82 intestinal infection in conventional mice

Previous work has demonstrated that persistent AIEC LF82 intestinal infection in mice requires depletion of gut microbiota or expression of specific host factors such as human CEACAM receptors (Benoit Chassaing, Koren, *et al.* 2014, Small *et al.* 2013). In this work, a murine model of persistent LF82 colonisation was utilised, that was established by Gillian Douce and Zoe Marjenberg (University of Glasgow; Institute of Infection, Immunity and Inflammation). In this model, conventional mice were first treated with streptomycin, to eradicate gut microbiota populations, followed by infection with streptomycin resistant AIEC LF82 strain, LF82::Strp^{R}. Persistent colonisation of murine intestine with LF82::Strp^{R} was facilitated by supplementation of drinking water with streptomycin throughout the experiment. Typically, colonisation by LF82 occurs in the murine intestine for 9 days with approximately 10^5 CFUs detected in faecal pellets on day 9 (Figure 4.2; Douce and Marjenbern, unpublished data). LF82 are detected in the caecum (approx. 10^6 CFUs) and colon (approx. 10^5 CFUs) 3 days after infection. At 9 days post-infection, approximately 10^5 CFUs and 10^4 CFUs are detected in the caecum and colon respectively (Figure 4.2c).

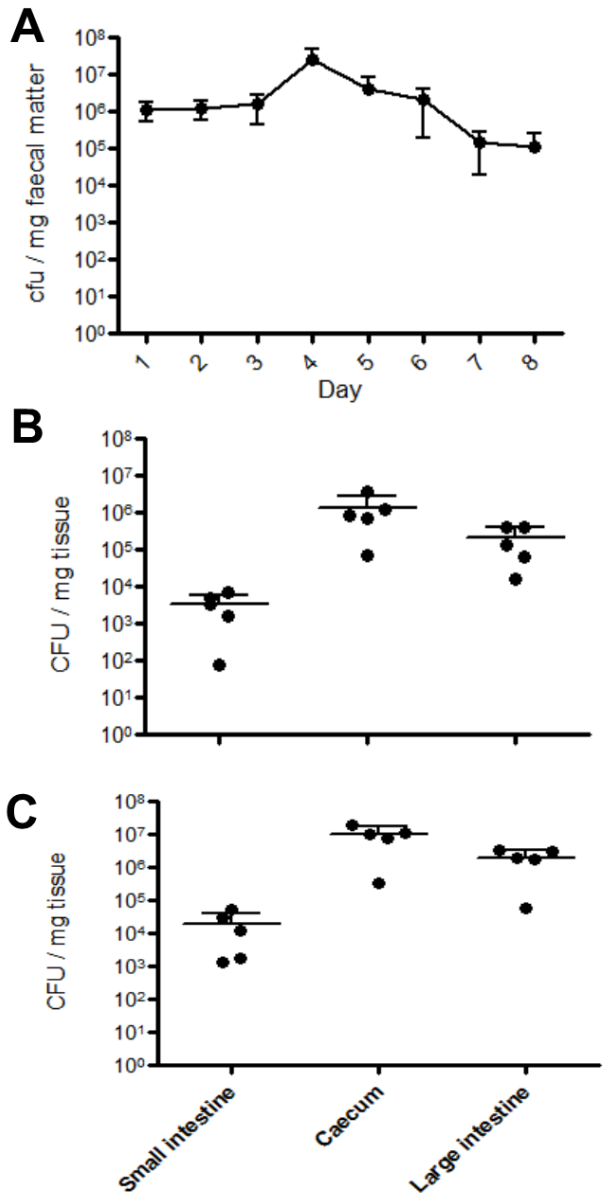


Figure 4-2: Persistent intestinal colonisation with LF82 in conventional CF-1 mice treated with streptomycin.

A) Figure shows LF82 faecal CFUs. CF-1 mice were treated with streptomycin (100 mg ml^{-1}) and infected with LF82::$Strp^R$ for 18 h and faecal pellets were collected for 9 days and serially diluted in PBS and plated on LB agar containing streptomycin (100 µg ml^{-1}) and erythromycin (500 µg ml^{-1}|). Plotted dot represents average CFU count for group (n=5). Error bars shown are SD. B) Figure shows LF82 CFUs detected in intestinal tissue samples. LF82 infected mice were culled at 3 days and tissues were extracted and homogenised and diluted to determine CFU counts. C) LF82 bacteria counts detected in intestinal luminal samples (3 days). Infected mice were culled and luminal contents were flushed from organs and plated to determine CFU counts. Each dot represents one mouse. Black line shown represents mean and error bars show SD. Data shown in figure obtained by Zoe Marjenberg (University of Glasgow; Unpublished).

4.2 Aims

The aim of the work described in this chapter was to test the ability of purified colicin to reduce bacterial numbers in a mouse model of AIEC intestinal colonisation. In order to achieve this, a delivery method was established in which colicin was encapsulated in pectin/ zein hydrogel beads. Encapsulated colicin was then delivered orally to the GI tract of AIEC colonised animals.

4.3 Results

4.3.1 Preparation of pectin / zein hydrogel beads

In order to test the suitability of pectin / zein hydrogel beads for delivery of colicin to the mouse colon, beads were prepared as previously described (LinShu Liu, Fishman, Hicks, *et al.* 2006). Pectin solution prepared from pectin (poly-D-galacturonic acid methyl ester; ≥74% esterified) from citrus peel (Sigma) was dropped manually by syringe into zein / $CaCl_2$ solution at room temperature. Beads formed immediately as droplets entered reception solution and were then washed and dried (Figure 4.3). Several parameters were optimised for this process including syringe needle diameter (23 gauge-30 gauge), pectin concentration (2-6%) and zein concentration (0.5-1%). Beads were stored at 4°C and were used within one week. Due to manual dropping by syringe, each round of bead production yielded approximately 200 beads. Beads were homogenous in appearance, were an average diameter of 1- 2mm and were stable in deionised water, PBS, 0.5% NaCl and 50 mM Tris-HCl (Figure 4.3). Beads also retained structure and rigidity for one month at 4°C. Beads produced by this method were used as control beads for all mouse experiments.

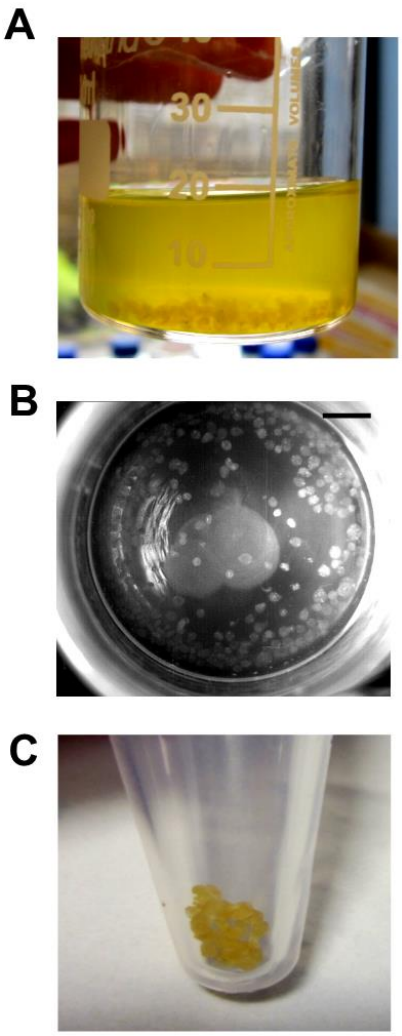


Figure 4-3: Preparation of pectin / zein hydrogel beads.

A) Pectin droplets in zein solution (1% zein; 85% EtOH; 0.5% $CaCl_2$) at room temperature. Beaker shown represents “one batch” production method. B) Light micrographs of washed pectin / zein hydrogel beads. Scale bar shown is 10 mm. C) Dried pectin / zein hydrogel beads. Following drying at 30°C beads showed highly robust consistency.

4.3.2 Colicin E1 encapsulated within pectin / zein hydrogel beads is active against LF82

For protein encapsulation, colicin E1 was purified as previously described and was diluted to 10 mg ml^{-1} in pectin solution and dropped into zein / $CaCl_2$ solution to form colicin-containing beads. As stated before approximately 200 beads were yielded from each production batch. To determine the efficiency of colicin encapsulation into pectin / zein hydrogel beads, dried beads were run on SDS-PAGE. A single bead (~15 mg) and five beads (~75 mg) were each dissolved in 0.5% NaOH, 20% Tween and diluted in SDS running buffer and run on SDS-PAGE (12% polyacrylamide). For every assay, bead samples were also run alongside known concentrations of colicin E1 to determine band intensities for standard concentrations (Figure 4.4).

For samples consisting of one colicin loaded pectin / zein bead, a band was detected at the corresponding size for colicin E1 (62kDa) and resolved at a similar intensity to band observed for colicin E1; 625 ng (Figure 3.4). Additionally, for 5 bead samples, a band that resolved at a similar intensity to band observed for colicin E1; 4 µg was detected (Figure 4.4). This indicated that approximately 700 ng of colicin E1 was encapsulated per one hydrogel bead. The encapsulation efficiency was calculated by: (actual E1 encapsulated / total E1 added) * 100 and was calculated as 1.4%. The encapsulation efficiency was determined for every batch of colicin containing hydrogel beads produced and utilised for further investigation.

In order to test the cytotoxic activity of colicin E1 beads, overlay spot plate assays were performed using both colicin E1 beads and control beads. For killing assays a single dried bead was placed on a growing lawn of LF82::$Strp^R$ and incubated overnight at 37°C. Colicin E1 beads showed good killing of LF82 and control beads failed to inhibit growth of LF82 lawn (Figure 4.4). This indicated that encapsulation of colicin within pectin / zein matrix does not affect the cytotoxic function of colicin E1.

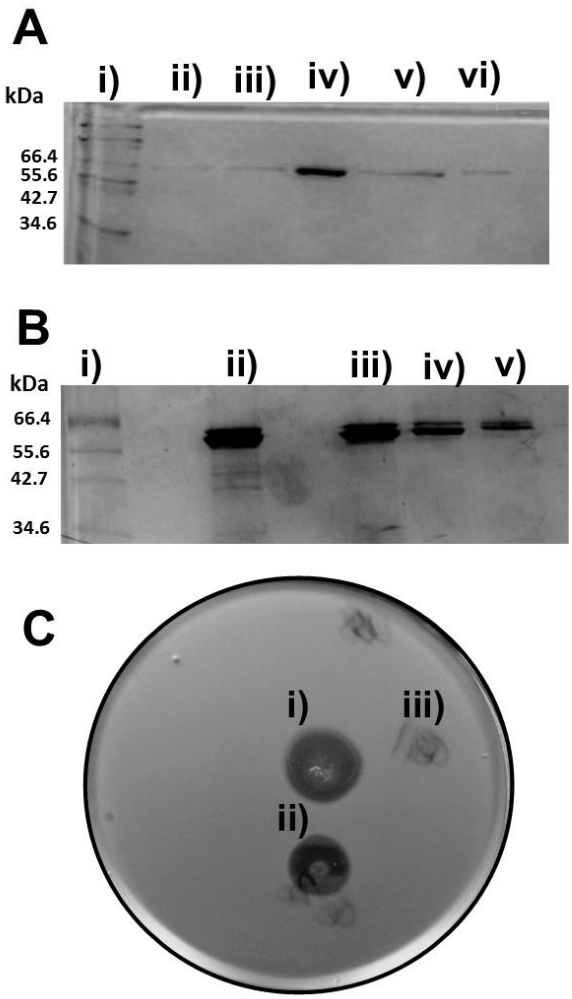


Figure 4-4: Encapsulation of colicin E1 in pectin / zein hydrogel beads.

A) Figure shows SDS-PAGE gel (12% polyacrylamide) for visualisation of colicin protein encapsulated in one pectin / zein hydrogel bead. ColE1 (10 mg ml^{-1}) was mixed with pectin $CaCl_2$ and dropped into zein solution to form beads. To determine concentration of colicin E1 encapsulated, beads were dissolved in 20% Tween, 0.5% NaOH, mixed with SDS running buffer, boiled and run on SDS PAGE. Lanes are: i) Protein molecular weight ladder; ii) ColE1 bead A, iii) ColE1 bead B; iv) ColE1 2.5 µg ; v) ColE1 1.25 µg; vi) ColE1 625 ng B) Figure shows SDS-PAGE gel for visualisation of colicin E1 encapsulated in five pectin / zein hydrogel beads. i) protein molecular weight ladder ii) protein band resolved from 5 ColE1 beads iii) E1 4 µg , iv) ColE1 2 µg, v) Col E1 1 µg. C) Overlay of hydrogel beads on LF82 bacterial lawn. i) + ii) Col E1 bead, iii) control bead.

4.3.3 Encapsulated colicin treatment does not eliminate LF82 intestinal infection

To determine if encapsulated colicin was active *in vivo* against intestinal LF82, colicin E1 loaded beads were administered to infected mice by oral gavage.
In an initial experiment, streptomycin treated mice (n=5) were infected with approximately 10^8 CFUs of LF82::Strp^R for 18 h and then received five colicin E1 loaded pectin/ zein beads in distilled water (100 µl) containing approximately 4 µg colicin E1. For the control group (n=5), mice received beads not loaded with colicin E1. Faecal pellets were collected after 18 h to determine bacterial load in all groups. Faecal pellets were homogenised, serially diluted and plated on LB agar containing streptomycin (100 µg ml^{-1}) and erythromycin (500 µg ml^{-1}) and incubated overnight at 37°C. Colicin-loaded bead treatment of mice showed significantly reduced bacterial counts relative to control bead treated mice (P=0.01) (Figure 4.5a).
From these data it was postulated that increased and prolonged doses of colicin-loaded beads would result in a larger reduction in LF82 intestinal infection. To investigate this, streptomycin treated mice were infected with LF82::Strp^R for 18 h as stated but then received a double dose of ten colicin loaded beads, containing approximately 8 µg of colicin E1. In addition to this, 10 colicin loaded beads were administered to mice every day, for 7 days. Bead treatments were administered approximately 18-22 h apart. Faecal pellets were collected every 18 h and were plated as described to determine bacterial load. LF82 was shown to colonise mice for 8 days and high bacterial numbers (approximately 10^4 – 10^6 CFUs) were detected (Figure 4.5b). In mock treated mice, peak CFU counts were observed for LF82 at day 4 (Figure 4.5b). These data showed that bacterial loads were not significantly different among colicin treated and control groups at all time points tested (P>0.05) (Figure 4.5b). At day 4 – day 7 a reduction in bacterial load was shown for colicin E1 bead treated group relative to control group but this was not significantly different (P> 0.05) (Figure 4.5c).

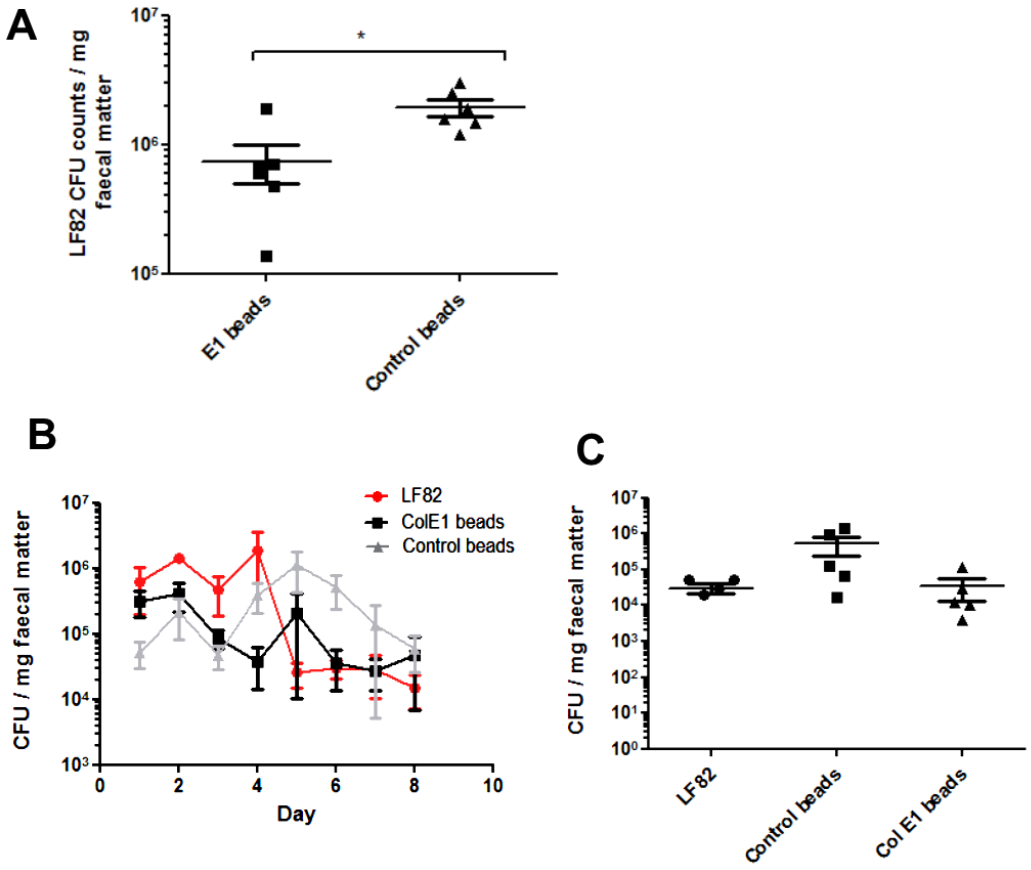


Figure 4-5: Treatment of persistent LF82 colonisation in conventional mice with colicin E1 loaded pectin / zein hydrogel beads.

A) Treatment of LF82 infection with colicin E1 encapsulated in pectin / zein hydrogel beads at 18 h post infection. Mice were infected with LF82::Strp^{R} for 18 h and treated with 5 colicin loaded beads (~75 mg) by oral gavage. Each bead contained approximately 700 ng purified colicin E1. For the control group, beads were not loaded with colicin. Faecal pellets were collected 18 h after colicin treatment to determine bacteria counts. Colicin bead treatment significantly reduced bacterial load (P=0.01). Each symbol represents one mouse. Black line and error bars shown are mean and SEM. * shows significant difference between groups (P < 0.05). Non-parametric Mann–Whitney U-test was carried out for statistical significance. B) Daily treatment of LF82 infection with double and prolonged dose of colicin loaded beads. Streptomycin treated mice were infected with LF82::Strp^{R} and received ten colicin E1 loaded beads by oral gavage 18 h after infection and then daily for 7 days. Dot represents mean CFU counts for treatment group (n=5). Day 0= day of infection. Day 1= first bead treatment. No significant difference in bacterial load was observed between all groups at all time points. C) Bacterial load detected at day 6 in all groups for daily treatment with colicin beads. Error bars shown are mean and SEM. No significant difference detected in bacterial load between colicin E1 and control bead treated groups (P=0.055). Non-parametric Mann–Whitney U-test was carried out for statistical significance.

4.3.4 Treatment of LF82 colonised mice with non-encapsulated colicin E1

As colicin E1 loaded beads (4-8 µg) did not show good efficacy against LF82 colonising the murine intestine, we tested the activity of purified colicin E1 solution. For this, streptomycin treated mice were infected with 10^8 CFUs of LF82::Strp^R for 18 h as previously described. Mice were administered with sodium bicarbonate and then received colicin E1 (10 mg ml $^{-1}$) in PBS (100 µl) by oral gavage. Mice received colicin E1 solution daily for 7 days. Mock-treated group received PBS only. Bacterial load was monitored by faecal output as stated. LF82 intestinal infection was significantly reduced at day 2 for colicin E1 treated mice compared to mock treated mice (P= 0.0195; Figure 4.6). A reduction in bacterial load was detected at day 4 for colicin E1 treated group relative to mock-treated group but this was not significantly different (P>0.05; Figure 3.6). These data indicate that colicin E1, when used in the form of purified protein and encapsulated within hydrogels is not an effective therapy against intestinal LF82 in this specific murine model of infection.

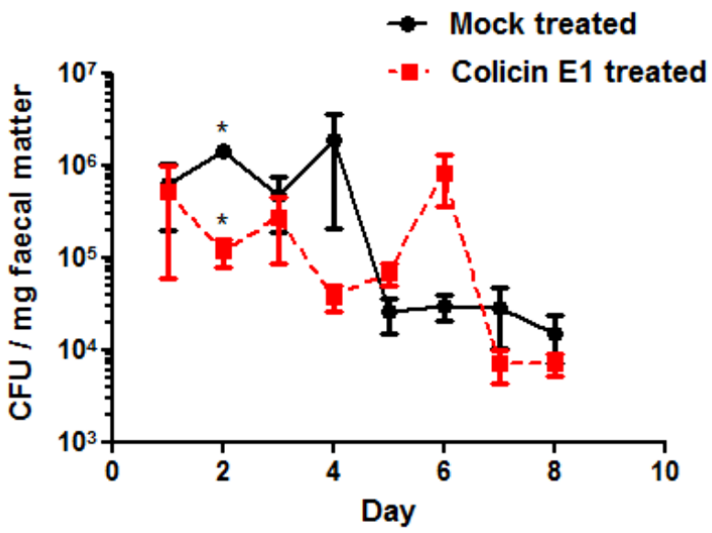
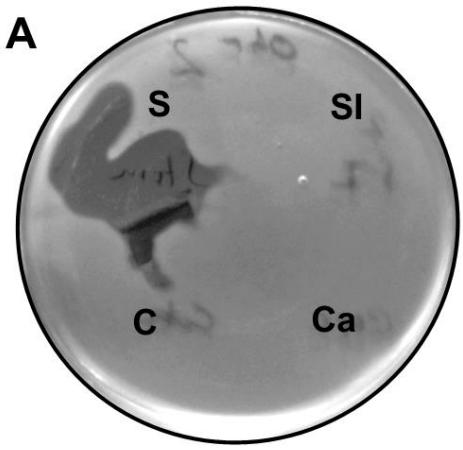


Figure 4-6: Treatment of LF82 intestinal infection with recombinant colicin E1 solution.
A) Mice were infected with LF82::Str^R for 18 h and received colicin E1 (10 mg ml^{-1}) by oral gavage (100 µl). Colicin E1 treatment was administered daily for 7 days. Bacterial loads were determined by plating homogenised faecal pellets on LB agar. Dot represents mean CFU count for treatment group (n=5). Day 0 = day of infection. Day 1= first colicin treatment. Scale bars shown represent SEM. To compare bacteria numbers non-parametric Mann–Whitney U-test was carried out at all time points for statistical significance. * shows significant difference between groups (P< 0.05). LF82 intestinal infection was significantly reduced at day 2 for colicin E1 treated mice compared to mock treated mice (P= 0.0195).

4.3.5 Assessment of colicin E1 stability in murine gastrointestinal tract

It was postulated that poor killing of colonic LF82 by colicin E1 could be explained by digestion or inactivation of colicin in the murine small intestine. To ascertain if colicin administered via the oral route remained active in the murine intestine, mice that had not been exposed to LF82, received 0.1 M sodium bicarbonate and were administered with colicin E1 in PBS (10 mg ml^{-1}) by oral gavage (100 µl). Mice were culled 30, 60 and 120 min post treatment and intestinal organs were extracted and homogenised. Colicin activity in organ samples was tested via overlay spot plate method and LF82::Strp^R was used as the indicator strain. Colicin

activity was detected in stomach samples at all time points tested. This was shown in the form of a clear and large zone of killing at 30 and 60 min and as a diffuse zone of killing at 120 min. No colicin activity was detected in the small intestine, cecum and colon tissue samples at all tested time points (Figure 4.7). This indicates that colicin E1 was inactivated in the small intestine.



B

Tissue	Time 30min	60min	120min
Stomach	+	+	~
Small intestine	-	-	-
Cecum	-	-	-
Colon	-	-	-

Figure 4-7: Colicin stability in murine gastrointestinal tract.

Colicin E1 is inactivated in the small intestine. Conventional mice were treated with colicin E1 (10 mg ml^{-1}) and were culled at 30, 60 and 120 min post treatment. A) Plate shows spot plate results from organs extracted from mice culled at 60 min. Intestinal tissues were extracted and homogenised and suspension was spotted on agar lawn of LF82::$Strp^R$. Zones of inhibition represent killing of LF82. S; stomach, SI; small intestine; Ca' cecum; C; colon. Image is representative image of three replicate spot assays from 5 intestinal tissue samples of 5 mice. B) Table showing colicin activity detected in intestinal organs 30, 60 and 120 min post treatment. + denotes large zone detected in spot plate assay, ~ denotes small diffuse zone and – denotes no zone.

4.4 Discussion

The use of colicins as a novel therapy for CD associated AIEC infection demonstrates several attractive attributes, one being the preservation of resident bacteria populations in a microbial community likely showing dysbiosis. Due to this, we were extremely interested to investigate the efficacy of colicins against AIEC growing in the gastrointestinal tract. For this purpose, we utilised an established model of persistent LF82 colonisation (Marjenberg and Douce, University of Glasgow) that involved infection of conventional mice following streptomycin depletion of gut microbiota species. For this model, LF82 colonisation was monitored by bacterial load in faecal pellets and infected mice did not display symptoms of colitis or illness. The method of encapsulation utilised in this work was adapted from previous work that represents the first investigation of pectin / zein hydrogel beads for colon specific drug targeting (LinShu Liu, Fishman, Hicks, *et al.* 2006). This specific method of encapsulation was selected for 2 reasons: 1) the simple encapsulation process involved and 2) beads could be administered orally to mice without a requirement for anaesthesia. Although data obtained in this chapter indicates that encapsulated colicin shows poor activity against intestinal AIEC, the method utilised in this pilot study prevents us from making valid conclusions on the prospect for using colicins against AIEC.
Firstly, due to the poor efficiency of colicin encapsulation, very low concentrations of colicin E1 were administered to infected mice. Taking into account the dilution of colicin E1 in the murine GI tract, it is likely that the final concentration of colicin E1 reaching the site of AIEC colonisation was extremely low. Furthermore, as drug release at the colon target site requires colonic microbiota pectinase activity, by utilising a streptomycin treated murine model, it is likely that the one component required for this delivery vehicle was absent or severely reduced. Indeed, the slight reductions in bacterial load detected in colicin treated groups indicates that colicin E1 may be reaching its target site. Delivery of encapsulated protein drug to its target site was determined by western blot detection in the pioneer study utilising pectin/zein hydrogel beads (Yan *et al.* 2011). This should be incorporated into future work utilising this colicin treatment model.

It must also be realised that for future assessment of colicin efficacy, alternative murine models of AIEC infection should be utilised that accurately depict the role of this *E. coli* pathovar in CD. This would ensure that both killing of intestinal AIEC and alleviation of AIEC-induced symptoms could be investigated. Good examples of such models include chronic colitis induced by AIEC infection in TLR-5 deficient (T5KO) mice that are raised in a GF environment (Benoit Chassaing, Koren, *et al.* 2014). Additionally, in mice that express human CEACAM receptors, LF82 persists in the intestine and induces severe colitis relative to wild type mice (Frédéric a Carvalho *et al.* 2009). Future work that utilises a more accurate model of CD associated AIEC infection will give greater insights into the applicability of colicins as a therapy for CD patients and other intestinal *E. coli* infections.

Encapsulated bacteriocins for the treatment of intestinal bacterial infections has been previously investigated whereby *Campylobacter jejuni* infection in chickens was reduced approximately one million fold following oral administration of encapsulated bacteriocins isolated from *Lactobacillus salivarius* and *Paenibacillus polymyxa* (Norman J Stern *et al.* 2005). In this work, purified bacteriocins were micro-encapsulated in polyvinylpyrrolidone (PVP) and added into chicken feed at 250 mg bacteriocin/kg feed, suggesting that in this case, alternative methods of encapsulation that facilitated better loading of colicin, should be attempted.

When considering long term and clinical implications, this therapy could be utilised as both a treatment and a prophylactic for AIEC infection in CD. CD patients could receive encapsulated colicin immediately after diagnosis to prevent abnormal colonisation by AIEC and further dysbiosis of gut microbiota. Indeed, for such a treatment to be utilised effectively, further information on the role of AIEC in CD is required to ensure accurate targeting of drug and that treatment begins before inflammation. This relies on determining whether AIEC is a primary cause of CD associated inflammation or is in fact, a commensal *E. coli* that behaves pathogenetically in the hostile CD gut environment. Key components of this will be investigated in the following chapter.

5 The prevalence of AIEC-associated phenotypes in *E. coli* isolated from HLA-B27 positive juvenile idiopathic arthritis (JIA) patients

5.1 Supporting evidence

5.1.1 Colonic *E. coli* are increased in HLA-B27 positive JIA patients

Juvenile idiopathic arthritis (JIA) is classified as a clinically heterogeneous group of chronic, inflammatory diseases characterised by arthritis that begins before the age of 16 (Weiss 2012). Cohort studies have demonstrated that 45% of enthetis related arthritis (ERA) JIA patients are HLA-B27 positive (+) (Stanevicha *et al.* 2010). A pilot study, performed at Yorkhill Hospital, showed increased colonic *E. coli* in JIA patients compared to healthy controls (Gerasimidis *et al.*, unpublished work) (Figure 5.1a). Additionally, following subdivision of JIA patients into HLA-B27 (-) and HLA-B27 (+) groups, it was reported that increased *E. coli* numbers were detected in HLA-B27 (+) patients compared to HLA-B27 (-) patients (Figure 5.1b). Results obtained from this pilot study also showed reduced *Lactobacillus* numbers in JIA patients and increased *Faecalbacterium prausnitzii* relative to healthy controls (Figure 5.1a). Additionally, faecal calprotectin measurements showed normal levels of this intestinal inflammatory marker in the majority of JIA patients relative to healthy controls indicating that increased colonic *E. coli* numbers are not a consequence of pre-existing inflammation.

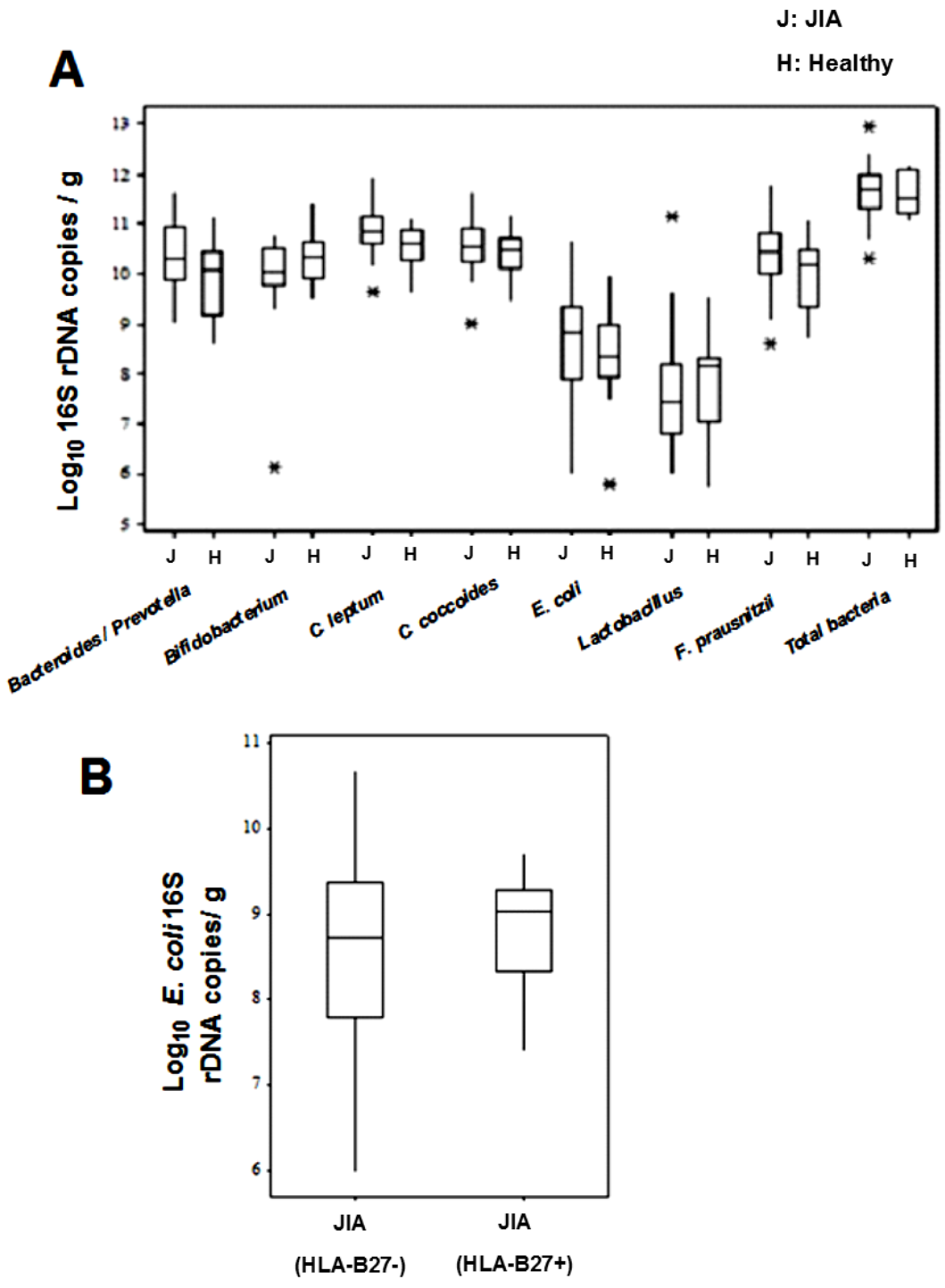


Figure 5-1: Intestinal dysbiosis and increased E. coli in HLA-B27 positive JIA patients.

A) Adapted from figure obtained from Yorkhill study investigating gut microbiota composition in JIA (Gerasimidis *et al.*, 2011-2012. Unpublished work). Box plots for faecal bacteria in healthy and JIA patients determined by quantitative polymerase chain reaction using primers targeted at *16S* rDNA. For each set of data, the horizontal line within the box is the median, the box ends are the interquartile range, and the whiskers represent the absolute range. B) Box plots for faecal *E. coli 16S* rDNA in JIA NULL (HLA-B27 negative) and JIA RISK (HLA-B27 positive) patients. Results show increased median bacteria counts for faecal *E. coli* in JIA RISK patients.

5.2 Background

5.2.1 The association between HLA-B27 and Crohn's disease

Ankylosing spondylitis (AS) is regarded as the prototype of seronegative spondyloarthropathies (SpAs), that are a group of chronic inflammatory diseases affecting the bowel, joints and the axial skeleton (Rashid *et al.* 2013). AS is predominantly described as chronic inflammation of joints of the spine. Other disease entities of SpA include reactive arthritis, psoriatic arthritis and arthritis associated with inflammatory bowel disease (IBD), that includes Crohn's disease (CD) (de Vlam *et al.* 2000). AS and spondylitis associated CD are interlinking conditions based on certain genetic, clinical, immunological and microbial features. For over 3 decades, human leukocyte antigen B27 (*HLA-B27*) gene has been associated with ankylosing spondylitis (AS) and related spondyloarthropathies (Brewerton *et al.* 1973). HLA-B27 is a cell-surface protein encoded by the B locus in the major histocompatibility complex (MHC) that functions in the presentation of peptide antigens to T cells (Joel D Taurog 2007). It's association with AS is one of the strongest known associations between an HLA molecule and disease. Approximately 95% of AS patients carry at least one HLA-B27 allele compared with <10 % of healthy individuals (Brewerton *et al.* 1973). The frequency of this molecule in the general IBD population is found to be comparable to the healthy control population (Figure 5.2)(J *et al.* 1988, Palm *et al.* 2002, Peeters 2004). However, IBD patients with AS often show a significantly higher association with HLA-B27 (25–78% being HLA-B27 positive), suggesting that HLA-B27 is a risk factor for the development of AS in these patients (Figure 5.2)(Palm *et al.* 2002, Steer *et al.* 2003, de Vlam *et al.* 2000). Furthermore, AS is frequently associated with IBD with 5-10% of cases having clinically overt IBD and approximately 70% of cases having subclinical bowel inflammation (Thomas and Brown 2010, De Vos *et al.* 1989). Additionally, approximately 30% of CD patients present with axial and peripheral arthritis (Hammer *et al.* 1990, Mielants *et al.* 2005, Orchard and Holt 2009). Furthermore, gut changes varying from acute to asymptomatic chronic inflammation, are observed in 60% of patients with SpA's, and disease remission is frequently preceded by the disappearance of gut inflammation (Demetter *et al.*

2002). These data indicate a pivotal role for gut inflammation and HLA-B27 for the development of SpA's, especially CD and AS.

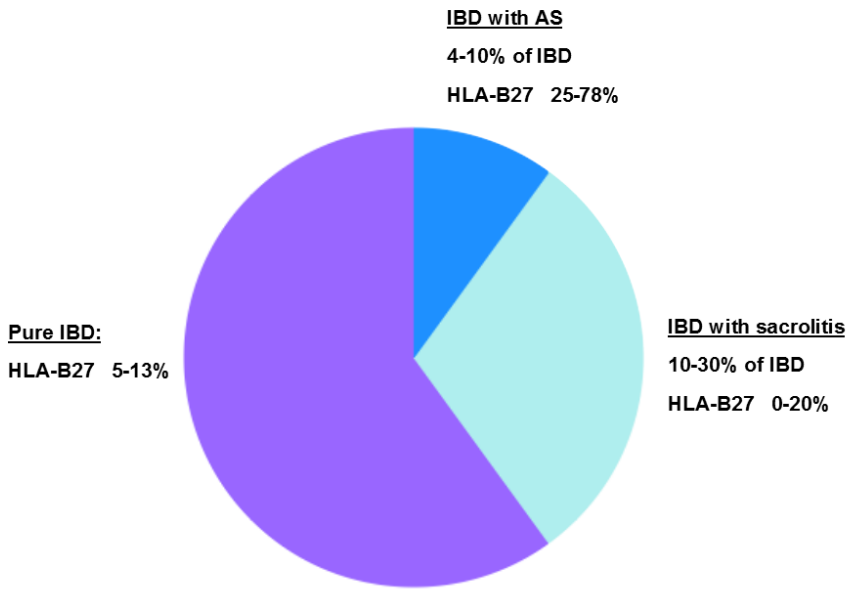


Figure 5-2: Proportion of phenotypic and genetic subsets in patients with IBD.
Figure shows approximate frequency of HLA-B27 in each subset of IBD. Figure adapted from Rudwaleit *et al.*(Rudwaleit and Baeten 2006).

5.2.2 HLA-B27 is associated with intestinal inflammation and dysbiosis

The HLA-B27 transgenic rat model represents a unique model to understand the role of the HLA-B27 gene in SpAs associated with IBD (Milia *et al.* 2009). Rats transgenic for HLA-B27 spontaneously develop chronic inflammatory disease that resembles the human SpA, involving colitis and peripheral arthritis (Hammer *et al.* 1990). Several important factors of this model have been established, including it's dependency on bacterial flora and the immune system (Milia *et al.* 2009). Typically, colitis and arthritis does not develop in HLA-B27 transgenic rats that are raised in a GF environment, although transfer of rats out of this environment causes spontaneous intestinal inflammation to develop (Hammer *et al.* 1990; Taurog *et al.* 1994). Intestinal inflammation detected in this model involves progressive chronic gastrointestinal inflammation (mostly involving the colon) and the pro-inflammatory cytokine pattern of HLA-B27 rats corresponds to that observed in CD (Faure *et al.* 2004).

Interestingly, increased *E. coli* and *Enterococcus* numbers were detected in HLA-B27 transgenic rats with chronic colitis (Onderdonk *et al.* 1998). Moreover in this model, authors failed to detect the presence of enterotoxin genes in resident *E.coli* populations, indicating an imbalance of commensal *E. coli* rather than pathogenic *E. coli* species. In other work, severe colitis was induced by reconstitution of germ free HLA-B27 transgenic rats with *Bacteroides vulgatus* and monocolonisation of rats with *E. coli* induced very mild colonic inflammation (Rath *et al.* 1999). It is yet to be determined if increased *E. coli* populations associated with HLA-B27 demonstrate any phenotypic similarity to CD associated AIEC.

5.3 Hypothesis and aims

5.3.1 Hypothesis

Although dysbiosis and increased levels of AIEC are strongly associated with CD, the exact role of the gut microbiota in CD is poorly understood. This is due to the inability of previous studies to establish whether alterations to the gut microbiota are a consequence of disease and not a primary factor in the development of CD. The hypothesis we attempted to test in this work was that JIA patients who are HLA-B27 positive and are at increased risk of developing CD, show increased levels of AIEC, that precede the development of intestinal inflammation. If this is the case then this would strengthen the idea that AIEC is an important primary factor in the development of CD.

5.3.2 Aims

- Isolate *E.coli* from faecal samples of JIA HLA-B27 (-) (JIA NULL), JIA HLA-B27 (+) (JIA RISK) patients and healthy patients using standard cultivation techniques.
- Determine if isolated *E. coli* demonstrate AIEC associated phenotypes and assess if these phenotypes are more frequently detected in *E. coli* isolated from JIA HLA-B27 (+) patients.

5.4 Results

5.4.1 Isolation of *E. coli* from patient faecal samples

5.4.1.1 Cultivation of *E. coli* on Gram-negative selective media

In order to isolate *E. coli* from faecal samples, standard cultivation methods on Gram-negative selective media were used. Faecal samples were homogenised, serially diluted and plated in triplicate on MacConkey agar and incubated overnight at 37°C. *E. coli* colonies (pink; purple) were counted and mean colony counts were determined for each patient stool sample. Three to five putative *E. coli* isolates that showed distinct colony morphology, were stocked for each stool sample. All isolates were restreaked on EMB agar and *E. coli* growth was detected by appearance of metallic green colouration around colonies (Figure 5.3). The majority of patient faecal samples contained very low numbers of viable *E. coli.* Median faecal *E. coli* counts for JIA RISK, JIA NULL and healthy groups were 81.20; 8.42 and 13.57 respectively (Figure 5.3). There was no significant difference between groups for median *E. coli* counts ($P > 0.05$).

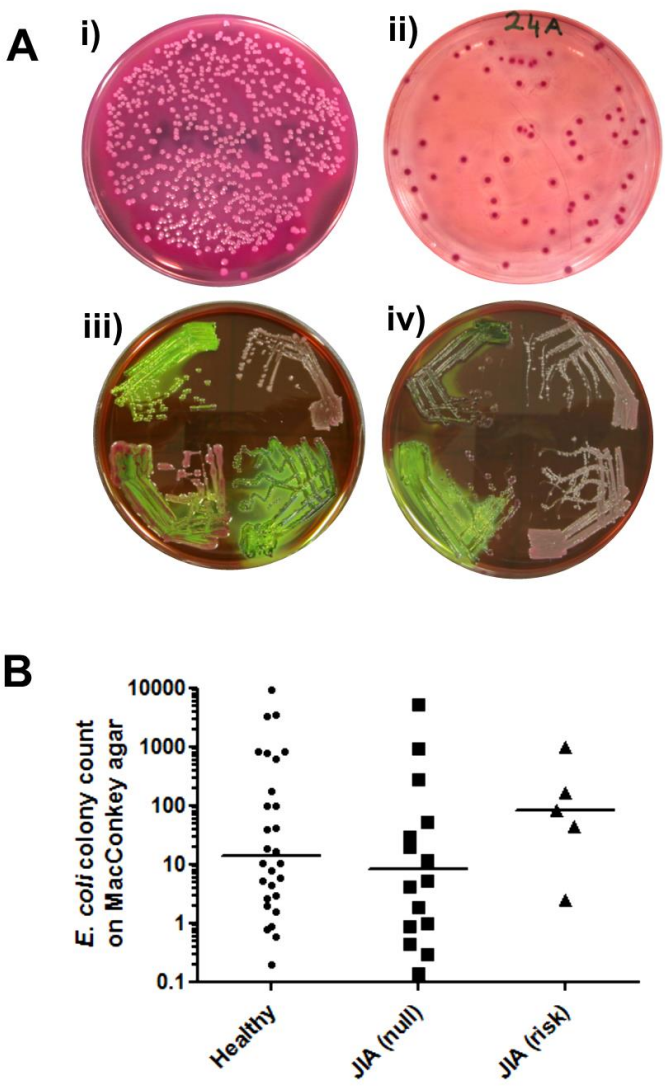


Figure 5-3: Isolation and selection of faecal *E.coli* strains.

A) Faecal samples were plated on Gram-negative selective media, MacConkey agar, and incubated overnight at 37°C. Growth of *E. coli* was shown by growth of pink (i) or purple colonies (ii). Isolates were stocked on LB agar and restreaked on eosin methylene blue (EMB) agar that indicates for the growth of *E. coli* by formation of metallic green colonies (iii; iv). B) Colony counts of *E. coli* on MacConkey agar for healthy, JIA NULL and JIA RISK faecal samples. Each point represents mean *E. coli* colony count for one patient stool sample and data represents mean value from three independent experiments. Black line shown represents median colony count for each group. Differences in bacterial counts between groups were tested by the Mann-Whitney U test. There was no significant differences observed between groups for median colony counts (P> 0.05).

5.4.1.2 PCR amplification of *16S* rRNA of faecal *E. coli* strains

In order to select strains for further assessment of the adherent and invasive phenotype it was important to confirm species identify as *E. coli*. This was achieved by sequencing of the *16S* rRNA gene. As the majority of strains showed identical growth phenotype on MacConkey and EMB agar, only half of the isolated strains were tested by this method (See Appendix E).
Extraction of genomic DNA was carried out using a freshly streaked colony on LB agar that was lysed as described. Universal primers targeting the V2 and V3 region of the *16S* rRNA gene sequence, 88F and 91R, were used in a standard PCR reaction giving a 300bp fragment for all tested strains of *E. coli.* Amplicons of *16S* rRNA gene were sequenced at Source Bioscience using 88F and 91R primers and the sequences were analysed using BLAST tool at National Centre for Biotechnology Information (NCBI). Sequencing of *16S* rRNA showed that all tested strains belonged to *E. coli* species. Strains that were not investigated for PCR amplification of *16S* rRNA but showed identical phenotypes to surveyed strains were also classified as *E. coli*. By this method 40 *E. coli* strains were isolated that comprised of 23 strains isolated from healthy patient samples; 12 strains isolated from JIA NULL patients and 5 strains from JIA RISK patients (Appendix E). These strains were used for all experiments described in the remainder of this chapter.

5.4.2 Investigation of the AIEC phenotype in faecal *E. coli* strains

5.4.2.1 Invasion of T84 intestinal epithelial cells by faecal *E. coli* strains

It has previously been shown that in contrast to most commensal *E. coli*, AIEC have the ability to invade intestinal epithelial cells (Boudeau *et al.* 1999). In order to compare the invasive ability of *E. coli* strains isolated from JIA RISK, JIA NULL and healthy patients, we performed invasion assays using T84 cell monolayers. T84 monolayers were infected with *E. coli* strains (MOI = 10) for 2 h and treated with gentamicin or kanamycin for 1 h to kill extracellular bacteria. MICs for antibiotics against faecal *E. coli* strains are shown in Appendix F. Lysed cells were then plated on LB agar to determine CFU counts. The ability to invade T84 cells was expressed as an invasion index (I_INV) that was calculated as: (number of

intracellular bacteria at 2 h / number of bacteria in original inoculum)* 100. As reported previously in the literature, to be classified as invasive *E. coli* strains must show I_INV value ≥ 0.1 (Martinez-Medina and Garcia-Gil 2014). The mean I_INV of the non- invasive control strain, *E. coli* F18, was 0.001 ± 0.0025 (Table 5.1). AIEC reference strain LF82, included in all of the assays as a positive control, showed mean I_INV of 1.25 ± 0.3. For the *E. coli* strain group isolated from healthy patients, the median I_INV was 0.0040. The median I_INV for the *E. coli* strain group isolated from JIA NULL patients was 0.02567. The median I_INV for *E. coli* strain group isolated from JIA RISK patients was 0.0027 (Figure 5.4). A significantly different median I_INV was shown between *E. coli* groups isolated from JIA NULL and JIA RISK patients (P = 0.0392) (Figure 5.4). There was no significant difference in the median I_INV values between *E. coli* groups isolated from JIA RISK and healthy patients (P=0.76). For *E. coli* strains isolated from healthy patients (n=23), 3 strains (13%) showed I_INV ≥ 0.1 (Figure 5.4). For *E. coli* strains isolated from JIA NULL patients, 2 (16.6%) strains showed I_INV ≥ 0.1. In this work, zero *E. coli* strains that were isolated from JIA RISK patients demonstrated I_INV ≥ 0.1. These data indicate that *E. coli* isolated from HLA-B27 positive patients do not show an increased ability to invade intestinal epithelial cells compared to *E. coli* isolated from HLA-B27 negative patients and healthy patients.

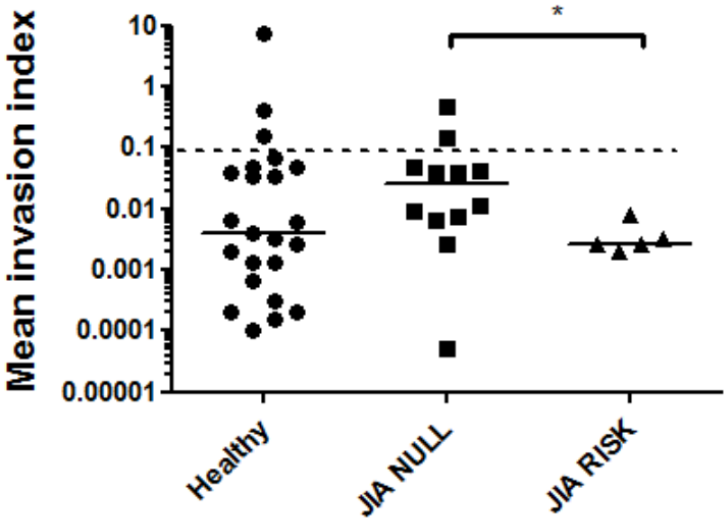


Figure 5-4: Mean invasion index values for faecal *E.coli* strains isolated from healthy, JIA NULL and JIA RISK patients.

Each data point represents mean I_INV value for one strain. Median is shown by black line. Strains that showed invasion index ≥ 0.1 (above or on dashed line) were classified as invasive. Data represents mean I_INV value calculated from three independent experiments. For invasion data, comparisons of unpaired median I_INVs were performed using the nonparametric Mann–Whitney U test. Differences were considered statistically significant at $P < 0.05$.

5.4.2.2 Intramacrophagic replication

AIEC bacteria survive and replicate within macrophages without inducing host cell death. This is proposed to induce production of pro-inflammatory cytokines (Glasser *et al.* 2001). In order to test bacterial replication within macrophages, gentamicin protection assays were performed using the murine macrophage-like cell line J774-1. *E. coli* (MOI=50) were added to confluent J774 cells for 2 h and treated with gentamicin or kanamycin to kill extracellular bacteria. Cells were plated on LB agar after 1 h and 4 h to determine intramacrophagic proliferation. The ability to replicate within macrophages was expressed as the replication index (I_REP) that was calculated as: (number of intracellular bacteria at 4 h / number of bacteria at 1 h) *100. AIEC associated replication was denoted by I_REP ≥ 100. AIEC reference strain LF82 showed a mean I_REP of 159 ± 21. The non-invasive commensal strain F18 showed a mean I_REP of 72 (Table 5.1). For *E. coli* strain

group isolated from healthy controls, the median I_REP was 52.87. The median I_REP for *E. coli* strain group isolated from JIA NULL patients was 62.88. For the *E. coli* group isolated from JIA RISK patients, the median I_REP was 41.67 (Figure 5.5). There was no significant difference in median I_REP values between all groups ($P>0.05$).

These data showed that 7 (30.4%) of the *E. coli* strains isolated from healthy controls showed I_REP ≥ 100. It was shown that 5 (41.6%) of the *E. coli* strains isolated from JIA NULL patients showed mean I_REP ≥100 and finally 2 (40%) of the *E. coli* strains isolated from JIA RISK patients, demonstrated mean I_REP ≥ 100 at 4 h (Figure 5.5).

Overall, these data indicate that *E. coli* strains isolated from HLA-B27 positive patients do not show an increased ability to proliferate within macrophages and so do not resemble AIEC more than *E. coli* strains isolated from JIA NULL patients and healthy controls. Furthermore, these data also demonstrate that *E. coli* strains that demonstrate intramacrophagic proliferation can be found in the colon of healthy individuals.

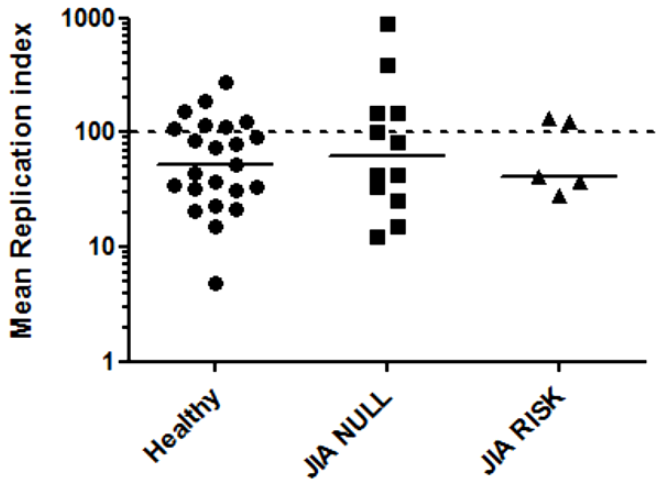


Figure 5-5: Mean replication index values for faecal *E.coli* strains isolated from healthy, JIA NULL and JIA RISK patients.

Each data point represents mean value for one strain. Median is shown by black line. Strains that showed replication index ≥ 100 (above or on dashed line) demonstrated AIEC associated phenotype of intramacrophagic proliferation. For intramacrophagic replication data, comparisons of median I_REPs for groups were performed using the nonparametric Mann–Whitney U test. Differences were considered statistically significant at $P < 0.05$.

5.4.2.3 Intramacrophagic persistence

In addition to the ability of AIEC to replicate within macrophages, other work has demonstrated persistence of AIEC within macrophages at 48 h (Dunne *et al.* 2013). In order to test the ability of *E. coli* strains to persist within macrophages at 48 h, gentamicin protection assays were performed using J774.1 macrophages. The ability of *E. coli* to persist within macrophages was expressed as a persistence index (I_PERS) that was calculated as: (number of intracellular bacteria at 48 h/ number of bacteria at 1 h)*100. Intracellular persistence was denoted by I_PERS ≥1. AIEC LF82 reference strain showed a mean I_PERS of 2.3 ± 1.2 at 48 h and commensal *E. coli* strain F18 showed a mean I_PERS value of 0 (Table 5.1). The majority of faecal *E. coli* strains investigated in this assay showed a mean I_PERS of 0 and did not persist in macrophages at 48 h (Figure 5.6a). *E. coli* strains that showed I_PERS value of 0 were removed from subsequent analysis. For *E. coli* strains that showed I_PERS > 0: the median I_PERS value for *E. coli* strains isolated from healthy controls was 1.17; the median I_PERS value for *E. coli* strains isolated from JIA NULL patients was 0.23

and the median I_PERS value for *E. coli* group isolated from JIA RISK patients was 3.75. Median I_PERS values were not significantly different between JIA NULL and healthy control groups (P=0.14). Due to the small sample size shown for *E. coli* group isolated from JIA RISK patients, Mann Whitney U test could not be performed to determine statistical significance between JIA RISK vs JIA NULL, healthy *E. coli* groups. These data showed that 5 (21.7%) of the *E. coli* strains isolated from healthy controls showed mean I_PERS ≥1. Two (16.6%) of the *E. coli* strains isolated from JIA NULL patients showed a mean I_PERS ≥ 1. Two (40%) *E. coli* strains isolated from JIA RISK patients showed a mean I_PERS≥ 1 (Figure 5.6a).

Data obtained in this work demonstrates that *E. coli* strains isolated from HLA-B27 positive patients do not show increased ability to persist within macrophages as shown by AIEC, relative to *E. coli* isolated from HLA-B27 negative patients and healthy controls. Additionally, these data demonstrate that the AIEC associated phenotype of intracellular persistence is shown by *E. coli* strains isolated from healthy patients.

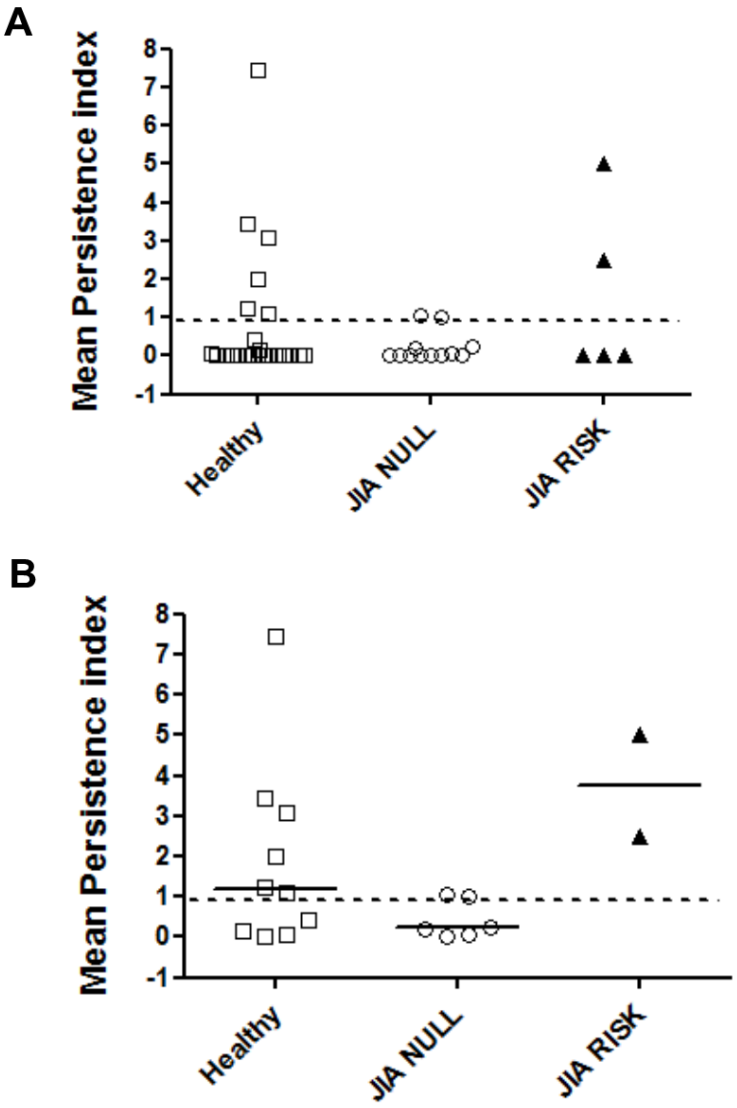


Figure 5-6: Mean persistence index values for faecal *E.coli* strains isolated from healthy, JIA NULL and JIA RISK patients.

A) Each data point represents mean I_PERS for one *E. coli* strain. Majority of *E. coli* strains show mean I_PERS of 0. Strains that showed persistence index ≥ 1 (above or on dashed line) were classified as intramacrophagic persister strains. B) Persistence index values for strains that showed mean I_PERS> 0. Median I_PERS value for each group is shown by black line. Comparisons of median I_PERSs for groups were performed using the nonparametric Mann–Whitney U test. There was no significant differences in median I_PERS values between all groups (P> 0.05).

A full summary of the mean I_INV, I_REP and I_PERS for all *E. coli* strains used in this chapter is listed in Table 5.1.

Table 5-1: AIEC associated invasive and intracellular survival phenotypes detected in faecal *E. coli* strains.

Strain	Type	I_INV	SD (±)	I_REP	SD (±)	I_PERS	SD (±)
F18	Commensal	0.0000	0.0000	72.00	0.00	0.00	0.00
LF82	AIEC	1.2500	0.0289	159.02	21.19	2.30	1.20
H2CA1	Healthy	0.0467	0.0115	187.81	146.99	0.00	0.00
H47AA1	Healthy	0.0400	0.0000	155.17	34.48	3.45	1.19
44BB2	Healthy	0.0000	0.0000	126.56	17.95	0.06	0.01
H42CA1	Healthy	0.0002	0.0003	116.54	82.08	0.00	0.00
H17CA1	Healthy	0.0033	0.0012	113.95	79.80	7.44	1.61
H51cA1	Healthy	0.4000	0.3464	107.14	0.00	0.00	0.00
H43CA1	Healthy	0.0013	0.0012	91.09	45.36	0.00	0.00
H44BA1	Healthy	0.0007	0.0012	85.71	69.99	0.14	0.03
H13AA1	Healthy	0.0333	0.0115	80.36	96.43	0.00	0.00
H54bA1	Healthy	0.0013	0.0012	74.35	60.03	3.08	3.08
H50aA1	Healthy	0.0333	0.0115	52.87	42.00	0.00	0.00
H14BA1	Healthy	0.0002	0.0000	44.65	7.77	0.00	0.00
H59cA1	Healthy	7.3333	1.1547	37.50	30.62	0.00	0.00
H45AA1	Healthy	0.0040	0.0020	34.57	9.55	0.00	0.00
H15AA1	Healthy	0.0467	0.0115	33.86	39.46	0.44	0.00
H7dA1	Healthy	0.1600	0.0200	32.50	8.66	0.00	0.00
H12bB1	Healthy	0.0020	0.0000	30.77	35.53	0.00	0.00
H11dA1	Healthy	0.0667	0.0306	22.90	26.66	1.24	1.14
H13AB1	Healthy	0.0067	0.0042	21.15	26.18	1.10	0.08
H18BA1	Healthy	0.0003	0.0000	20.31	13.86	0.01	0.01
H53bA1	Healthy	0.0060	0.0040	14.89	17.40	0.00	0.00
H12bA1	Healthy	0.0027	0.0012	4.84	1.71	0.00	0.00
JR25aA1	JIA risk	0.0080	0.0053	131.25	94.37	5.00	2.17
JR39AA2	JIA risk	0.0033	0.0023	28.13	13.01	0.00	0.00
JR24aA1	JIA risk	0.0027	0.0012	124.03	12.00	2.51	0.40
JR29bA1	JIA risk	0.0027	0.0012	41.67	14.43	0.00	0.00
JR40bA1	JIA risk	0.0020	0.0035	37.40	26.97	0.00	0.00
J22eA1	JIA	0.4667	0.0577	384.62	314.04	0.00	0.00
J64dA1	JIA	0.1433	0.0321	15.34	11.64	0.00	0.00
J80cA1	JIA	0.0467	0.0115	42.86	52.49	0.26	0.15
J23aA1	JIA	0.0403	0.0560	897.22	1625.25	1.06	0.00

J83bA1	JIA	0.0400	0.0200	102.63	119.21	0.04	0.03
J66cA1	JIA	0.0400	0.0000	33.33	27.22	0.00	0.00
J82aA1	JIA	0.0113	0.0012	25.00	28.87	0.05	0.07
J35aA1	JIA	0.0093	0.0012	0.13	0.25	0.00	0.00
J26aA1	JIA	0.0073	0.0012	150.00	101.00	0.00	0.00
J84dA1	JIA	0.0067	0.0031	42.86	38.97	1.02	0.40
J36aA1	JIA	0.0027	0.0012	146.15	63.43	0.20	0.18
J62b3	JIA	0.0000	0.0000	82.90	56.38	0.00	0.00

Ability to invade intestinal cells was expressed as invasion index (I_INV) that was calculated as: (number of intracellular bacteria at 2 h/ number of bacteria in original inoculum)*100. Ability to replicate within macrophages was expressed as replication index (I_REP) that was calculated as: (number of intracellular bacteria at 4 h / number intracellular of bacteria at 1 h)*100. Ability to persist within macrophages was expressed as persistence index (I_PERS) that was calculated as: (number of intracellular bacteria at 48 h/number of intracellular bacteria at 1 h)* 100. Data shown is mean index value and SD determined from 3 independent assays.

5.4.3 Serotyping of *E. coli* O antigen

AIEC are clonally diverse and belong to different groups of serotypes (Martinez-Medina, Mora, *et al.* 2009). Interestingly, previous work has reported that AIEC prototype strains LF82 and NRG857c belong to O83:H1 serotype (Nash *et al*. 2010). Other work reported that approximately 40% of surveyed AIEC strains belonged to O6 or O22 serogroups (Martinez-Medina, Aldeguer, et al 2009). In order to determine serogroups of faecal *E. coli* isolates from JIA patients and healthy controls, serotyping of somatic O antigen was performed at the Gastrointestinal Bacteria Reference Unit, Public Health, Colindale. These data showed that a high variability in O antigen serotypes was detected in all groups (Table 5.2). This work did not report increased prevalence of serogroups O22, O6 or O83 in *E.coli* strain group isolated from JIA RISK patients relative to strains isolated from JIA NULL or healthy patients.

Table 5-2: Serogroups detected in *E. coli* strain groups isolated from JIA RISK, JIA NULL and healthy patients.

O antigen	Number of isolates		
	JIA NULL	JIA RISK	Healthy
O1	0	1	5
O18	0	1	0
O2	0	1	0
O21	0	1	0
O8	0	1	2
O6	0	0	2
O11	0	0	1
O19A	0	0	1
O159	0	0	1
O146	0	0	1
O55	0	0	1
O88	1	0	1
O125c	1	0	0
O18Ac	1	0	0
O20	1	0	0
O25	1	0	0
O32	1	0	0
O81	1	0	0
O9A	1	0	0
Ond	4	0	8

Ond; O antigen for *E. coli* strain was not identified by this method.

5.4.4 Detection of AIEC associated virulence genes in *E. coli* faecal isolates

In this work, our first approach that investigated the prevalence of AIEC associated phenotypes in faecal *E. coli* through measurement of invasion and intracellular replication assays failed to show differences in the frequency of AIEC in JIA NULL, JIA RISK and healthy *E. coli* groups. Therefore, we decided to next investigate the prevalence of genetic markers associated with AIEC in faecal *E. coli* strains. AIEC have been shown to carry different sets of virulence genes that are characteristic of ExPEC strains (Martinez-Medina, Aldeguer, *et al.* 2009). In this work, two virulence genes that have been previously associated AIEC were investigated; *lpfA* and *malX* (Martinez-Medina, Mora, *et al.* 2009). PCR products for *lpfA* and *malX* were approximately 540 bp and 1600 bp respectively (Figure 5.7). Boiled and lysed bacterial colonies were utilised for extraction of genomic DNA and amplification of *lpfA* and *malX* was performed by standard PCR reaction utilising primers lpfAF, lpfAR and malXF, malXR. These data showed that 7 (30.4%) strains of the *E. coli* group isolated from healthy controls, 4 (33.3%) strains of the *E. coli* group isolated from JIA NULL patients and one (20%) strain from the *E. coli* group isolated from JIA RISK patients possessed *lpfA*. The *malX* gene was detected in 8 (34.7%) strains of the *E. coli* group isolated from healthy patients. Four strains (33.3%) from the *E. coli* group isolated from JIA NULL patients and 4 strains (80%) of the JIA RISK *E.coli* group possessed *malX*. Overall, these data show that AIEC associated virulence genes, *malX* and *lpfA* are detected in *E. coli* strains isolated from healthy controls, HLA-B27 positive and HLA-B27 negative patients.

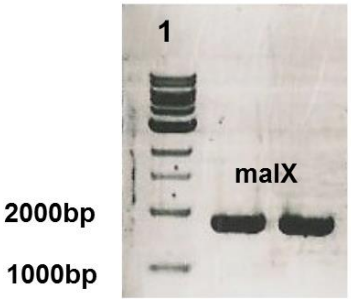


Figure 5-7: PCR for detection of AIEC associated virulence factor *malX*.

PCR product visualised by agarose gel electrophoresis for AIEC LF82. Lane one shows DNA ladder.

A full summary of the detection of *malX* and *lpfA* in all *E. coli* strains isolated from healthy controls and JIA patients is shown in Table 5.3. The table shows that *lpfA* and *malX* are detected in *E. coli* strains isolated from JIA RISK, JIA NULL and healthy patients. It is shown Table 5.3 that some *E. coli* strains possess both *lpfA* and *malX.*

Table 5-3: AIEC associated virulence genes detected in faecal *E. coli* strains isolated from JIA NULL, JIA RISK and healthy patients.

Type	Strain	Serotype	AIEC genes detected (*lpfA, malX*)
Healthy	H44bB2	OND	-
	H2cA1	O:88	-
	H7dA1	O:19A	*lpfa*
	H11dA1	OND	*lpfA, malX*
	H15aA1	OND	*lpfa*
	H18Ba1	O11	-
	H20Da1	O:1	*lpfA, malX*
	H42cA1	O:1	*malX*
	H43cA1	O:6	*malX*
	H45aA1	O:55	-
	H47cA1	OND	*lpfa*
	H50aA1	O:1	*malX*
	H51cA1	OND	-
	H53cA1	O:8	*lpfa*
	H54bA1	O:6	*malX*
	H59cA1	OND	*lpfA*
	H12ba1	OND	-
	H12bB1	OND	-
	H13aa1	O:1	*malX*
	H13AB1	O:1	*malX*
	H14BA1	O:146	-
	H17CA1	O:8	-
	H44bA1	O:139	-

JIA NULL	J22eA1	OND	*lpfa*
	J23aA1	OND	-
	J26aA1	O:81	-
	J35aA1	OND	*malX*
	J36aA1	O:125C	-
	J64dA1	OND	*lpfA*
	J66cA1	O:25	*malX*
	J80cA1	O:20	-
	82aA1	O:88	*lpfA*
	83bA1	O:32	-
	J84dA1	O:18AC	*malX*
	J62B3	O:9A	*lpfA, malX*
JIA RISK	JR24aA1	O:18	*malX*
	JR25aA1	O:1	*malX*
	JR29aA1	O:2	*lpfA, malX*
	JR39aA2	O:21	-
	JR40bA1	O:8	*malX*

OND; O antigen was not identified. – shows that neither *malX* nor *lpfA* were detected in *E. coli* strain.

5.4.5 Genotypic profile of intramacrophagic *E. coli* strains

The data obtained in this chapter did not report increased prevalence of AIEC associated phenotypes in *E. coli* strains isolated from HLA-B27 positive patients. However, phenotypes associated with AIEC, including survival in macrophages, were detected in *E.coli* strains isolated from all patient groups, including healthy controls. Additionally, AIEC associated virulence genes were detected in *E. coli* strains isolated from healthy patients. This indicates that phenotypes associated with pathogenic *E. coli,* such as AIEC, may also be associated with specific commensal strains, therefore undermining the current definition for AIEC. In order to further investigate the existence of pathogenic traits in commensal *E. coli*, the presence of other pathogenic *E. coli* associated virulence factors was investigated. Four *E. coli* strains isolated from healthy patients (H13aB1; H47aA1) and HLA-B27 negative patients (J36aA1; J84Da1) were assessed using multiplex PCR that was performed for 10 virulence factor gene regions associated with extraintestinal *E.*

coli. Investigated genes were *pap, fimH, ibeA, fyuA, iutA, ompT, traT, k1 and uidA*, a molecular marker for *E. coli*. Multiplex PCR assays were performed at Gastrointestinal Bacteria Reference Unit, Public Health, Colindale (work by Holly Ciesielczuk). These data showed that different virulence factors were detected in the 4 surveyed commensal *E. coli* strains. It was shown that J84dA1, isolated from HLA-B27 negative JIA patient, and H13aB1, isolated from a healthy patient, possessed *fyuA* and *k1* that encode iron siderophore and K1 capsule respectively (Table 5.4). J84dA1 also possessed *iutA*, that encodes the aerobactin siderophore and *ompT* that is involved in serum resistance. H13aB1 possessed *pap,* that encodes pyelonephritis associated pili and *traT* that is involved in serum resistance (Table 5.4). These data also showed that strain J36aA, isolated from HLA-B27 negative JIA patient, possessed *ibeA*, an invasive protein found in APEC and low numbers of ExPEC (Germon *et al.* 2005) *(*Table 5.4). These data highlight the existence of pathogen associated virulence factors in commensal strains that reside in the human gut.

Table 5-4: Presence of pathogenic *E. coli* associated virulence factors in commensal *E. coli* strains.

	E. coli Strain			
Virulence factor	H13aB1	J36aA1	H47aA1	J84Da1
pap	+	-	-	-
fimH	+	+	+	+
ibeA	-	+	-	-
fyuA	+	-	-	+
iutA	-	-	-	+
ompT	-	-	+	+
traT	+	-	-	-
k1	+	-	-	+
uidA	+	+	+	+

Presence of virulence genes was tested by multiplex PCR that was carried out at Gastrointestinal Bacteria Reference Unit, Public Health, Colindale. + denotes presence of gene, - denotes absence of gene.

5.5 Discussion

Over 2000 years ago Hippocrates, referred to as 'the father of medicine', stated that "all disease begins in the gut". Much work carried out over the last decade now highlights the significance of this observation. In an attempt to draw further conclusions on the prevalence of AIEC in gut-associated inflammatory disorders and further establish its role in the development of intestinal inflammation, this chapter aimed to investigate the frequency of AIEC occurring in gut *E. coli* populations in JIA HLA-B27 (+) patients relative to HLA-B27 (-) patients and healthy controls. Increased prevalence of AIEC in CD patients has been demonstrated in a multitude of independent studies (Martin *et al.* 2004, Martinez-Medina, Aldeguer, *et al.* 2009) and recent work has also shown increased AIEC in newly diagnosed, paediatric CD patients compared to healthy controls (Conte *et al.* 2014). For the purpose of this pilot study, previously sampled faecal samples (Gerasimidis *et al.* 2012; unpublished data) obtained from healthy, JIA HLA-B27 (-) and JIA HLA-B27 (+) patients were used to isolate viable *E. coli* bacteria by traditional cultivation methods. A key limitation of this method was that the majority of patient faecal samples contained very low numbers of viable *E. coli*. Previous work investigating the prevalence of AIEC bacteria in specific patient groups frequently utilised fresh biopsy samples for isolation of *E. coli* bacteria (Martin Baumgart *et al*. 2007, Martin *et al*. 2004, Martinez-Medina, Aldeguer, *et al*. 2009). This has shown to be extremely effective for the isolation of large numbers of clinical *E. coli* isolates and through extensive washing procedures can be used to specifically isolate mucosa-associated *E. coli* strains, that is a feature of AIEC colonisation (Prorok-Hamon *et al*. 2014). In order to make valid conclusions on the prevalence of AIEC in HLA-B27 (+) patients and indeed other patient groups, future work should include fresh biopsy samples obtained from patients for which extensive clinical information has been obtained (Guy *et al.* 2014, Ibekwe and Grieve 2003, Kang *et al.* 2010, Martinez-Medina *et al.* 2014).

Overall, this work did not show increased prevalence of AIEC associated phenotypes in *E. coli* strains isolated from HLA-B27 (+) patients relative to HLA-B27 (-) patients and healthy controls. A key limitation in this chapter was the low number of *E. coli* strains isolated from HLA-B27 positive patients. However, invasion of intestinal epithelial cells and survival within macrophages was

demonstrated by *E. coli* strains isolated from healthy patients. Indeed, the ability of commensal *E. coli* strains to invade colonic epithelial cells has been previously demonstrated *in vitro* using SW-480 cells whereby several commensal bacteria including *E. coli* JCM 1649T but not probiotic strains were shown to invade SW-480 cells (Ohkusa *et al.* 2009). In this work 13% of the *E. coli* strains isolated from healthy patients were classified as invasive. This indicates that *E. coli* capable of inducing pathology via invasion of intestinal epithelial cells exist in the gut microbiota of healthy patients. These particular strains are unlikely to induce disease in healthy individuals who possess effective gut barrier functions. However, in compromised hosts such as CD patients, where impaired autophagy and a leaky intestinal barrier is reported, invasion of intestinal epithelial cells would be a far more frequent event (Buhner *et al.* 2006, Caprilli *et al.* 2010, Gibson 2004, Homer *et al.* 2010).

The detection of *E. coli* strains which show the ability to survive within macrophages in healthy patients, also demonstrates the capacity for commensal *E. coli* to induce pathology following disruption of host mucosal immune responses. Intramacrophagic survival has been demonstrated for several distinct *E. coli* pathovars including *E. coli* O157:H7 (Poirier *et al.* 2008); *E. coli* K1 (Sukumaran *et al.* 2003), uropathogenic *E. coli* (Bokil *et al.* 2011) and indeed, AIEC (Bringer *et al.* 2012). Several virulence factors have been implicated in this mechanism including the production of shiga toxin that causes downstream effects on host cells (*E. coli* O157:H7); OmpA (*E*. coli K1) and expression of specific virulence factors utilised for intracellular survival including *dtrB* and *htrA* (AIEC LF82) (Bringer *et al*. 2005, Poirier *et al*. 2008, Sukumaran *et al*. 2003). Furthermore, recent work has demonstrated the ability of commensal *E. coli* strains to adapt genetically to the intramacrophagic environment. Following prolonged co-culture with host macrophage cells, commensal *E. coli* evolved virulent clones that were able to evade phagocytosis and showed increased pathogenesis in mice *in vivo* (Miskinyte *et al.* 2013). This was mediated by transposon insertion into promotor region of the *E. coli yrfF* gene (Miskinyte *et al.* 2013).

These data obtained in this work therefore support a pathobiont role for specific commensal *E. coli* strains, where pathology is induced by host factors or an

environmental trigger. This is further supported by the detection of multiple virulence factors in commensal *E. coli* strains surveyed in this work.

A theory that can derived from this work is that the term ‘AIEC’ can be recognised as a catch all title for certain commensal *E. coli* strains that have the ability to induce pathology. In this new working model, AIEC exist as commensal *E. coli* in healthy hosts, however in patients that show impaired bacterial clearance and recognition functions, these unique commensal *E. coli* strains are able to proliferate, interact with host cells, induce pathology and become AIEC. It is likely that in the healthy host these behaviours are still demonstrated by a small number of these AIEC strains but, due to effective mucosal immune responses are not inductive of inflammation. Yet, it is possible that AIEC may also exhibit a unique and unknown behaviour that may enhance their interaction with host cells compared to other commensal strains (Appendix G and H). Indeed, this unique attribute is yet to be identified.

Pathogenic *E. coli* pathovars have been widely investigated and their roles in human disease confirmed. Commensal *E. coli* on the other hand are not investigated extensively and in much work they are grouped as a single entity. It is clear from the results obtained in this work that commensal *E. coli* strains are a diverse group, with specific strains having the capability to induce disease in certain individuals. Therefore, rather than invent new *E. coli* pathovars for strains that display seemingly pathogenic behaviours, I recommend that we first distinguish unique pathogenic determinants prior to grouping of these strains. This work has shown that the *in vitro* phenotypical traits associated with AIEC are detected readily in *E. coli* isolated from healthy individuals. Therefore, one must question the role of the host in the evolution of these novel *E. coli* pathovars? Are alterations in gut barrier function, induced by antibiotic exposure and western diet, making our commensal strains pathogenic? Of course, long term and large cohort studies are required to confirm the role of AIEC in CD pathogenesis. However, as they are currently recognised as a diverse group, it is likely that AIEC strains associated with specific CD patients would behave similar to a commensal strain when reintroduced into the healthy gut environment.

6 Final conclusion and future perspectives

Crohn's disease is a highly complex disorder that is associated with distinct aetiological agents in different groups of patients. In this work we recognise that for a subset of patients, intestinal inflammation is associated with an abnormal microbiota profile involving enriched AIEC (Arlette Darfeuille-Michaud 2002, Martinez-Medina, Aldeguer, *et al.* 2009). For this particular subset of patients, *E. coli*-targeted antibiotics such as colicins represent a promising therapy.

AIEC is a diverse and specialised *E. coli* pathotype (Arlette Darfeuille-Michaud 2002) that shows abnormal association with the gut mucosa and can adopt an intracellular lifestyle (Bringer *et al.* 2006, Eaves-Pyles *et al.* 2008). Additionally, multidrug resistance is frequently detected in *E. coli* isolated from ileal CD patients and resistance to macrophage-penetrating antimicrobials, including ciprofloxacin is common (Dogan *et al.* 2013). Evidently, alternative therapies to traditional antibiotics are required that show activity within eukaryotic cells without inducing cytotoxicity.

In this work, we report good *in vitro* efficacy of colicins against AIEC that was adherent to intestinal epithelial cells and growing inside macrophages. As a putative *E. coli* pathotype associated with CD, the most striking phenotypic feature of AIEC is its ability to persist within macrophages that is associated with granuloma formation (Meconi *et al.* 2007). Indeed, the development of a therapeutic that has the ability to prevent and / or target granuloma formation is highly beneficial. Currently, granulomatous CD is managed by the administration of anti-inflammatory and immunoregulatory therapies including infliximab and mesalamine (Bandzar *et al*. 2013). However, previous work reporting *E. coli* DNA in 80% of CD granulomas indicates that antimicrobial therapies such as colicins would be far more suitable therapies in these subsets of patients.

The intracellular activity of colicins within eukaryotic cells is promising with regards to the use of colicins as a therapeutic for intracellular colon-cancer associated *E. coli* detected in tumours and colon tissue (Swidsinski *et al.* 1998; Raisch *et al.* 2015). Future work must ascertain the activity of colicins against AIEC growing intracellularly within granulomas. A good basis for this work is an investigation of colicin penetration into granuloma tissue *in vitro.*

Although, this study did not report good *in vivo* activity of colicins against AIEC growing in the murine intestine, these data alone should not be used to predict colicin efficacy in human subjects. For example, we showed that following encapsulation within pectin / zein hydrogel beads, colicin showed potent activity against AIEC *in vitro.* This highlights the potential of this method of delivery following optimisation of colicin encapsulation efficiency that was calculated as 1.4% in this work. Furthermore, we show here that prolonged colicin treatment of mammalian cells failed to induce production of pro-inflammatory cytokines or cytotoxicity. These data support the use of colicins as a clinical therapeutic in CD patients however further work investigating colicin immunogenicity *in vivo* is required.

Based on results on from earlier work in the Walker lab a patent was filed titled "Colicins as a treatment for bacterial infections" (WO2014009744 A1) that is supported by the promising work described in this thesis. This innovative treatment method holds remarkable possibilities for the future as a form of personalised medicine. Personalised medicine has the potential to dramatically alter the way we perceive medicine, for example by switching the emphasis on prevention rather than reaction (Gurwitz 2013). Screening for Crohn's-associated *E. coli* in early childhood and hence administration of specific and patient matched colicins could function as a preventative measure for CD development. Of course, the success of this relies on further investigation into the pathogenic mechanisms of these mucosa-associated *E. coli* in CD.

An interesting study that will provide additional support for *E. coli* targeting in CD and potentially CRC, is the recently implemented clinical trial at Royal Liverpool University Hospital (Royal Liverpool University Hospital 2014). The pilot study will investigate the use of ciprofloxacin, doxycycline and dydroxychloroquine with budesonide for treatment of AIEC overgrowth and alleviation of inflammatory symptoms in CD patients. Hydroxychloroquine in combination with doxycycline and ciprofloxacin were selected on the basis of their ability to kill intramacrophagic AIEC. The clinical outcome of this study has the potential to demonstrate the pathogenic capability of mucosa-associated *E. coli* in these intestinal disorders. Alongside the development of a narrow spectrum therapeutic for AIEC, this study also investigated the prevalence of this *E. coli* pathotype in other inflammatory disorders associated with CD. Increased prevalence of AIEC was not reported in

HLA-B27 (+) JIA patients compared to HLA-B27 (-) patients and healthy controls. However, the identification of AIEC-associated phenotypes, namely persistence within macrophages in *E. coli* strains isolated from healthy and JIA patients clearly highlights that bacteria capable of inducing pathogenesis occur frequently in the gut. These data demonstrate the clinical potential for microbiota manipulating therapeutics such as the colicin-like bacteriocins, that can be administered frequently and throughout life to maintain a 'healthy microtype' in the gut of risk patients.

Due to their multifunctional potential as a prophylactic and antimicrobial therapeutic, colicin-like bacteriocins deserve extreme merit in the clinical sector. Enormous effort must be placed upon the development and utilisation of these innovative and targeted antibiotics as a primary treatment method for bacterial infection and dysbiosis.

Appendices

A. Alignment of peptide sequences for colicin E9 and mutant colicin E9H575A

Mutant colicin E9H575A was constructed by PCR mutagenesis of pcS4 template using primers E9H575AF and E9H575AR. PCR product was sequenced and nucleotide sequences were aligned with wild-type colicin E9 protein sequence that showed mutation at residue 575, Histidine to Alanine.

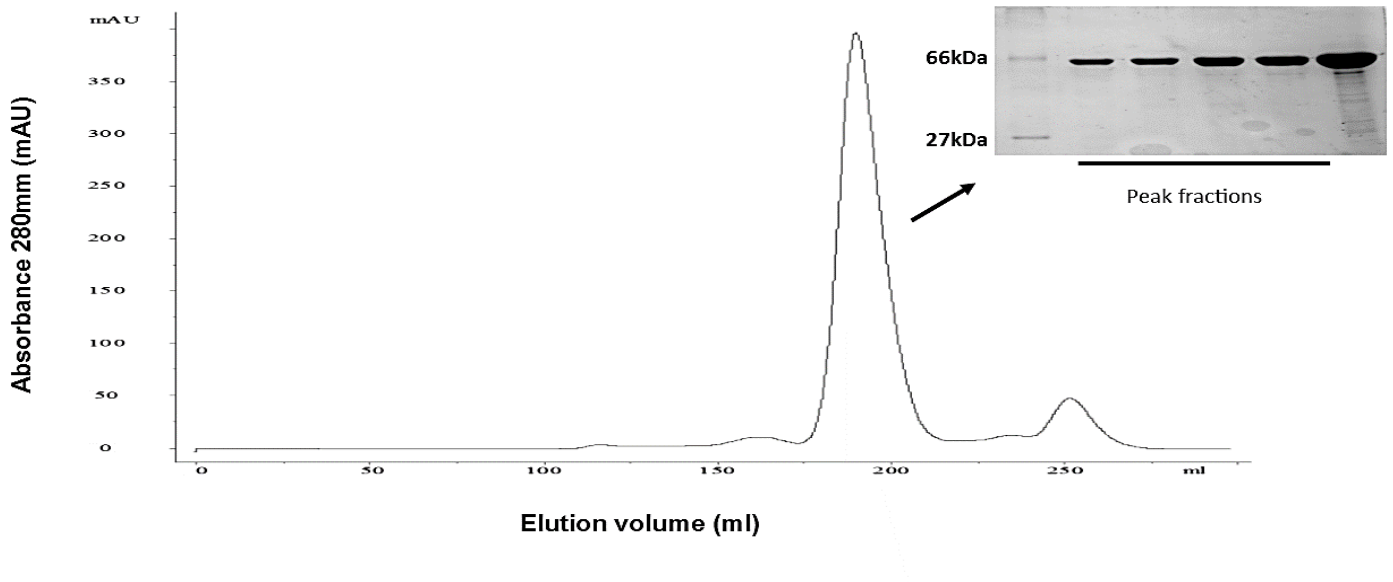
```
                533                                                 582
MUTANT E9       VGGRKVYELHHDKPISQGGEVYDMDNIRVTTPKRAIDIHRGK
WT E9           VGGRKVYELHHDKPISQGGEVYDMDNIRVTTPKRHIDIHRGK
                ********************************** *******
```

Figure A-1: Peptide sequence alignment of WT colicin E9 and mutant colicin E9H575A showing mutation at residue 575.

B. Purification of colicin E9

Figure B-1: Purification of colicin E9

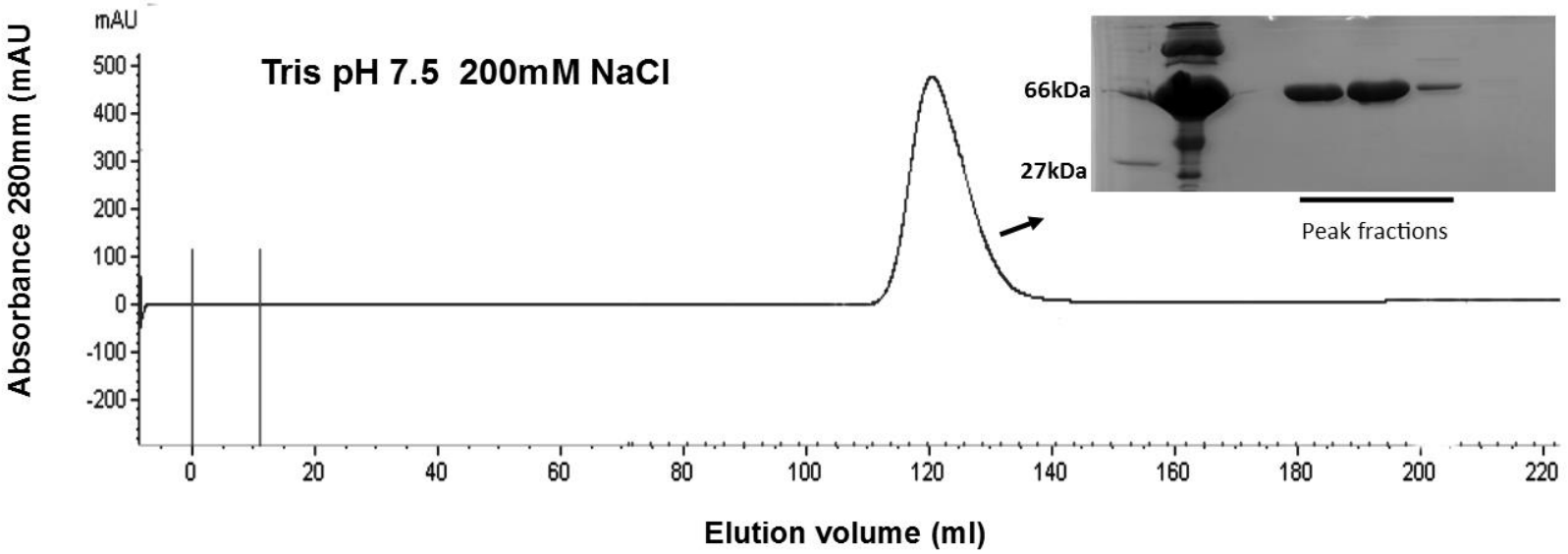
Purification of colicin E9 by SEC (Superdex S275 26/60 column), post-purification with nickel affinity chromatography. Protein eluted into 50 mM Tris-HCl, 200 mM NaCl, pH 7.5. This method gave highly purified colicin E9. Figure shows peak and corresponding SDS-PAGE (12% polyacrylamide) gel of purified colicin E9 (60kDa).



C. Purification of colicin E1

Figure C-1: Purification of colicin E1

Purification of colicin E1 by SEC (Superdex S200 26/60 column), post anion exchange chromatography. Protein eluted into 50 mM Tris-HCl, 200 mM NaCl, pH 7.5. This method gave highly purified colicin E1. Figure shows peak and corresponding SDS-PAGE gel of purified colicin E1 (62kDa).



D. Determination of minimum inhibitory concentrations of AIEC strains for colicin E1 and E9.

The MIC profiles of ileal AIEC reference strain LF82 and colonic AIEC strains against colicin E1 and E9 were determined in liquid broth.

Table D-1: Minimum inhibitory concentrations (MICs) for AIEC isolates against colicin E1 and colicin E9.

Strain	MIC (µg ml^{-1})	
	Colicin E1	**Colicin E9**
LF82	≤ 0.122	≤ 0.122
HM95	0.244- 0.48	0.122 - 0.244
HM154	≥ 250	≥ 250
HM419	0.48 - 0.976	<0.122
HM580	≥ 250	250-125
HM605	≥ 250	≥ 250
HM615	0.122 - 0.244	0.122 - 0.244

E. Faecal *E. coli* strains used for invasion and intracellular proliferation assays for determination of AIEC phenotype

Selection of *E. coli* strains was performed on MacConkey agar and EMB agar. Species identify was confirmed by sequencing of *16S* rRNA gene. Activity of *E. coli* strains against LF82 indicator strain was investigated by overlay spot plate assay. *E. coli* strains were grown in LB broth and spotted on LB agar containing mitomycin C, lysed and then LF82 soft agar lawn was grown on top. Killing was shown by zone of inhibition around producer *E. coli* strain. A wide range of *E. coli* strains were shown to produce antimicrobial compound active against LF82.

Table E-1: Summary of *E. coli* strains utilised for invasion and intracellular proliferation assays.

Table shows strain name, appearance on selective media and species identity determined by *16S* rRNA sequencing of V2 and V3 region and activity of strains against LF82 determined by overlay spot plate assay. Strains that were not investigated by PCR amplification of *16S* rRNA but showed identical colony morphology to sequenced strains were classified as *E. coli.* MAC: MacConkey, EMB: Eosin methylene blue agar. NT: Not tested.

Type	Strain no	Pink / purple on MAC	Metallic green on EMB	*16S* rRNA sequence identity	Activity against LF82
Healthy	H2ca1	+	+	*E. coli*	
	H7da1	+	+	NT	-
	H11da1	+	-	*E. coli*	+
	H12ba1	+	+	*E. coli*	-
	H12bb1	+	+	*E. coli*	-
	H13aa1	+	+	*E. coli*	-
	H13ab1	+	+	*E. coli*	-
	H14ba1	+	+	NT	-
	H15aa1	+	+	NT	-
	H17ca1	+	+	*E. coli*	-
	H18ba1	+	+	NT	-

	H20da1	+	+	NT	-
	H42ca1	+	+	NT	+
	H43ca1	+	+	NT	-
	H44ba1	+	+	*E. coli*	+
	H44bb2	+	+	NT	-
	H45aa1	+	+	NT	-
	H47aa1	+	+	*E. coli*	-
	H50aa1	+	+	NT	+
	H51ca1	+	+	*E. coli*	-
	H53ba1	+	+	*E. coli*	-
	H54ba1	+	+	NT	-
	H59ca1	+	+	*E. coli*	-
JIA NULL	J22ea1	+	+	*E. coli*	
	J23aa1	+	+	*E. coli*	+
	J26aa1	+	+	NT	+
	J26ab3	-	+	*E. coli*	-
	J35aa1	+	+	*E. coli*	-
	J36aa1	+	+	*E. coli*	-
	J62b3	+	+	*E. coli*	-
	J64da1	+	+	NT	+
	J66ca1	+	+	NT	+
	J80ca1	+	+	NT	-
	J82aa1	+	+	NT	+
	J83ba1	+	+	*E. coli*	-
	J84da1	-	-	*E. coli*	-
JIA RISK	JR24aa1	+	+	NT	-
	JR25aa1	+	+	NT	-
	JR29ba1	+	+	NT	+
	JR39aa2	+	+	*E. coli*	+
	JR40ba1	+	+	*E. coli*	-

F. MICs of antibiotics against faecal *E. coli* strains isolated from JIA NULL, JIA RISK and healthy patient stool samples

To determine MICs of antibiotics against *E. coli* strains, MIC assays were performed in 96 well plates in LB broth.

Table F-1: MICs for antibiotics against *E. coli* faecal isolates for antibiotics gentamicin, ampicillin and kanamycin.

Type	Strain	MIC (µg ml^{-1})		
		AMP	GEN	KAN
Healthy	H2ca1	1.56- 3.125	50-100	12.5-25
	H7da1	1.56- 3.126	12.5-25	6.25- 12.5
	H11da1	0.39- 0.78	50-100	12.5-25
	H12ba1	0.78- 1.56	25-50	12.5-25
	H12bb1	1.56 - 3.125	25-50	12.5-25
	H13aa1	0.78 - 1.56	≥200	50-100
	H13ab1	3.125 - 6.25	≥200	25-50
	H14ba1	1.56 -3.125	25-50	6.25-12.5
	H15aa1	0.78 - 1.56	50-100	6.25-12.5
	H17ca1	1.56-3.125	100-200	25-50
	H18ba1	≥200	25-50	50-100
	H20da1	≥200	25-50	50-100
	H42ca1	≥200	50-100	25-50
	H43ca1	6.25-12.5	25-50	6.25-12.5
	H44ba1	1.562-3.125	12.5-25	Not tested
	H44bb2	50-100	12.5-25	Not tested
	H45aa1	1.562-3.125	50-100	6.25-12.5
	H47aa1	>200	50-100	6.25-12.5
	H50aa1	0.78-1.562	6.25-12.5	3.125-6.25
	H51ca1	≥200	≥200	25-50
	H53ba1	3.125-6.25	12.5-25	6.25-12.5
	H54ba1	1.562-3.125	6.25-12.5	6.25-12.6
	H59ca1	≥200	>200	50-100
JIA NULL	J22ea1	0.78- 1.56	25-50	6.25-12.5
	J23aa1	0.78-1.56	50-100	12.5-25
	J26aa1	1.56-3.125	50-100	12.5-25
	J26ab3	3.125-6.25	50-100	12.5 - 25
	J35aa1	1.56 - 3.126	50-100	25-50
	J36aa1	1.56 - 3.127	12.5-25	25-50
	J62b3	3.125-6.25	50-100	12.5-25
	J64da1	≥200	≥200	50-100

	J66ca1	≥200	12.5-25	Not tested
	J80ca1	3.125-6.25	3.125-6.25	Not tested
	J82aa1	3.125-6.26	25-50	25-50
	J83ba1	3.125-6.27	6.25-12.5	Not tested
	J84da1	3.125-6.28	6.25-12.5	Not tested
JIA RISK	JR24aa1	≥200	12.5-25	Not tested
	JR25aa1	1.56-3.125	50-100	12.5-25
	JR29ba1	1.56 - 3.125	12.5-50	Not tested
	JR39aa2	0.78-1.562	50-100	12.5-25
	JR40ba1	1.56-3.125	25-50	12.5 - 25

AMP; ampicillin, GEN; gentamicin; KAN; kanamycin.

G. Differential expression of Ag43 by AIEC and commensal *E. coli* strains

By western blotting it was shown that increased expression of Ag43 α-domain was detected in AIEC LF82 compared to commensal *E. coli* strains K12 and F18 (Figure G1). Furthermore results also showed increased expression of α-Ag43 in AIEC HM95, HM605, HM615, HM154, HM580 compared to *E. coli* K12 and F18.

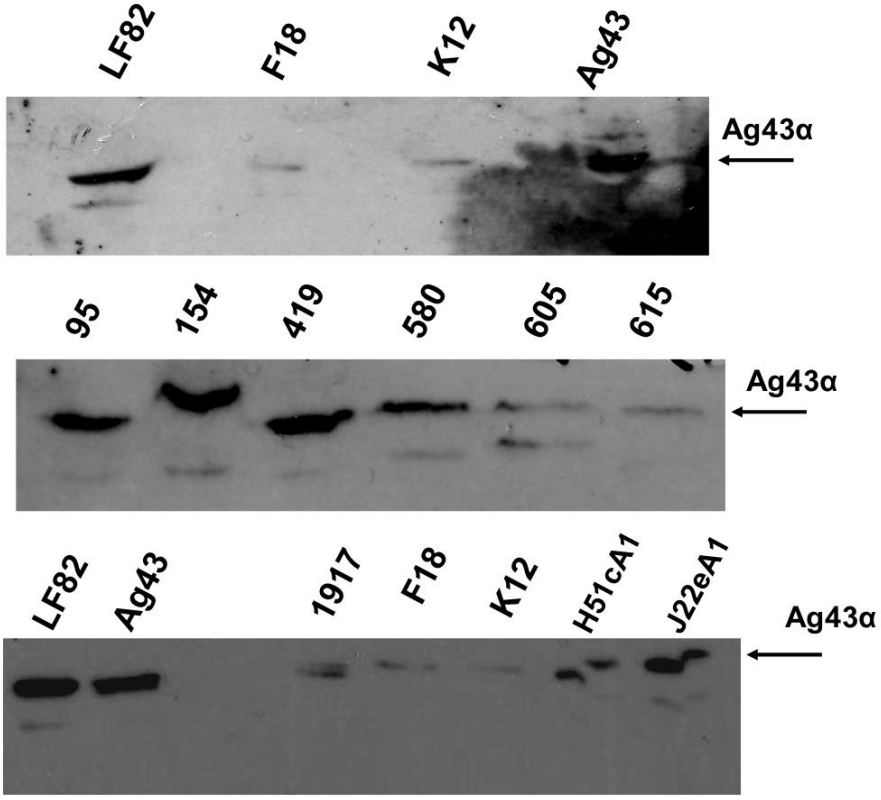


Figure G-1: Increased Ag43 production by AIEC bacteria.

Western blotting, with polyclonal antiserum against the Ag43 passenger domain, of α domains liberated from *E. coli* strains by brief heat treatment. The position of the Ag43 α-subunit protein is indicated by arrow.

H. Incubation of LF82 with Ag43α increases intramacrophagic proliferation.

Log phase cultures were incubated with 1 mg ml^{-1} purified α- Ag43 for 3-4 h at 4°C. Where required anti- α-Ag43 antibody was added to bacterial suspension and incubated for 3-4 h at 4°C mixing. Bacteria- Ag43α suspension was then added to J774.1 macrophages (MOI= 50) and incubated for 2 h. Macrophages were then treated with gentamicin for desired time point to kill extracellular bacteria. Pre-incubation of LF82 with α-Ag43 significantly increased bacterial survival within J774 macrophages at 24 h (P=0.0284). Pre-incubation of LF82 with anti-Ag43α antibody prior to macrophage invasion was also shown to reduce intracellular survival at 24 h but this was not significant (P=0.064). Pre-incubation of *E. coli* commensal strain F18 with Ag43α did not significantly increase macrophage survival at 24 h (P=0.54).

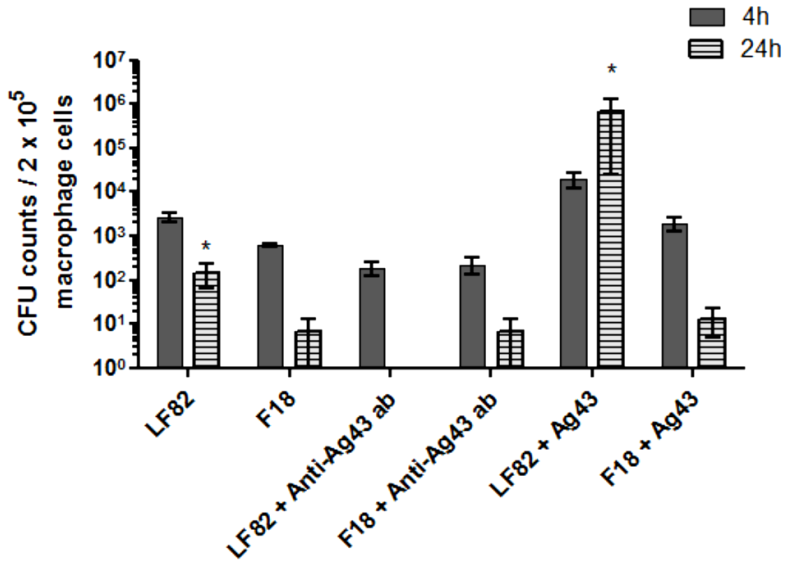


Figure H-1: Pre-incubation of LF82 with Ag43 protein enhances intracellular macrophage survival.

LF82 was pre-incubated with 1 mg ml^{-1} Ag43 α-fragment and added to J774-1 macrophages for 4h or 24 h. Bacteria were also pre-incubated with antibody against α-Ag43 and used to infect macrophages. Intracellular bacteria were determined by CFU counts. *E. coli* commensal F18 strain was used as negative control. Data shown represents mean from two independent experiments. Error bars show SEM. Non parametric Mann-Whitney U test used for comparison of bacteria CFUs. Significant difference was reported as $P < 0.05$. * denotes signficant difference.

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