INTERACTIONS OF INFLAMMATION AND *E. COLI* IN CROHN'S DISEASE

ANTIBIOTICS AND INTESTINAL INFLAMMATION INCREASE HOST SUSCEPTIBILITY TOWARDS CROHN'S DISEASE-ASSOCIATED ADHERENT-INVASIVE *ESCHERICHIA COLI*

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LAY ABSTRACT

Crohn's disease is a type of inflammatory bowel disease that affects many young adults in Canada. It causes a wide range of symptoms including nausea, pain, and diarrhea. While the disease can be treated with surgery and medications, it is considered incurable and affects most individuals for life. The exact cause of Crohn's disease is not known, but it is thought to be caused by a combination of factors including genetics, environmental exposures, and changes in the number and types of bacterial species in the intestine. Intestinal bacteria play an important role in preventing inflammation in the intestine. An unusual strain of bacteria called adherent-invasive *E. coli* is found more commonly in Crohn's disease patients than in healthy individuals. This strain does not cause the disease on its own, but it may interact with other environmental factors that are also associated with Crohn's disease, such as taking antibiotics. Antibiotic use is a risk factor for developing Crohn's disease later in life and antibiotics have previously been shown to promote the growth of other *E. coli* strains in the intestine. In a mouse model of Crohn's disease, we found that antibiotics made mice more vulnerable to infection with this *E. coli* strain. This increased vulnerability was because the antibiotics caused inflammation, and we also found that other sources of inflammation benefitted this *E. coli* strain. These findings help us understand how gut bacteria and other Crohn's disease risk factors might interact to cause the disease.

ABSTRACT

Crohn's disease (CD) is an inflammatory bowel disease characterised by chronic inflammation with a complex pathophysiology involving host, environmental, and microbial factors. The intestinal microbiota is an important regulator of inflammation within the intestine, and a disruption of the interplay between gut bacteria and host immunity is a key factor in CD development. Intestinal inflammation itself is known to cause changes to the intestinal physiology that affect the ability of various bacteria to survive. Additionally, certain environmental risk factors for CD such as antibiotics are also known for their ability to impact the intestinal microbiota. CD is associated with various changes in the intestinal microbiome including increased colonisation with a group of bacteria known as adherent-invasive *Escherichia coli* (AIEC). The purpose of this study is to investigate how AIEC interact with antibiotics and intestinal inflammation *in vivo*. Multiple classes of antibiotics were found to increase the colonisation of AIEC and to increase its persistence. These antibiotics caused a loss diversity in the intestinal microbiome, but this did not explain the increased infectivity of AIEC. Antibiotic-induced inflammation was found to produce metabolites that benefitted AIEC growth in the intestine and similar results were found with chemically-induced inflammation. These results show that AIEC can benefit from both antibiotics and other sources of inflammation through inflammation-derived metabolites, which contributes to a greater understanding of the interactions between AIEC and CD.

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LIST OF ABBREVIATIONS AND SYMBOLS

AG	Aminoguanidine
AIEC	Adherent-invasive *E. coli*
AMP	Antimicrobial peptide
ATG16L1	Autophagy-related protein 16-1
CD	Crohn's disease
CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6
CFU	Colony forming units
EHEC	Enterohemorrhagic *Escherichia coli*
FimH	Type 1 fimbrin D-mannose specific adhesin
GF	Germ-free
GipA	Gifsy-1 prophage GlpA
Gp96	Heat shock protein 90kDa beta member 1
IbeA	Invasion protein IbeA
IBD	Inflammatory bowel disease
IFN-γ	Interferon gamma
IL12B	Interleukin-12 subunit beta
IL-1β, Il-1β	Interleukin 1 beta
IL-23	Interleukin 23
IL23R	Interleukin-23 receptor
IRGM	Immunity-related GTPase family M protein
JAK2	Janus kinase 2
M-cell	Microfold cell
Mig-14	Antimicrobial resistance protein Mig-14
NF-κB	Nuclear factor kappa light chain enhancer of activated B cell
NO	Nitric oxide
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NOS2, Nos2, iNOS	Inducible nitric oxide synthase
OmpA	Outer membrane protein A
OmpR	Outer membrane protein R
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SCFA	Short-chain fatty acids
SPF	Specific pathogen free

STAT3	Signal transducer and activator of transcription 3
STm	*Salmonella enterica* serovar Typhimurium
UPEC	Uropathogenic *Escherichia coli*
Vat-AIEC	Vacuolating autotransporter toxin

DECLARATION OF ACADEMIC ACHIEVEMENT

The thesis entitled "Antibiotics and Intestinal Inflammation Increase Host Susceptibility Towards Crohn's Disease-Associated Adherent-Invasive *Escherichia coli*" and the work presented within it are my own. Chapter 2 includes several experiments which were conducted along with other researchers, and their individual contributions are outlined at the beginning of the chapter. In Chapters 2 and 3, 16S sequencing and data processing was performed by the McMaster Genomics Facility. The remaining experiments, analyses, and written sections of all other chapters are my own work.

INTRODUCTION

The intestine is an organ with a functional microbial component

The human gastrointestinal tract contains a massive bacterial ecosystem comprised of trillions of bacteria[1]. This ecosystem is also highly diverse and it is estimated that 500-1000 unique bacterial species colonise an individual at any point in time, the vast majority of which are considered commensals[2]. Furthermore, each of these bacterial species can possess a unique repertoire of genes that provides far more genetic diversity between individuals than the makeup of their somatic cells. By adulthood, the majority of bacteria are anaerobes found within the lumen belong to the phyla Firmicutes or Bacteroides[3]. While bacteria phyla such as Actinobacteria, Proteobacteria, and Verrucomicrobia are comparatively rare, due to the high densities of bacteria in the intestine these phyla also represent major components of the ecosystem.

The intestinal microbiota provides several metabolic advantages to the host. Bacteria can convert poorly digestible carbohydrates to useable energy sources, such as short-chain fatty acids (SCFAs). The major SCFAs produced by the gut microbiota are acetate, propionate, and butyrate, which are all absorbable by the host. Over 95% of SCFAs are absorbed by the host and can contribute to 5-10% of the daily host energy requirements[4]. Additionally, intestinal bacteria can synthesise vitamins, eliminate exogenous toxins, and regulate intestinal endocrine signalling[5]. The gut microbiota has been shown to be important in augmenting host nutrition not only in humans but also in many vertebrate hosts, and it is hypothesised that the gut microbiota co-evolves with their host to adapt to new dietary niches[6].

Commensal bacteria promote immune tolerance and maintain intestinal homeostasis

In addition to the metabolic benefits that intestinal commensals provide to the host, they are also important in the regulation of the intestinal immune system. The innate immune system has evolved to detect bacteria through conserved microbial signatures collectively called pathogen-associated molecular patterns (PAMPs), which despite the term "pathogen" are also found on commensal bacteria[7]. PAMPs are detected by a variety of different membrane-bound and cytosolic sensors called pattern-recognition receptors (PRRs). PAMPs include components of common bacterial structures such as flagellin, lipopolysaccharides, lipoteichoic acids, and peptidoglycan and their detection in the majority of compartments within the body results in the induction of antimicrobial inflammation. In contrast to most regions of the body which generally do not contain microbes, the intestine contains high levels of potentially inflammatory bacteria and PAMPs. The intestine also contains an enormous number of innate and adaptive immune cells, located primarily in the sub-epithelial compartment called the lamina propria. Despite the presence of a large number of immune cells and potentially inflammatory signals, in normal circumstances intestinal immune cells adopt immunotolerant phenotypes[8].

Bacteria and their PAMPs are restricted to the lumen of the intestine by several host factors. Peristaltic movement pushes luminal contents distally in the intestine which prevents bacterial overgrowth, particularly in the small intestine[9]. Antimicrobial peptides (AMPs) are released from specialised enterocytes called Paneth cells in the small intestine

and limit the ability of bacteria to colonise these regions[10]. Enterocytes transcytose immunoglobulin A into the intestinal lumen which can neutralise bacterial antigens[11]. Mucins are highly glycosylated proteins released from goblet cells that coat the apical surface of enterocytes and physically restrict most bacteria from coming into direct contact with enterocytes[12].

Enterocytes present another barrier separating the microbiota from the lamina propria despite being only a single layer in thickness and play an important role in the compartmentalisation of microbial signals. Enterocytes can detect PAMPs on both basolateral and apical surfaces. Basolateral exposure to PAMPs leads to canonical proinflammatory nuclear factor kappa light chain enhancer of activated B cell (NF-κB) signalling; however, apical stimulation leads to inhibited NF-κB signalling and hypo-responsiveness to other PAMPs[13]. Apical PAMP signalling by commensal bacteria promotes the secretion of AMPs[14], the secretion of immunoglobulin A[11], and the integrity of tight junctions[15] which all help to maintain barrier function.

Professional antigen presenting cells located both within the intestinal epithelium and in the lamina propria also play key roles in promoting intestinal homeostasis. Enterocytes secrete cytokines such as thymic stromal lymphopoietin, transforming growth factor beta, and retinoic acid to promote the formation of tolerogenic macrophages and dendritic cells[16]. These cytokines alter the activity of dendritic cells, which are the primary antigen presenting cells involved in developing adaptive immunity, to promote the development of anti-inflammatory regulatory T cells[17]. Similarly, enterocyte signalling promotes the tolerogenic function of CX3CR1^{hi} macrophages, which

phagocytose commensals that pass through the epithelium and promote the activity of regulatory T cells[16].

Intestinal inflammation is detrimental to the survival of most commensal bacteria. Inflammation causes the release of additional AMPs which can damage microbial cell membranes, although some commensals such as Bacteroidetes have evolved considerable AMP resistance[18]. Inflammation results in the release of calprotectin and lipocalin into the intestinal lumen, which sequester zinc and iron respectively and cause nutrient limitation[19,20]. Inflammation increases oxygen tension and also produces reactive oxygen (ROS) and nitrogen (RNS) species[21,22], which are particularly toxic to many obligate anaerobe commensals[23]. As a result, it is likely that commensals have evolved with selective pressure to decrease activation of host inflammation. Commensal promote immune tolerance through several mechanisms. Some commensals possess modified PAMPs that are less immunogenic, such as pentacylated lipid A found in many *Bacteroides*[24]. Most commensal microbes do not penetrate the inner mucin layer and do not come into direct contact with host cells[12].

The intestinal microbiota can also directly modulate the host immune system to promote tolerance. Multiple commensals have been shown to directly downregulate NF-κB signalling through secreted factors[25–27], and the metabolic production of SCFA through fermentation by commensal microbes is also known to have a variety of immunoregulatory roles. SCFA can inhibit histone deacetylation, which decreases the transcription of several pro-inflammatory cytokines in macrophages[28]. This mechanism has also been shown to inhibit effector T cells and to promote regulatory T cell

proliferation. SCFA can also directly bind to certain receptors present on leukocytes such as G-protein coupled receptor 43, however the expression and effect of this receptor on immune cells is controversial[29,30]. In addition to the impact of SCFA on immune cells, the SCFA butyrate is the preferred energy source of colonocytes. The metabolism of butyrate leads to the consumption of oxygen, which in turn deoxygenates the mucosa on the apical surface of colonocytes[31]. Butyrate has also been shown to directly bind to peroxisome proliferator-activated receptor gamma, a nuclear receptor and transcriptional regulator, resulting in decreased production of reactive nitrogen species[32,33]. As a result, during homeostasis the lumen of the intestine is primarily anaerobic.

In addition to modulating host immunity, commensal bacteria can also suppress the ability of newly acquired bacteria, including pathogens, from colonising the gut which is known as colonisation resistance[34]. Commensal bacteria compete with pathogens for nutrient availability. Commensal *E. coli* has been shown to compete with pathogenic *Salmonella* for iron during a mouse infection[35]. Some bacterial metabolic products can directly inhibit pathogenic bacteria, such as the SCFA propionate which has been shown to directly inhibit the growth of *Salmonella*[36]. The commensal *Bacteroides thetaiotaomicron* has been shown to secrete a factor that inhibits the virulence of enterohemorrhagic *E. coli*[37]. Many gut bacteria produce bacteriocins, which are soluble proteins which kill other bacteria. For example, *Bacillus thuringiensis* produces a bacteriocin which targets *Clostridium difficile*[38]. Many Gram-negative bacteria have type 6 secretion systems (T6SS), which can be used to deliver toxic effectors to other bacteria. T6SS are common both among pathogens and are found in around half of all sequenced

Bacteroidales species[39]. These systems are highly active in the gut, are required for the ability of many commensals to colonise, and can kill both commensals and pathogens. However, the exact magnitude of the T6SS in contributing to colonisation resistance is hard to measure as there are likely many T6SS-containing species within each host and many T6SS effectors have not been characterised.

In addition to the roles that commensal bacteria play in preventing infection, commensals are important during the resolution of infection and inflammation. Fecal transplants containing healthy commensals have been shown to effectively treat *Clostridium difficile* infection[40], and animal models have shown that nutrient competition is necessary for the clearance of some intestinal pathogens[41]. Commensal microbes maintain intestinal homeostasis passively by avoiding contact with the immune system competing for resources with pathogenic microbes, and actively by producing compounds that directly inhibit host immunity and other bacteria.

Crohn's disease is associated with chronic intestinal inflammation

Crohn's disease (CD) is an inflammatory bowel disease (IBD) which is characterised by relapsing inflammation both within and outside of the intestine[42]. CD causes a wide range of symptoms including abdominal pain, diarrhea, intestinal obstructions, and an increased risk of cancer. CD is most commonly diagnosed in young adults and it is typically a lifelong condition with cycles of relapse and remission. CD imposes significant direct and indirect costs on the health care system in addition to the debilitating impact on patient quality of life[43].

CD is associated with severe intestinal inflammation that often fails to resolve without the use of anti-inflammatory medications. The initial trigger of CD inflammation is unknown; however, CD lesions are characterised by the infiltration of a variety of different immune cells which are thought to contribute to the disease. Neutrophils, which are nearly absent from the lamina propria during homeostasis, traffic to the intestine in high numbers during the acute and chronic phases of CD[44]. Neutrophils migrate not only to the lamina propria but cross the epithelium into the intestinal lumen. Activated neutrophils produce large amounts of ROS and RNS in the lumen, as well as calprotectin which is commonly used as a marker of CD disease activity[45].

Mononuclear cells, which act in an immunotolerant capacity under homeostatic conditions, adopt pro-inflammatory phenotypes within inflamed tissue. CD patients have been shown to have increased $CD14^+$ macrophages in the lamina propria which produce interleukin 6, interleukin 23 (IL-23), and tumour necrosis factor alpha (TNFα), and contribute to interferon gamma (IFN-γ) production by local mononuclear cells[46]. Dendritic cells also show increased PRR expression, altered cytokine profiles, and appear to be in an activated state. Regulatory T cells appropriately expand in the lamina propria of CD patients and appear to be functionally active, however for unclear reasons they are insufficient to resolve inflammation[47]. The secretion of inflammatory cytokines such as IFNγ and TNFα have both been shown to disrupt epithelial barrier function by reorganising actin filaments[48] and the transit of neutrophils across the intestinal epithelium requires a transient disruption of epithelial integrity[44]. The exposure of commensal bacteria to the lamina propria in CD patients leads to a loss of tolerance to

commensal bacterial antigens[49], which causes further inflammation. The inability to resolve intestinal inflammation differentiates CD from uncomplicated forms of intestinal inflammation such as infectious gastroenteritis, and it is thought that both host susceptibility factors and intestinal microbiota composition are important factors.

Genetic predispositions in CD occur in pathways for bacterial detection and immune regulation

Genetic susceptibility factors are one of the major risk factors for CD. The familial aggregation of CD has been recognised for decades[50] and more recently genome-wide association studies have identified over 70 loci which are associated with an increased risk of developing CD[51,52]. Several well-studied mutations occur in pathways involved in innate immunity and bacterial recognition. Loss-of-function mutations in the gene coding for nucleotide-binding oligomerization domain-containing protein 2 (*NOD2)*, which acts as a cytosolic sensor for the bacterial PAMP muramyldipeptide, are among the best studied mutations associated with CD[53]. NOD2 signalling appears to promote hypo-responsiveness of enterocytes to other bacterial PAMPs and leads to decreased inflammation. *NOD2* mutations have also been implicated in the dysregulation of autophagy, which is one process used by cells to eliminate intracellular pathogens[54]. Mutations identified in the genes *ATG16L1* and *IRGM*, two other proteins also involved in autophagy, further suggest that autophagy represents another mechanism of controlling intestinal inflammation[55,56]. Multiple mutations have been found to contribute to CD risk in the IL-23 signalling pathway, including *IL23R, IL12B, STAT3,* and *JAK2*[57]. IL-23

signalling has been shown to be an important component in T_H17 signalling, and aberrant signalling through this pathways is believed to be a contributor to chronic inflammation and fibrosis seen in CD[58].

Although many CD risk alleles have been identified, most alleles have an odds ratio of <1.5 indicating that these alleles confer only a modest susceptibility to CD[51]. Furthermore, it is thought that all high-risk alleles have already been discovered in European populations[59]. Finally, the concordance rate between identical twins with CD is ~30%[60] suggesting that while genetic factors are likely important for creating susceptibility to CD, non-genetic factors are also highly important for CD pathogenesis.

Environmental factors in high-income countries affect both CD risk and the intestinal microbiome

Population studies have revealed that environmental changes that occur in high-income countries play a significant role in CD incidence[61]. CD has been typically considered to be a disease of high-income countries with Canada, Northern Europe, and Australia having the highest prevalences of CD worldwide. CD was once a rare occurrence in developing nations, but it is becoming increasingly common in countries with rising incomes, especially in urban environments. A high-income environment appears to have an age-dependent effect on CD risk. One study found that immigrants migrating from regions with low CD incidence to Canada have an increased risk of developing CD which inversely correlates with the age they arrived in Canada[62].

Industrializing countries are associated with environmental changes such as decreased exposure to livestock, increased sanitation, changes in diet, and increased exposure to pollutants. These factors have all been correlated to an increased risk of developing CD[63] and have also been shown to alter the intestinal microbiome[64–67]. Similarly, rising incomes are associated with increased medical interventions such as antibiotic use[68], infant bottle feeding, and caesarian sections, which have all been shown to delay the maturation of the intestinal microbiome[64], and these same risk factors are also associated with increased CD risk[69–71]. While these various environmental factors likely have a wide variety of effects on people which may contribute of CD risk, it is likely that these factors play a major role in the loss of bacterial species and functional diversity observed in the intestinal microbiomes of individuals from developed nations[72].

Antibiotic use is a risk factor for CD

Among the many changes that occur in countries undergoing developing countries is a better access to medications including antibiotics. Multiple population studies in Western countries including populations from different countries, occupations, and age groups have generally found a positive association between antibiotic use and a risk of subsequent CD diagnosis[73–76]. The classes of antibiotics associated with CD are broad and include nitromidazoles, tetracyclines, quinolones, penicillins, and cephalosporins. There are discrepancies between which antibiotics are associated with CD risk between studies, however this may be related to both differences in patient populations and in prescribing practices between regions. Some studies have shown a dose dependency between the

number of antibiotic prescriptions and the risk of developing CD[73,75]. Whether antibiotics are also associated with ulcerative colitis, a related IBD, is unclear as some studies have shown no increased risk[75,77] while others have shown a positive association[78]. Furthermore, a study of patients from Asian countries found that antibiotic use decreased the risk of developing IBD[79], which may indicate that antibiotics are a risk factor for IBD only in the context of other environmental or genetic factors found in Western countries.

Due to the observational nature of these studies that have linked antibiotic use to CD risk, it is difficult to show causality between antibiotics and CD. It is possible that patients with susceptibility towards CD are also more susceptible towards infections requiring antibiotics. Supporting this, one study found that CD patients are at an increased risk of developing pneumonia independently of taking immunosuppressive medications[80]. Even if CD patients are at an increased risk of infection prior to CD diagnosis, it is possible that the combination of genetic susceptibility to infection and a subsequent increased exposure to antibiotics to treat infections act in concert in the development of CD. In addition to the potential role of antibiotics in contributing to CD, certain antibiotics are paradoxically also used in the treatment of CD, although the effectiveness of these antibiotics seems to be limited to certain complications of the disease such as abscesses and bacterial overgrowth[81]. Furthermore, microbiome comparison between CD patients with and without exposure to antibiotics show that antibiotics magnify the dysbiosis signature associated with CD[82]. Relatively short courses of antibiotics can lead to dramatic shifts and loss of diversity on the gut microbiome, which can persist for years[83]. Antibiotics have also been shown to independently cause a decline in bacterial

diversity in IBD patients[84]. It is possible that antibiotic exposure may trigger an unfavourable microbial community structure that increases the potential for long-term dysbiosis and risk of CD in susceptible individuals, however the exact mechanism of this has not been elucidated.

Crohn's disease is associated with changes to the intestinal microbiota and increased prevalence of *Escherichia coli*

Given the importance of bacteria in the regulation of intestinal inflammation, many researchers have speculated that changes in the intestinal bacteria may contribute to the development of CD. Multiple studies have investigated the intestinal microbiota of CD patients and have found various bacterial taxa that are increased or decreased in the microbiota[85]. However, it is often difficult to draw broad conclusions between different studies. Differences in patient populations, disease severity, treatments, sampling locations, sequencing technologies, and data analysis all contribute to large amounts of heterogeneity between studies. It is also difficult to determine the direction of the causal relationship between inflammation and the intestinal microbiota.

Despite these challenges, some common microbial changes have been observed. Several studies have shown an overall decrease in bacterial species richness in CD patients[86,87]. At the phylum level, several changes in taxa have also been shown with reasonable consistency. CD is associated with decreased relative abundance and diversity of the Firmicutes phylum[88], and the commensal *Faecalibacterium prausnitzii* has been specifically shown to be decreased in multiple studies[89–91]. *F. prausnitzii* is a butyrate

producer[92] and has also been shown to directly modulate host inflammation[27]. Low abundance of the bacterium has been linked with higher risk of postoperative recurrence of ileal CD[89]. While decreased *F. prausnitzii* alone is unlikely to cause CD inflammation, animal studies suggest that this bacterium can reduce intestinal inflammation[93] and several clinical trials are investigating prebiotics to increase *F. prausnitzii* in CD patients.

Multiple studies have also found an increased abundance in the Proteobacteria phylum[84,88]. In particular, *Escherichia* have been specifically shown to be more prevalent in the fecal and tissue microbiota in multiple studies of CD patients[94–96]. Culturing of *E. coli* isolates from Crohn's disease patients found that many isolates had unusual adherent and invasive phenotypes both *in vitro* and *in vivo.* Surprisingly these isolates did not encode for toxins or adhesins found in typical pathogenic *E. coli* pathovars[97,98]. These isolates have been collectively called adherent-invasive *E. coli* (AIEC).

Adherent-invasive *E. coli* are pathobionts commonly isolated from the mucosa of CD patients

Several studies have now confirmed that AIEC are enriched in humans with CD, where they are six times more likely to be isolated from ileal and colonic samples compared to healthy controls, and are sometimes the dominant bacterial species present[97–99]. AIEC show genetic and phenotypic diversity and have been isolated from adults[94,99–101], children[102,103], and pets[104]. The presence of AIEC in CD patients is an independent risk factor for both a penetrating disease phenotype and severe post-operative recurrence[105]. The ability of AIEC to exacerbate intestinal inflammation in animal models

suggests that these bacteria may have a pathogenic role in CD[106,107]. AIEC have also been isolated in ulcerative colitis patients, however the prevalence of AIEC in ulcerative colitis and their correlation with disease progression has not been as well studied as with CD [108].

Despite the association between CD disease progression and AIEC, AIEC are not considered pathogens as they are occasionally isolated from healthy individuals without causing overt illness. The dichotomous nature of terms such as "commensal" and "pathogen" oversimplifies the complex relationships between hosts and microbes[109]. Many bacteria are only pathogenic in certain host backgrounds, and even many classical pathogens show wide variability in their ability to cause disease in different hosts. For example, *Pseudomonas aeruginosa* is commonly associated with cystic fibrosis patients[110]. *P. aeruginosa* is an environmental bacterium that typically does not cause disease in an immunocompetent host. However, many cystic fibrosis patients are colonised by *P. aeruginosa* at a young age and later experience chronic, reoccurring lung infections[111]. Since *P. aeruginosa* is only able to cause acute disease in immunocompromised hosts, it is called an opportunistic pathogen.

AIEC are classified as pathobionts, a term which has been coined to describe bacteria which are associated with chronic inflammatory conditions[112]. However, there is often not a clear distinction between what defines a bacteria as a pathogen, an opportunistic pathogen, or a pathobiont[113]. *Helicobacter pylori* has be classified as an overt pathogen due to its ability cause acute ulceration and a pathobiont due to its ability to induce chronic inflammation in some patients[112]. Similarly, *Clostridium difficile* has been called both an opportunistic pathogen and a pathobiont as it can cause both acute

and chronic disease in a susceptible host[112,114]. The lack of agreement in terminology reflects the varied relationships between microbes and their hosts, both in terms of pathogenic potential of the microbe and the susceptibility of the host. Since AIEC is not known to directly cause acute manifestations of disease, it is generally considered a pathobiont rather than an opportunistic pathogen. While AIEC can infect the mucosa in some CD patients[97], the term "AIEC colonisation" rather than "AIEC infection" will be used in the remainder of this thesis to reflect that AIEC does not appear to cause pathology in the absence of host inflammation.

AIEC are a genetically heterogeneous group with multiple origins

While the evolution of several other *E. coli* pathotypes is well understood, the evolutionary origins of AIEC are less clear. The identification of AIEC isolates is difficult as it is based on the exclusion of virulence factors found in other *E. coli* pathovars such as Shiga toxin or a type III secretion system, and *in vitro* phenotype assays that are laborious and not well standardised. Most AIEC isolates are from the B2 or D phylotypes of *E. coli*[115,116]. The B2 phylotype, and to a lesser extent the D phylotype, contains many extraintestinal *E. coli* isolates including pathogens from uropathogenic *E. coli* (UPEC), neonatal meningitis-causing *E. coli*, and avian pathogenic *E. coli*. However, AIEC from this phylotype do not have distinguishing genetic features from other B2 members, nor is there a genetic feature specific to CD AIEC isolates. The absence of a genetic marker or clear phylogenetic distribution of AIEC suggests that the AIEC pathotype likely evolved through convergent evolution from different commensal *E. coli* progenitors. Interestingly,

most *E. coli* from phylotypes A and B1 are transient and colonise the lumen, whereas resident *E. coli* which are more likely to be found to the mucosa mainly belong to the B2 and D phylotypes[108]. This suggests that resident *E. coli* from B2 and D phylotypes may be the ancestors of AIEC within CD patients.

While the genetic diversity of AIEC isolates is unusual compared to most overt pathogens, several other bacteria associated with disease show similar patterns of genetic diversity with convergent evolution towards a pathogenic phenotype. UPEC, which are the causative agents of most community acquired urinary tract infections[117], are generally thought to arise from intestinal *E. coli* strains that are usually unique to each patient[118]. Patients with UPEC often experience recurrent urinary tract infections with the same UPEC isolate, suggesting that some isolates are proficient at evading both antibiotic treatment and host clearance[119]. UPEC strains typically have large, horizontally acquired pathogenicity islands that are required for several common virulence traits, such as adhesion to the epithelial lining, intracellular survival, iron uptake, and immune evasion[120]. AIEC isolates likely also arise from a diverse population of commensal *E. coli*, which similarly experience different selective pressures in each patient due to differences in host genetics, intestinal microbiota, and therapeutic treatments. As a result, it is not surprising that AIEC neither show a common phylogeny nor show the same genetic adaptations.

AIEC have evolved traits for colonisation of the intestine in CD patients

Despite the genetic diversity of AIEC, many strains share common virulence traits which are also occasionally found in commensal *E. coli* strains. One of the defining features of AIEC strains is the ability to adhere to intestinal epithelial cells, which is facilitated by several bacterial surface adhesins. The best-characterised AIEC adhesin is the type I pilus. FimH, the adhesive tip of the type I pilus, binds to mannose residues which are commonly found on glycosylated host proteins and has been shown to bind to carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6)[121,122]. Unlike most commensal *E. coli* strains, FimH in AIEC has accumulated a small number of non-synonymous mutations that may facilitate binding to CEACAM6 which has been shown to be highly expressed in the ileum of CD patients[123].

Additional AIEC adhesins include chitinase, which has been shown to similarly contain point mutations that differ from commensal *E. coli*[124]. AIEC chitinase facilitates binding to chitinase-3-like protein 1, another host epithelial protein which is upregulated during inflammation. AIEC can also express long polar fimbriae, which have been shown to mediate AIEC attachment to Peyer's patches found in the terminal ileum[125]. Finally, the AIEC protein OmpA has been shown to bind to glycoprotein 96, which has similarly been shown to be upregulated during inflammation in the ileum[126].

While the invasive properties of AIEC are less well understood than other invasive pathovars such as enteroinvasive *E. coli*, several factors involved in the penetration through the mucous layer and cell invasion have been identified. The transcription factor GipA found in 31.4% of AIEC strains appears to sense reactive

oxygen and acid stress[127]. The deletion of *gipA* is detrimental to intracellular survival and invasion of Peyer's patches. Vat-AIEC is a vacuolating toxin related to one found in avian pathogenic *E.coli* that has proteolytic activity towards mucin which may allow AIEC to penetrate through the mucous layer[128]. Multiple AIEC strains also encode IbeA, a putative invasin that is important for invasion of Caco-2 and M-cells and survival within macrophages[129]. AIEC has also been shown to exploit cell phenotypes associated with genetic CD risk factors, such as defects in autophagy[130].

Whether AIEC can directly translocate effectors to the host cytosol is unclear as AIEC lack type 3 secretion systems found in other *E. coli* pathovars. However, the genomes of multiple AIEC strains encode for T6SS components.[115] The T6SS is a surface structure related to contractile phage tails and used by Gram-negative bacteria to translocate effector proteins into both bacterial and eukaryotic cells. In other bacteria, the T6SS has been shown to dampen host immunity[131], promote intracellular growth in macrophages[132], and interact with the host microtubule network to promote bacterial internalisation[133]. However, to date no T6SS effectors have been identified in AIEC that target host cells.

AMP resistance is another trait found in AIEC strains. Since the expression of AMPs is increased during intestinal inflammation[10], many enteric pathogens evade AMP mediating killing by altering the composition of the outer membrane which reduces the affinity of AMPs, and by producing outer membrane proteases which cleave and inactivate AMPs. The AIEC strain NRG857c has high levels of resistance towards several AMPs that are common in the gut due to ArlC, an outer membrane protease, and ArlA, a

Mig-14 family protein with an unknown mechanism of action[134]. Finally, AIEC are enriched for antibiotic resistance. One study found that *E. coli* isolates that were isolated from CD patients were commonly resistant to a variety of antibiotics[135]. Since CD patients are commonly treated with antibiotics, this likely creates selective pressure towards favouring antibiotic resistance.

While these studies have identified the mechanisms of action of some factors involved in the AIEC phenotype, no single genetic element defines the AIEC pathovar[116]. The variable presence of these identified factors further suggests that there are multiple evolutionary paths from commensal *E. coli* to AIEC[136].

AIEC from CD patients possess metabolic adaptations for growth in a dysbiotic and inflamed intestine

In addition to the collection of either horizontally acquired or evolved virulence factors which contribute to AIEC attachment and invasion, AIEC possess several metabolic pathways that favour growth during intestinal inflammation. Many of these pathways are common amongst *Enterobacteriaceae* and have been well-characterised in commensal *E. coli* and *Salmonella*. Clinical studies have shown that *Enterobacteriaceae* are elevated in a number of inflammatory conditions other than CD, including colorectal cancer and necrotising enterocolitis suggesting that members of this family have increased fitness during a variety of inflammatory conditions[137].

One mechanism that supports higher *Enterobacteriaceae* during inflammation is increased oxygen tension. Intestinal inflammation has been shown to increase oxygen

tension in the intestinal lumen in concert with dysbiosis of the microbiome. In a mouse infection model, both *Salmonella*-induced inflammation and streptomycin have been shown to deplete butyrate-producing *Clostridia*[21]. This results in a shift of colonocyte metabolism from the aerobic process of butyrate metabolism to the anaerobic glycolysis of glucose to lactate. This decrease in oxygen consumption leads to a relative increase in available oxygen in the intestinal mucosa, which allows aerobic bacteria to expand. Both *Salmonella* and commensal *E. coli* have been shown to require aerobic respiration for colonisation, and both posses three oxygen utilising cytochromes which are required for different niches[138]. The bo_3 oxidase is used for aerobic respiration in high oxygen environments and is dispensable for intestinal colonisation[21,139]. The *bd*-I *and bd*-II oxidases are expressed under low oxygen tension, have a higher affinity for oxygen, are resistant to microbiota-derived H_2S inhibition, and are required for intestinal colonisation[140]. Since AIEC also possess these same cytochromes, it is possible that it also exploits increased oxygen tension during inflammation or antibiotic treatment.

In addition to increased oxygen tension, inflammation can also alter the redox balance of the intestine through the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS are generated by the epithelium in response to IFN-γ from the enzymes NADPH oxidase 1 and dual oxidase 2[141]. RNS are produced in the intestine by host expression of inducible nitric oxide synthase (iNOS), which has be shown to be expressed by both colonocytes and inflammatory cells within the lamina propria[142]. iNOS expression is increased in IBD and iNOS gain-of-function variants are associated with early onset CD[143,144]. iNOS uses L-arginine, molecular oxygen, and

NADPH to produce nitric oxide (NO)[145]. In many tissues, physiological levels of NO can act as a signalling molecule and control both neuronal function and vascular tone. NO itself can be directly toxic to cells due to its affinity towards protein-bound iron, such as cytochromes[146]. The reaction of NO with the ROS superoxide anion produces the potent oxidant peroxynitrite. Peroxynitrite is highly antimicrobial and will oxidise proteins, lipids, and nucleic acids[147]. Peroxynitrite can also oxidise organic sulfides and tertiary amines to S-oxides and N-oxides, and will spontaneously isomerise into nitrate[22]. NO can also react with water and oxygen to form nitric acid.

E. coli possess three nitrate reductases which are all capable of utilising nitrate as a terminal electron acceptor, which is then reduced to nitrite. This ability provides a major fitness advantage for commensal *E. coli* colonisation in mice[138]. *E. coli* also possess reductases for nitrite, S-oxides, and N-oxides, although these are dispensable for the colonisation by a commensal strain in mice[138]. The ability of *Enterobacteriaceae* to utilise alternative electron acceptors provides several advantages over anaerobic commensals undergoing fermentation. During fermentation, the redox balance is maintained by transferring electrons to endogenous organic compounds to produce metabolites such as SCFAs[148]. These compounds are relatively poor electron acceptors compared to oxygen or nitrate, and as a result fermentation generates less energy than oxidative phosphorylation. Furthermore, this process is metabolically expensive as bioavailable carbon is lost from the cell. As a result, bacteria that can use aerobic or anaerobic respiration can gain a growth advantage.

Enterobacteriaceae can also adapt to other changes in nutrient availability during inflammation. During inflammation, RNS-derived nitric acid reacts with glucose and galactose to produce glucarate and galactarate respectively. *E. coli* and *Salmonella* can uptake and metabolise these sugars and gain a fitness advantage[149]. Metal sequestration is another important mechanism of innate immunity. During CD inflammation, neutrophils transcytose into the intestinal lumen and release calprotectin upon cell lysis which sequesters zinc and manganese[19]. *E. coli* and *Salmonella* both possess the *znu* operon, which has been shown to overcome calprotectin-mediated zinc sequestration during a *Salmonella* infection.

While all these metabolic pathways are common amongst both commensal *E. coli* and AIEC, AIEC often contain additional metabolic adaptations that may provide further advantages for growth during inflammation. AIEC is enriched for the *pdu* operon which is involved in propanediol metabolism[150]. This metabolite is produced by the microbiota and has recently been shown to be important for the fitness of *Salmonella* during intestinal inflammation, which also possesses the same metabolic cluster[151]. Metabolism of propanediol requires the presence of oxygen or alternative electron acceptors which are similarly more abundant during inflammation.

AIEC are also enriched for genes involved in iron acquisition, such as genes that promote uptake of siderophore yersiniabactin[100,150]. While the primary siderophore for *Enterobacteriaceae* is enterobactin[152], during intestinal inflammation lipocalin is released into the intestinal lumen by the host which sequesters iron-bound enterobactin. However, some siderophores such as yersiniabactin do not bind to lipocalin, and the uptake of

yersiniabactin may represent an evolutionary adaption during iron-limiting inflammation. The prototypical AIEC strain NRG857c also has a receptor for aerobactin, which is similarly resistant to lipocalin and provides a fitness advantage during mouse colonisation[100]. This strain also encodes for the *sit* operon that is also involved in iron uptake. This pathway is found in all clinical *Shigella* isolates but is absent from most commensal *E. coli*, and appears to be important for intracellular iron and manganese acquisition[153].

AIEC possesses several metabolic pathways that are common among other *Enterobacteriaceae* that provide fitness advantages over the largely anaerobic commensal microbiota. The acquisition of additional metabolic pathways that provide a fitness advantage during inflammation further strengthens the idea that AIEC likely evolved from commensal *E. coli* during CD-associated intestinal inflammation. However, most research on AIEC has focused on isolates from CD patients and it is not known if AIEC isolated from ulcerative colitis or healthy patients carry similar adaptations. These metabolic adaptations to inflammation have been studied in commensal *E. coli* and *Salmonella* and the relative importance of these adaptations have not been studied in AIEC.

Purpose and Goals of the Present Study

While some factors that influence AIEC colonisation have been previously explored, the effects of changes in microbial composition and altered nutrient availability after insults to intestinal homeostasis have not been investigated. The purpose of this study is to investigate how antibiotics and chemically-induced inflammation influence the intestinal microbiota and AIEC colonisation.

The hypothesis of this thesis is that AIEC benefit from antibiotic and inflammation induced changes to the intestinal metabolites and the microbiome. The specific aims of this work will be outlined in the following chapters:

1. Antibiotics potentiate adherent-invasive *E. coli* infection and expansion

    Streptomycin dramatically reduces colonisation resistance towards AIEC.

    Multiple classes of antibiotics promote AIEC colonisation without a common change to the intestinal microbiota.

    Anaerobic respiration and alterative carbohydrate metabolism provide a fitness advantage during antibiotic treatment.

2. Streptomycin-depleted intestinal microbiota promotes adherent-invasive *E. coli* persistence.

    Streptomycin causes a loss of bacterial species richness and evenness.

    Reconstituting a germ-free mouse with a streptomycin-depleted microbiota results in a unique microbiome with low diversity.

    A streptomycin-depleted microbiome is resistant to initial AIEC colonisation but favours AIEC persistence.

3. Adherent-invasive *E. coli* expansion during DSS colitis is mediated by Nos2 metabolites

DSS causes AIEC expansion that is diminished with aminoguanidine treatment.

A nitrate respiration-deficient AIEC mutant shows reduced fitness that is not rescued with aminoguanidine.

Aminoguanidine treatment reduces markers of intestinal inflammation.

CHAPTER 2: ANTIBIOTICS POTENTIATE ADHERENT-INVASIVE *E. COLI* INFECTION AND EXPANSION

Chapter 2: Co-authorship statement

Chapter II is adapted from a publication in the journal *Inflammatory Bowel Diseases*, which is cited below:

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Manuscript was written by AMO and BKC.

The following experiments were performed by collaborators other than myself:

(1) 16s sequencing and the processing of data through the s1p pipeline was performed by the McMaster Genomics Facility.

(2) 16s profiling data was partially organised and analysed by CNT (Figure 2.2 C-F).

(3) One replicate of vancomycin drinking water experiment was performed by AAF (Figure 2.3A-C)

Antibiotics potentiate adherent-invasive *E. coli* infection and expansion

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Abstract

Background: Crohn's disease (CD) is an inflammatory bowel disease with a complex etiology. Paradoxically, CD is associated with the use of antibiotics and with an increased abundance of an unusual phenotypic group of *Escherichia coli* known as adherent-invasive *E. coli* (AIEC). However, the impact of antibiotics on AIEC infection has not been well studied in controlled models of infection.

Methods: We infected mice with AIEC before or after treatment with a variety of different classes of antibiotics. We assessed levels of AIEC in the feces and tissues, AIEC localisation by immunofluorescence microscopy, and tissue pathology.

Results: We found that a wide range of antibiotic classes strongly potentiated initial AIEC infection and expanded AIEC in chronically infected mice. We found that the ability of antibiotics to potentiate AIEC infection did not correlate with a stereotyped shift in the gut bacterial community, but was correlated with a decrease in overall diversity and a divergence from the pre-antibiotic state. We found that antibiotic-induced inflammation provided a fitness advantage for AIEC expansion through their use of oxidized metabolites in the post-antibiotic period.

Conclusions: Our results show that antibiotics can render hosts more susceptible to initial AIEC infection and can worsen infection in previously infected hosts. AIEC appears to exploit host inflammatory responses that arise in the post-antibiotic period, highlighting a previously unknown interaction between CD risk factors.

Keywords: Adherent-invasive *E. coli*, Crohn's disease, antibiotics

Introduction

Crohn's disease (CD) is an inflammatory bowel disease characterised by transmural intestinal inflammation and a range of debilitating symptoms. The etiology of CD is manifold and includes genetic, microbial, and environmental factors. Although the genetic component of CD has been explored in many studies, most risk loci impart a relatively small risk towards developing CD[154]. Furthermore, the rapid rise in CD incidence in industrialising countries such as China suggests that non-genetic factors are an important driver in CD pathogenesis[155]. Among the many changes that occur in countries with rising economic status is better access to medicines including antibiotics[156]. Antibiotic use has been correlated with an increased risk of CD in several epidemiological studies of high income countries[69]. The relative risk of CD onset following antibiotic use appears to be strongest within a few months after antibiotic exposure and this relationship is generally not found for ulcerative colitis, a related inflammatory bowel disease. The classes of antibiotics associated with CD risk vary between studies; however, this may be due to differences in patient populations and prescribing practices[69]. Certain antibiotics are also used in the treatment of CD, although their effectiveness appears to be limited to certain manifestations of the disease[81].

While epidemiological studies correlating antibiotic use to CD do not prove causality, antibiotics are known to cause changes within the microbiome that can lead to mild inflammation and altered nutrient availability within the intestine[149,157]. Furthermore, one study found that CD patients who were treated with antibiotics displayed microbial dysbiosis with increased levels of several Proteobacteria families including

Enterobacteriaceae[82]. The *Enterobacteriaceae* family includes *Escherichia coli*, which has been found to be elevated in the intestinal microbiome of CD patients[94]. In particular, many of the *E. coli* found in CD patients have been phenotypically classified as adherent-invasive *E. coli* (AIEC)[98]. AIEC have the ability to adhere to intestinal epithelial cells, survive within macrophages, and stimulate the secretion of pro-inflammatory cytokines. AIEC are found in over half of CD patients and are typically found within the mucosa of patients with ileal involvement. The presence of AIEC in CD patients is an independent risk factor for both a penetrating disease phenotype and severe post-operative recurrence[105]. Despite the lack of a conserved genetic marker for AIEC, most strains are able to adhere to inflamed intestinal epithelia, evade inflammation-associated antimicrobial peptides[134] and are resistant to multiple classes of antibiotics[135].

Antibiotic use and AIEC infection are both CD-associated factors; however, the interaction between these two features has not been investigated. Here we show that multiple classes of antibiotics can promote initial AIEC infection in mice with remarkably low infectious doses. We found that the ability of different classes of antibiotics to promote AIEC infection was not dependent on a characteristic shift in the intestinal microbiome and show that vancomycin treatment of mice with existing AIEC infection permits bacterial expansion. We additionally demonstrate that AIEC derive a fitness advantage from using alternative carbon sources and electron acceptors that become available after antibiotic treatment[149]. These data provide new insights into how xenobiotics might influence expansion of CD-associated pathobionts, either through *de*

novo acquisition due to a susceptible host state that antibiotics engender, or through expansion of resident microbes that derive advantage from a post-antibiotic state.

Materials and Methods

Ethics statement

Animal experiments were conducted according to guidelines from the Canadian Council on Animal Care. The Animal Review Ethics Board at McMaster University approved all procedures under Animal Use Protocol #17-03-10.

Bacterial strains

AIEC strain NRG857c (serotype O83:H1) was isolated from an ileal tissue biopsy from a CD patient in Charite Hospital (Berlin, Germany) and its genome sequence was determined previously[100]. AIEC mutants are listed in Table 2.1. *E. coli* HS was originally isolated from a healthy adult and has been shown to colonise the human intestine without causing symptoms[158]. Primer sequences used for cloning and mutant generation are listed in Table 2.2. Allelic exchange was used to delete the genes *moaA*, *garD*, *napA*, and *catL*[159]. Briefly, two pairs of primers and overlap-extension PCR were used to make constructs containing ~500 bp regions flanking each gene. These constructs were ligated into a modified pRE112 plasmid conferring gentamicin resistance and transformed into *E. coli* S17-1 λ*pir*. The construct was conjugated into NRG857c and deletion mutants were

screened by PCR after counter-selection on sucrose. All other mutants were made using Lambda Red recombination[160]. Due to the extensive antibiotic resistance profile of AIEC NRG857c, we first constructed a modified plasmid system for use with gentamicin selection. To generate the Lambda Red recombinase-expressing plasmid, a kanamycin resistance cassette was amplified from pKD4 using primers neoFwXmnI and neoRvXmnI. The amplified product was digested with XmnI and ligated into pKD46 to generate pKD46_km. To generate a template for knockout constructs, primers GmFrtFwNcoI and GmFrtRvNcoI were used to amplify a gentamicin resistance cassette from pSAM_Gm[161]. The PCR product was digested with NcoI and ligated into pCDF-1b to generate pCDF_GmFrt. Knockout constructs were amplified from pCDF_GmFrt using primers with extensions complementary to 48 bp in-frame regions at the 5' and 3' ends within each gene of interest. The knockout constructs were transformed into AIEC containing pKD46_km in the appropriate genetic background. Transformants were selected on LB media with 15 μg/mL gentamicin and were confirmed by PCR. The Flp recombinase system was cloned by amplifying pCP20 using primers cp20FwBamHI and cp20RvBamHI, and by amplifying a kanamycin resistance cassette from pKD4 using primers neoKD4FwBamHI and neoKD4RvBamHI. Both DNA products were digested with BamHI then ligated together to generate pCP20_km. When needed, the gentamicin resistance cassette was deleted by the transformation and expression of the FLP recombinase from pCP20_km.

When appropriate, chloramphenicol (BioShop, Burlington, Canada) (34 μg/mL), ampicillin (BioShop, Burlington, Canada) (100 μg/mL), and gentamicin (Gibco) (15

μg/mL) were used in LB (BioShop, Burlington, Canada) broth or agar plates to provide selection. Prior to mouse infections or *in vitro* assays, AIEC strains were grown in LB containing selective antibiotics for 16–18 h at 37 °C with shaking. For mouse infections, AIEC was resuspended in phosphate buffered saline (PBS) at the indicated density. For *in vitro* competitive assays, strains were sub-cultured into NCE media supplemented with mucin with or without nitrate as previously described[22], or in NCE media containing 50 mM glucose, glucarate, or galactarate (Sigma Aldrich) and 0.1% yeast extract (BioShop, Burlington, Canada), and were grown for 24 h at 37 °C in anaerobic jars without shaking.

Animal infections

Six to eight-week-old female C57BL/6N or CD1 mice were purchased from Charles River Laboratories or acquired from in-house breeding colonies. For each experiment, groups of animals were from the same supplier and subject to the same animal husbandry practices. Animals were housed in a specific pathogen-free barrier unit under level 2 conditions at the Central Animal Facility at McMaster University. When delivered as a pre-treatment, the antibiotics streptomycin (BioShop, Burlington, Canada) (200 mg/mL), vancomycin (BioShop, Burlington, Canada) (100 mg/mL), and gentamicin (12 mg/mL) were dissolved at the indicated doses in PBS, filter sterilised, and given as a 100 μL oral gavage 24 h before infection. Metronidazole (Sigma Aldrich) was suspended in PBS with 1% methylcellulose and given in a 100 μL oral gavage. Vancomycin (50 mg/L) used for drinking water treatment was dissolved in water, filter sterilised, and

given to mice *ad libitum*. Mice were infected with the indicated doses of AIEC in a 100 μL suspension by oral gavage.

Bacterial enumeration

Fecal pellets were weighed, homogenised in 1 mL PBS, serially diluted, and plated onto LB agar plates containing selective antibiotics. Intestinal tissues were flushed with PBS to remove luminal contents, homogenised with a sterile metal bead, and plated in the same manner as feces. Plates were incubated overnight at 37°C and colonies were counted to determine colony-forming units (cfu) per gram of tissue or feces. Competitive assays were enumerated by replica plating total isolated AIEC onto selective plates to discriminate the two competing strains and normalising cfu counts to the input ratio.

Histopathology and immunofluorescence staining

At various times following infection, cecal sections were fixed in 10% neutral-buffered formalin overnight and were processed into paraffin blocks. The blocks were cut into two to three 5 μm sections and stained with H&E, and for each block 9 high power field of view regions were used for histology scoring. The histology scoring system was described previously[162], and has been reproduced as Table 2.3 in detail. The pathology scores within this manuscript reflect the total pathology from all regions (lumen, lamina propria, mucosa, and submucosa).

For immunofluorescence staining, paraffin-embedded blocks were processed as described[163]. AIEC were stained using rabbit anti-O83 (1:200; Statens Serum Institute)

followed by anti-rabbit Alexa 568 (1:200, Abcam ab175475). Coverslips were mounted with ProLong™ Diamond Antifade mounting medium with DAPI (Invitrogen). Slides were imaged on an EVOS FL Auto 2 Cell Imaging System using a 40× objective. To count tissue-associated AIEC, regions of interest (ROI) were manually drawn around tissue sections on ImageJ. AIEC within each tissue ROI were counted by thresholding, watershed analysis, and particle analysis using Fiji (1.51n) as described in the ImageJ manual[164]. Representative images were taken with a Zeiss Axio Imager 2 at 40× objective magnification.

16s rRNA gene profiling

DNA was isolated from feces or cecal contents as described[165] with the exception that the homogenates were not lyophilised but were instead diluted 1:2 into CTAB buffer. Extracted DNA was used to amplify the v3 region of the 16s rRNA gene by PCR. Fifty ng of DNA was used as template with 1U of Taq, 1x buffer, 1.5 mM $MgCl_2$, 0.4 mg/mL BSA, 0.2 mM dNTPs, and 5 pmoles of Illumina adapted primers 341F as described[166]. The following PCR cycling conditions were used: 94 °C for 5 minutes; 25 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 30 seconds; and a final extension of 72 °C for 10 minutes. PCR products were visualised on a 1.5% agarose gel. DNA was normalised using the SequalPrep normalization kit (ThermoFisher) and sequenced on the Illumina MiSeq platform at the McMaster Genomics Facility. Resulting sequences were processed with the sl1p pipeline using the default settings[167]. Briefly, FASTQ reads were trimmed with Cutadapt, aligned with PANDAseq (v2.9), quality trimmed with sickle

(v1.33), and chimeric sequences were removed with USEARCH. OTUs were assigned with AbundantOTU+ 0.93b and taxonomic assignments were made using RDP Classifier (v2.2) against the database Greengenes (Feb. 2011). OTU tables, phylogenies, and preliminary analyses are conducted using QIIME and R (v3.3.1).

Rarefaction curves of observed species and Shannon index are shown in Fig. S2.3. Shannon index was rarefied at 28398 reads for Fig. 2.2C. Relative abundances of taxa were compared within samples before and after antibiotic treatment, and taxa with >1% abundance showing a >2 fold increase or decrease in relative abundance after treatment were screened. The two largest fold changes that met these criteria were *Bacteroides* and *Ruminococcaceae* which are shown in Figure 2.5 H-I. While *Escherichia* in Figure 2.5J did not meet these criteria due to its low abundance, we also showed this OTU as it is the most phylogenetically similar to AIEC.

qPCR

RNA was isolated from tissues by Trizol (Ambion) extraction. Briefly, samples were homogenised in 900 µL Trizol with a metal bead. Samples were mixed with 400 µL chloroform, centrifuged, and the supernatants were added to tubes with 500 µL isopropanol. RNA was precipitated at -20 °C , dried, and treated with DNase I (Quantabio). RNA was re-precipitated using LiCl, dissolved in water, and cDNA was synthesised using qScript cDNA Supermix (Quantabio). First strand cDNA was diluted 1:10 and used in qPCR reactions for various genes and the housekeeping gene *tbp* (Table

2). Quantitative PCR reactions were carried out in 96-well plates in a LightCycler 480 (Roche) with Quanta Perfecta SYBR Green Supermix (Quantabio).

Nitrate quantification

Nitrate was measured in cecal content homogenates using a Nitrate/Nitrite Fluorometric Assay Kit (Caymen Chemical). Cecal contents were centrifuged at 15,000 *g* for 5 min and 500 µL of supernatants were transferred to 10 kDa microcentrifuge columns which were centrifuged at 15,000 *g* for 5 min at 4 °C. Ten µL of the resulting filtrate was used to assay total nitrate + nitrite as described in the kit.

Statistical analysis

Data were analyzed using GraphPad Prism software v. 6.0 (GraphPad Inc., San Diego, CA). Statistical significance was calculated using Mann-Whitney tests, Student T-tests, one-way ANOVA with Kruskal-Wallis tests, or two-way ANOVAs with Holm-Sidak tests as indicated in each figure.

Results

Antibiotics increase host susceptibility to initial AIEC infection

Although AIEC are commonly found in CD patients, the precise mechanisms underlying initial AIEC colonisation are not well understood. The intestinal niche is protected, in part, by microbe-mediated colonisation resistance, which restricts the ability

of orally ingested microbes to colonise the gut through both direct competition and host immune modulation[168]. However, colonisation resistance can be compromised by antibiotic administration, which has been shown to lower the infectious dose of many bacterial species such as *Clostridium difficile*[169]. Experimentally, antibiotics have been incorporated into multiple animal models of infection to permit bacterial colonisation, for example, in the *Salmonella* colitis model that uses streptomycin pre-treatment[170]. We have previously shown that streptomycin treatment allows chronic infection with AIEC strain NRG857c in several mouse lines[106], and other groups have used antibiotics to facilitate mouse infections with different AIEC strains[171]. However, the degree to which antibiotics increase host susceptibility to *de novo* AIEC infection or potentiate existing AIEC infections is not known.

To explore the impact of streptomycin on short and long-term infectivity of AIEC, we infected mice with various doses of AIEC one day after treatment with streptomycin or PBS control. In PBS-treated mice, the dose of AIEC required to infect half of the animal cohort (ID_{50}) was 6.3×10^2 cfu. In this control group, mice infected with the lowest dose carried a lower fecal burden and cleared AIEC infection by day 7 (Fig. 1A). Following a single dose of streptomycin, as few as 20 bacterial cells could establish robust infection of mice. In contrast to AIEC fecal burdens that were proportional to the infective dose in the control group, streptomycin treatment resulted in dose-independent AIEC expansion and persistent colonisation *in vivo.* To determine if the ability of streptomycin to promote infection was specific to AIEC or common among other *E. coli* strains, we infected mice with various doses of the commensal *E. coli* strain HS, with or

without streptomycin (Fig. 1B). Similar to AIEC, streptomycin significantly reduced the infectious dose of *E. coli* HS needed to colonise mice. However, a much higher dose of HS was needed to provide transient colonisation in control mice, and streptomycin treatment did not promote the persistence of HS. Taken together, these results show that streptomycin allows for host infection with low doses of *E. coli*, demonstrating reduced colonisation resistance that is not specific to AIEC. However, unlike *E. coli* HS, AIEC persists at relatively high levels in the post-antibiotic period, suggesting additional adaptations that allow it to thrive following antibiotic treatment.

Multiple classes of antibiotics potentiate AIEC infection with diverse changes to the microbiome

To determine if the potentiating effect of antibiotic treatment on AIEC infection was specific to an antibiotic class, we examined other antibiotics used in the clinical setting of Crohn's therapy, including vancomycin, gentamicin, and metronidazole. These antibiotics differ in mechanism: vancomycin is a cell wall-targeting antibiotic used primarily in the treatment of Gram-positive bacteria and is administered orally to treat colitis[172]; gentamicin is a broad-spectrum aminoglycoside antibiotic targeting the ribosome; and metronidazole is used to treat certain manifestations of Crohn's disease and targets nucleic acid synthesis in anaerobic bacteria[81]. Doses were selected based on human dosages corrected by body surface area. We orally treated mice with a single dose of either vancomycin, gentamicin, metronidazole, or streptomycin or PBS as a control. The following day, mice were infected with a low dose of AIEC (2×10^3 cfu), which we

previously showed results in transient infection of untreated mice (Fig. 1A). Compared to untreated controls, mice treated with streptomycin and vancomycin showed significantly increased fecal AIEC burden at day 1 post-infection that persisted out to day 14 (Fig. 2A). The effect of gentamicin was more variable, showing AIEC potentiation at day 1 that did not reach statistical significance. By day 14, mice treated with gentamicin had AIEC outputs that were indistinguishable from the untreated controls. Mice treated with a single oral dose of metronidazole showed no AIEC potentiation at either time point, however in separate experiments, prolonged exposure to metronidazole in the drinking water did lead to AIEC expansion (Figure S2.1). We considered the possibility that the ability of some antibiotics to potentiate AIEC might be related to a reduction in total intestinal bacteria following antibiotic treatment, or a characteristic shift in the microbial composition. We measured total fecal bacteria present before and after antibiotic treatment using 16s qPCR and found that only streptomycin was associated with a significant reduction in fecal bacteria (Fig. 2B). This data suggests that while multiple classes of antibiotics can potentiate AIEC, this does not strictly correlate with a reduction in total bacterial numbers.

To profile the shifts in microbiome composition following antibiotic treatment, we performed 16S profiling on fecal samples from all treatment groups before and after antibiotic treatment. We observed a significant decrease in the Shannon index in the streptomycin, vancomycin, and gentamicin treatment groups, consistent with a loss of species richness and evenness (Fig. 2C). We measured how microbial composition changed after treatment using Bray-Curtis analysis and found that Bray-Curtis

dissimilarities between before and after antibiotic treatment significantly increased in streptomycin, vancomycin, and gentamicin treated mice. These data suggest a significant increase in the variability of microbiome composition after treatment (Fig. 2D), and that the fecal microbiome of antibiotic treated mice was dominated by a select few taxa (Fig. 2E). Principle coordinate analysis on the operational taxonomic units (OTU) revealed that the AIEC-potentiating antibiotics clustered separately suggesting that, despite being able to potentiate AIEC infection, these treatments resulted in unique changes to bacterial composition (Fig. 2F).

We did not find evidence for a common change in relative abundance of common (>1% relative abundance) bacterial taxa for AIEC potentiating antibiotics, which may reflect the diverse spectrum of activity of these antibiotics. The ratio of Firmicutes:Bacteroidetes, which is occasionally used as a measure of dysbiosis and tends to be lower in CD patients[90], was significantly decreased only in gentamicin treated mice (Fig. 2G). Gentamicin resulted in a relative expansion of taxa within *Bacteroides* (Fig. 2H), whereas streptomycin resulted in a significant increase in *Ruminococcaceae* (Fig. 2I). Interestingly, vancomycin significantly enriched for members of the genus *Escherichia* (Fig. 2J). Although it is possible that there were common changes to rare bacterial taxa which were not detected using our methodology, we overall found that several antibiotics that potentiate AIEC infection cause an overall loss in bacterial richness and evenness and produce non-similar shifts in microbial composition.

Vancomycin leads to AIEC expansion in chronically infected mice

CD is often treated with immunosuppressants, which have been shown to predispose patients to bacterial infection and thus increase the likelihood of antibiotic exposure[173]. We tested the effect of antibiotic exposure in mice that were already infected with AIEC. We used vancomycin in these experiments because it is used clinically to treat CD patients with *Clostridium difficile* infections[172], and our profiling data suggested that vancomycin supports the expansion of *E. coli*. We established AIEC infections using streptomycin pre-treatment as previously described[106] and after 8 days, treated half of the mice with drinking water containing vancomycin while maintaining regular drinking water for the control cohort. Vancomycin exposure caused an almost immediate expansion of AIEC that reached statistical significance within 48 h as measured in AIEC fecal burden (Fig. 3A). The expansion of AIEC following vancomycin treatment was also observed in the tissue-associated AIEC population in the colon, cecum, and ileum on day 13 and 28 (Fig. 3B). Vancomycin-treated mice exhibited mild cecal pathology at day 13 which resolved by day 28 in the uninfected group. Both groups infected with AIEC showed increased cecal pathology by day 28, suggesting that AIEC induced modest inflammation without additional antibiotics (Fig. 3C). Inflammation is known to increase luminal nitrate through *Nos2* expression[22,157]. Accordingly, we found that total cecal nitrate was significantly elevated in both vancomycin treatment groups at day 13 (Fig. 3D). In line with these observations, *Nos2* expression was significantly increased in AIEC infected mice treated with vancomycin at day 13 (Fig. 3E). Both vancomycin treatment groups displayed elevated *Il1b*, an established marker of intestinal inflammation (Fig.

3F). Using immunofluorescence microscopy, we observed significantly increased numbers of tissue-associated AIEC in vancomycin-treated mice compared to controls (Fig. 4A and B). Taken together, these data are consistent with vancomycin inducing mild cecal inflammation and increased nitrate availability, which coincides with AIEC expansion in both the luminal and tissue-associated populations.

Anaerobic respiration and alternative sugar metabolism provide a fitness advantage for AIEC following antibiotic exposure

Streptomycin causes moderate inflammation and *Nos2* upregulation in the mouse gut[157]. This inflammation results in the production of reactive nitrate species (RNS) and the generation of metabolites such as nitrate that can be harnessed for anaerobic respiration, and the production of oxidised sugars such as glucarate and galactarate[149]. Previous work has shown that commensal *E. coli* can expand its metabolic niche to use carbon and energy sources made available by streptomycin treatment[149,157]. The previous experiment indicated that vancomycin caused a transient increase in cecal *Nos2* expression and nitrate content (Fig. 3). Thus, we considered that metabolites derived from antibiotic-induced inflammation might drive AIEC expansion. We constructed mutants of AIEC that were deficient in glucarate and galactarate utilisation (Δ*garD*/*gudDXP*), nitrate reduction (Δ*narG/narZ/napA*), and the synthesis of molybdenum co-factor used for multiple anaerobic respiration pathways (Δ*moaA*). Through competitive growth assays *in vitro*, we first confirmed that the loss of these metabolic pathways resulted in the expected growth phenotypes. The Δ*garD*/*gudDXP* mutant had reduced fitness in media

containing glucarate or galactarate as the sole carbon source, and the Δ*narG/narZ/napA* and Δ*moaA* mutants had reduced fitness in media supplemented with nitrate (Fig. S2.2). As expected, these mutants had similar *in vitro* growth rates in both aerobic and oxygen-limited conditions in rich media where use of these metabolites is dispensable (Fig. S2.2). To determine if antibiotic-induced AIEC expansion required the metabolic capacity to consume oxidised products of inflammation, we first quantified the fitness of the Δ*garD*/*gudDXP* mutant in competition with wild type AIEC in mice treated with streptomycin or PBS. In the absence of antibiotic treatment, Δ*garD*/*gudDXP* bacteria were as fit as wild type, competing equally or slightly better than wild type by day 8 of infection. In contrast, following streptomycin treatment the Δ*garD*/*gudDXP* mutant was significantly compromised for competitive infection beginning on day 3 after infection (Fig. 5A). We then tested the effect of vancomycin treatment on wild type and AIEC mutants that were infected for 8 days before antibiotics were initiated, modeling the previous experiments investigating the effects of antibiotics on existing AIEC infection. To do these competitive infections, we constructed a chloramphenicol-sensitive wild-type strain ($wt^{Cm\text{-}S}$) to differentiate it from the unmarked AIEC mutants, and to ensure the absence of competitive infection bottlenecks. Total numbers of AIEC in the feces (which includes both mutant and wild type) were similar in all competitive infections before antibiotic treatment, and AIEC expanded after vancomycin treatment began on day 8 (Fig. 5B). In the presence of vancomycin, the Δ*moaA*, Δ*garD*/*gudDXP*, and Δ*narG/narZ/napA* mutants were significantly attenuated for infection of gut tissue compared to wild type in the colon, cecum, and ileum (Fig. 5C). Interestingly, the

attenuation of the Δ*moaA* and Δ*garD*/*gudDXP* mutants was normalised to near wild type levels in the absence of vancomycin treatment, whereas the Δ*narG/narZ/napA* mutant remained highly attenuated in tissues (Fig. 5D). The $wt^{Cm\text{-}S}$ was recovered to similar levels as wild type AIEC, indicating that this strain was equally fit and there were no significant infection bottlenecks under the conditions of these experiments. These data suggest that AIEC expansion after antibiotics is mediated by the ability to use inflammation-derived metabolites.

Discussion

CD is a multifactorial illness that arises from complex interactions between genetic and environmental factors. The interactions of AIEC with other CD factors has been investigated with some CD-associated genotypes as well as with infectious gastroenteritis[162]. However, the interaction between AIEC and different antibiotics in the setting of host colonisation has not previously been explored. Here we show that antibiotics promote AIEC colonisation and expansion by the production of inflammation-derived metabolites. We found that streptomycin, vancomycin, and gentamicin, but not metronidazole, promoted initial AIEC infection. In contrast to the potentiating antibiotics, a single dose of metronidazole had a minimal impact on AIEC infectivity and the intestinal bacterial community. Metronidazole has high intestinal absorption compared to the other antibiotics tested, which may have resulted in sub-therapeutic levels of the drug within the intestinal lumen when given as a single dose although we did not measure the levels of active drug in our model[174]. Indeed, recurrent exposure to metronidazole in

drinking water did lead to AIEC expansion over several days (Fig. S2.1). Our data suggest that antibiotic-promoted AIEC expansion is correlated with a loss of bacterial richness and evenness, and a disruption from the pre-antibiotic state. This is consistent with early studies in *Salmonella* models that first demonstrated robust intestinal colonisation in the mouse following streptomycin or vancomycin treatment[175]. However, like in the *Salmonella* model, we do not yet know whether there is a common microbial signature in the post-antibiotic state that accounts for this reduced colonisation resistance.

It has been previously suggested that streptomycin reduced colonisation resistance to Proteobacteria by selectively reducing the number of competing facultative anaerobes while sparing obligate anaerobes[139]. We found that streptomycin increased the abundance of obligate anaerobes within the family *Ruminococcaceae*, in agreement with the findings of a previous study[176]. Furthermore, our data also show that levels of endogenous facultative anaerobes, such as those belonging to the phylum Proteobacteria, were already extremely low in all mice before antibiotic treatment. Proteobacteria, specifically *Escherichia*, increased dramatically in vancomycin treated mice yet these mice were equally susceptible to subsequent AIEC infection. These results suggest that the presence of closely related bacteria might not impart colonisation resistance to AIEC. AIEC, including the strain used in this study, are enriched for genes needed for propanediol metabolism, which was shown to provide a fitness advantage for *Salmonella* in the inflamed gut[150,151]. In addition to possessing additional metabolic clusters, it is also possible that AIEC can colonise a niche that allows access to additional sources of nutrition that are not available to commensal *E. coli* by adhering to the mucosal surface,

which we commonly observed after antibiotic treatment. Whether AIEC can use carbon sources more similarly to commensal or to pathogenic *E. coli* has not been studied, however the ability of AIEC to persist in both inflamed and healthy hosts suggests that it may vary its lifestyle and adapt to diverse environments.

The ability of AIEC to benefit from inflammation-derived metabolites following antibiotic treatment is consistent with previous work in commensal *E. coli* and *Salmonella*, which showed that streptomycin causes mild inflammation and the upregulation of *Nos2*[149,157]. Our data showed that vancomycin treatment also causes mild cecal inflammation and a transient upregulation of *Nos2* expression, and that AIEC capable of using inflammation-derived metabolites outcompete mutants lacking these capacities following vancomycin treatment. This suggests that antibiotic-induced inflammation is not specific to streptomycin. While Nos2-derived metabolites appear to contribute to AIEC expansion after antibiotic treatment, AIEC levels did not decline at later time points even though *Nos2* levels do normalise to pre-antibiotic levels. This suggests that there are additional uncharacterized mechanisms by which antibiotics maintain high levels of AIEC burden. Antibiotics are also known to promote bacterial translocation through the formation of goblet cell-associated cell passages[177]. This effect is strongest following a single, high dose antibiotic treatment or for a continuous low dose antibiotic treatment similar to the conditions we used in our study. As such, the formation of goblet cell-associated cell passages may represent one mechanism by which AIEC are able to persist during vancomycin treatment and to gain access into tissues. Additional experiments will be required to address this possibility.

A complete understanding of the role that antibiotics play in CD disease progression is likely hampered by the heterogeneous nature of host and environmental factors driving the disease. A variety of antibiotics are commonly prescribed in the treatment of CD; however, only a few specific CD manifestations have good quality evidence supporting their use. For example, metronidazole has been shown to be effective at preventing post-operative disease reoccurrence in CD patients, whereas vancomycin has been shown to be ineffective at treating CD exacerbations[81]. This has lead to concerns of antibiotic overuse in CD patients, which may be a factor in the selection for antibiotic resistance in AIEC from CD patients[135]. Accordingly, the acquisition of antibiotic resistance genes diminishes the possibility of using antibiotics to eradicate AIEC in CD patients. Clinical data regarding the behaviour of AIEC in healthy individuals or CD patients who are taking antibiotics is lacking. Similarly, the temporal dynamics of AIEC acquisition, and whether CD patients were carriers before diagnosis, is still unknown. Our work shows that AIEC and antibiotic usage may act synergistically to promote gut inflammation and create a susceptible host state. Deciphering the interaction between AIEC and antibiotics in the gut will increase our understanding of the interplay between environmental and microbial factors that lead to CD manifestation.

Acknowledgments

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Figure 2.1. Streptomycin promotes chronic AIEC infection with a low infectious dose.

C57BL/6N mice (n=3-8) were infected with various doses of AIEC or *E. coli* HS 24 h after treatment with PBS or streptomycin. A, Fecal AIEC cfu counts from mice treated with PBS or streptomycin and infected with the indicated dose of AIEC at days 1, 7, or 14 post infection. B, Fecal *E. coli* cfu counts from mice treated with PBS or streptomycin and colonised with the indicated dose of *E. coli* HS at days 1, 7, or 14 post infection. Dotted lines show the limit of detection. n, number of mice per group.

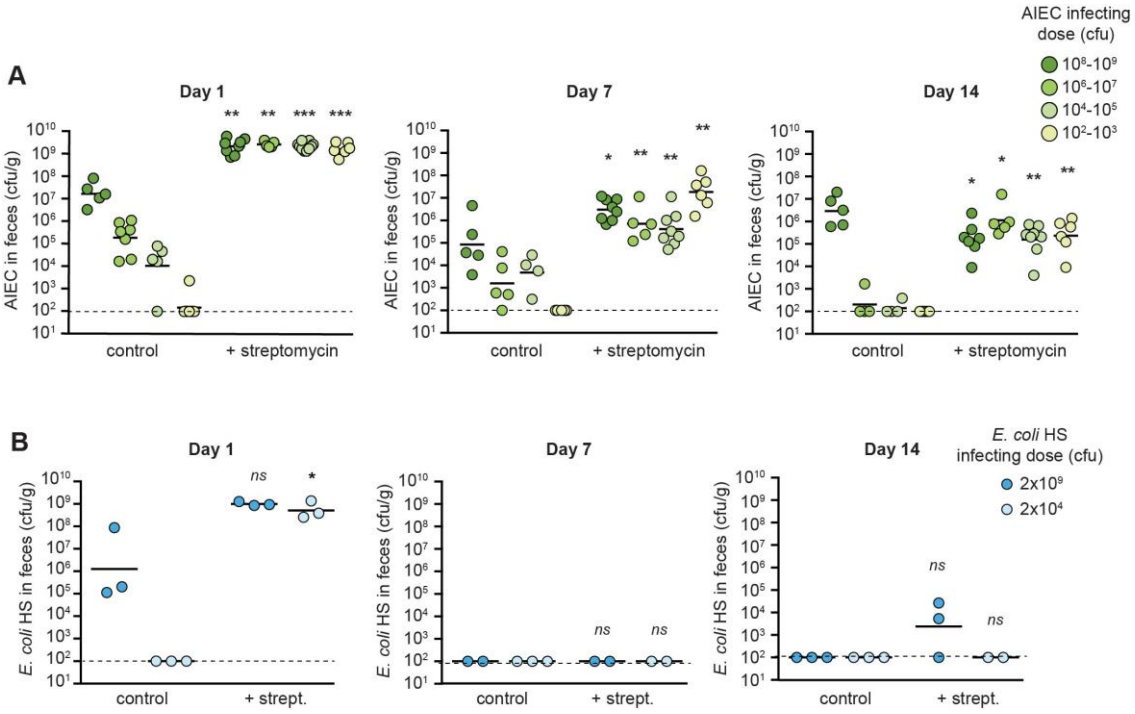

A
Day 1
Day 7
Day 14
AIEC infecting dose (cfu)
10⁸-10⁹
10⁶-10⁷
10⁴-10⁵
10²-10³
AIEC in feces (cfu/g)
control
+ streptomycin
B
Day 1
Day 7
Day 14
E. coli HS infecting dose (cfu)
2x10⁹
2x10⁴
E. coli HS in feces (cfu/g)
ns
control
+ strept.

Figure 2.2. Diverse classes of antibiotics promote AIEC infection and cause unique changes to the fecal microbiome.

CD1 mice (n=8) were treated with PBS, 20 mg streptomycin, 10 mg vancomycin, 1.2 mg gentamicin, or 4.8 mg metronidazole by oral gavage and were infected with $2x10^3$ AIEC. A. Fecal AIEC at days 1 and 14 post infection. B. Total fecal bacteria measured by 16s rDNA qPCR 24 h after antibiotic treatment (one-way ANOVA with Kruskal-Wallis test). C. Shannon index of fecal 16s profiles from mice one day before or one day after antibiotic treatment (two-way ANOVA with Holm-Sidak test). D. Bray-Curtis dissimilarities of fecal samples before or after antibiotic treatment (Mann–Whitney test). E. Stacked bar chart showing relative proportions of bacterial phyla from mice before or after treatment. F. Bray-Curtis principal coordinate analysis plot of fecal 16s profiling data. G. Ratio of the proportion of Firmicutes to Bacteroidetes phyla from mice treated with various antibiotics (two-way ANOVA with Holm-Sidak test). H-J. Relative abundances of *Bacteroides*, *Ruminococcaceae*, and *Escherichia* respectively from mice treated with various antibiotics (two-way ANOVA with Holm-Sidak test). *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

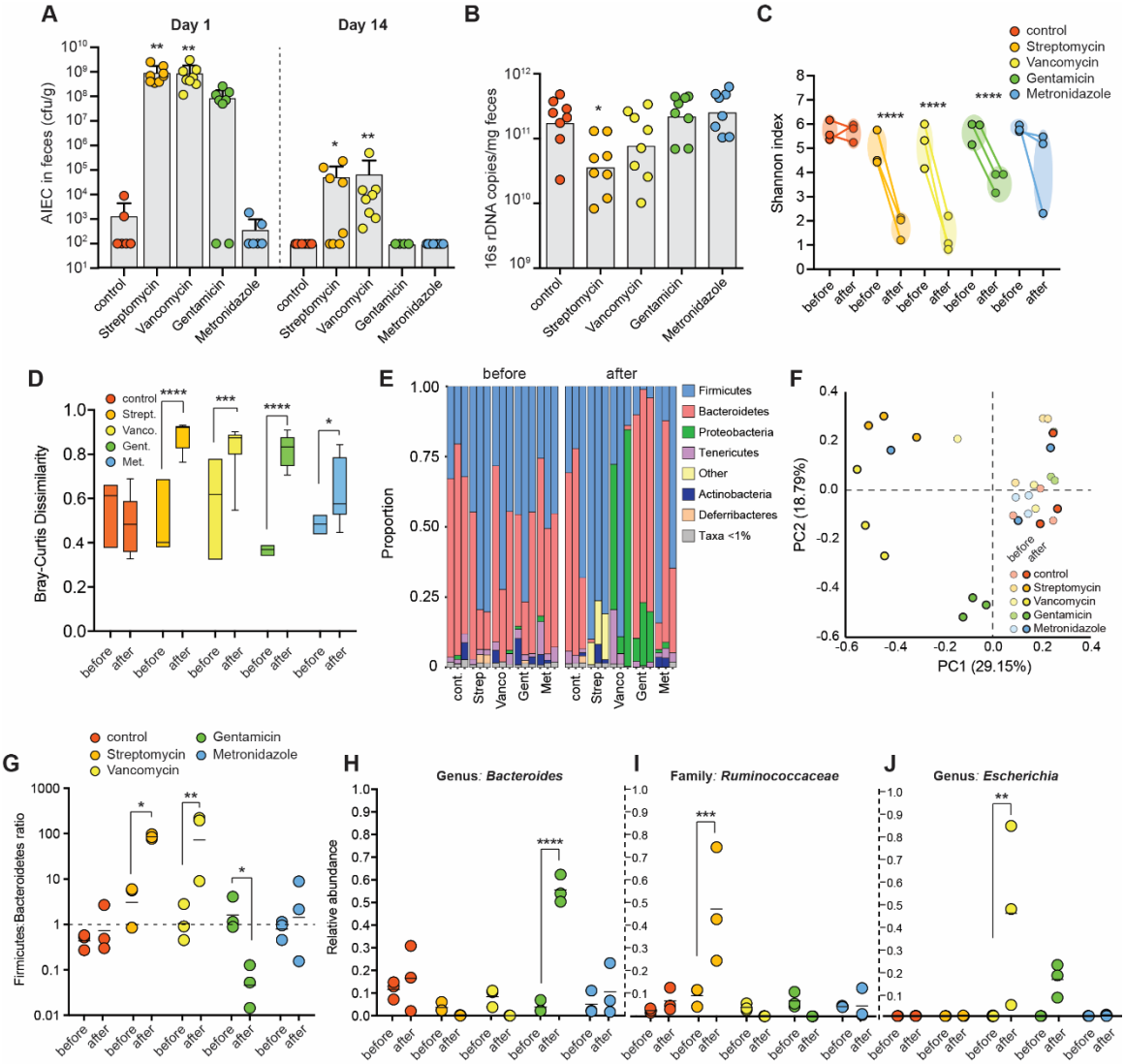

A
Day 1
Day 14
AIEC in feces (cfu/g)
control
Streptomycin
Vancomycin
Gentamicin
Metronidazole
B
16s rDNA copies/mg feces
C
Shannon index
control
Streptomycin
Vancomycin
Gentamicin
Metronidazole
before
after
D
Bray-Curtis Dissimilarity
control
Strept.
Vanco.
Gent.
Met.
E
before
after
Proportion
Firmicutes
Bacteroidetes
Proteobacteria
Tenericutes
Other
Actinobacteria
Deferribacteres
Taxa <1%
cont.
Strep
Vanco
Gent
Met
F
PC2 (18.79%)
PC1 (29.15%)
G
Firmicutes:Bacteroidetes ratio
H
Genus: Bacteroides
Relative abundance
I
Family: Ruminococcaceae
J
Genus: Escherichia

Figure 2.3. AIEC expands in chronically infected mice following vancomycin treatment.

C57BL/6N mice (n=13-14) were pre-treated with streptomycin followed by infection with 2×10^9 cfu AIEC. At 8 days post-infection, mice in the indicated groups received vancomycin in drinking water. A. Fecal AIEC at various days post infection (T-tests with Holm-Sidak). B. Tissue-associated AIEC at 13 and 28 days post infection (T-tests with Holm-Sidak). C. Cecal histopathology scores of H&E stained sections at 13 or 28 days post infection (one-way ANOVA with Kruskal-Wallis test). D. Total nitrate in cecal contents at day 13 post infection (one-way ANOVA with Kruskal-Wallis test). E. Cecal *Nos2* expression relative to water treatment group at day 13 post infection measured by qPCR (one-way ANOVA with Kruskal-Wallis test). F. Cecal *Il1β* expression relative to water treatment group at day 13 post infection measured by qPCR (one-way ANOVA with Kruskal-Wallis test). Error bars show standard deviation. Dotted line shows Firmicutes:Bacteroidetes ratio of 1. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

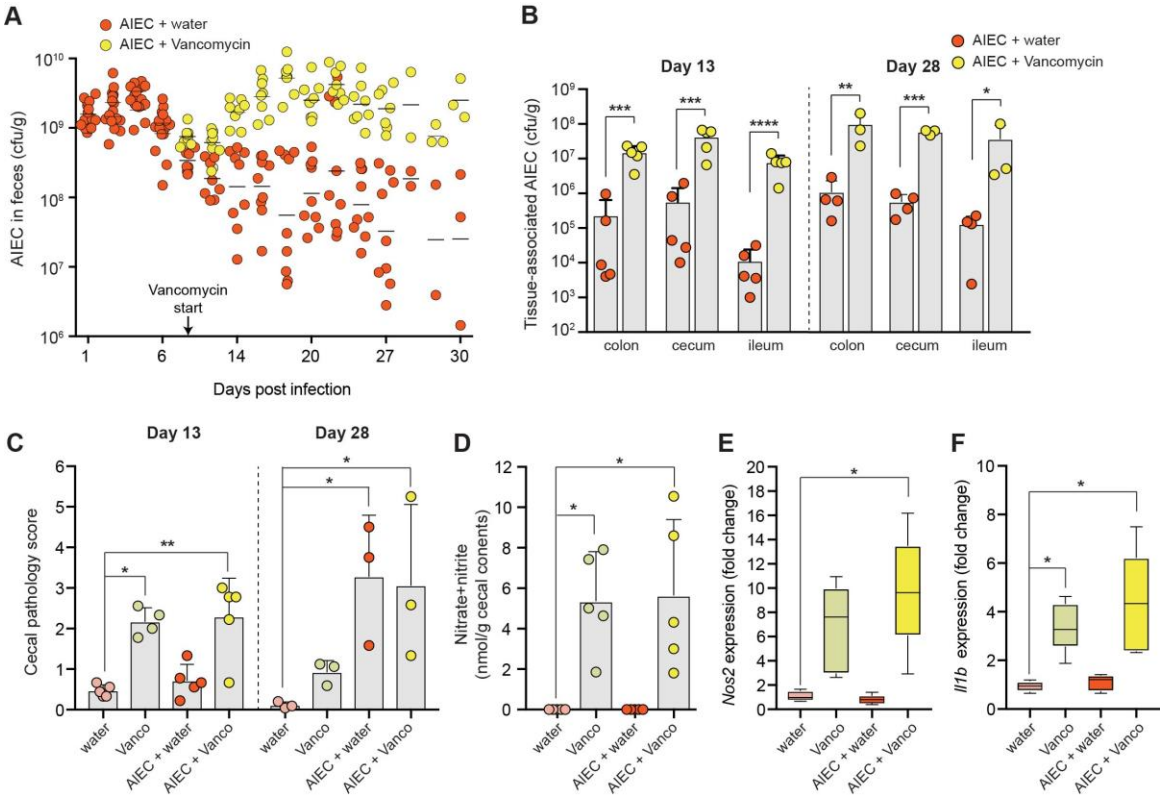

A
AIEC + water
AIEC + Vancomycin
AIEC in feces (cfu/g)
Vancomycin start
Days post infection
B
Day 13
Day 28
AIEC + water
AIEC + Vancomycin
Tissue-associated AIEC (cfu/g)
colon
cecum
ileum
C
Day 13
Day 28
Cecal pathology score
D
Nitrate+nitrite (nmol/g cecal conents)
E
Nos2 expression (fold change)
F
Il1b expression (fold change)
water
Vanco
AIEC + water
AIEC + Vanco

Figure 2.4. Tissue expansion of AIEC following vancomycin treatment.

A. Representative immunofluorescence images (red=AIEC, blue=nuclei) of ceca at day 28 showing AIEC tissue infiltration following water or vancomycin treatment. Two representative micrographs are shown for vancomycin-treated mice and water controls. Left panels are immunofluorescent images; right panels include differential interference contrast image overlay. White arrows indicate AIEC. Luminal AIEC are not indicated by arrows in the vancomycin-treated mice due to the large number of AIEC foci. B. Enumeration of AIEC tissue invasion using automated ImageJ counting (Mann–Whitney test). Data are compiled from 5 individual animals per group. Error bars show standard deviation. **$p<0.01$.

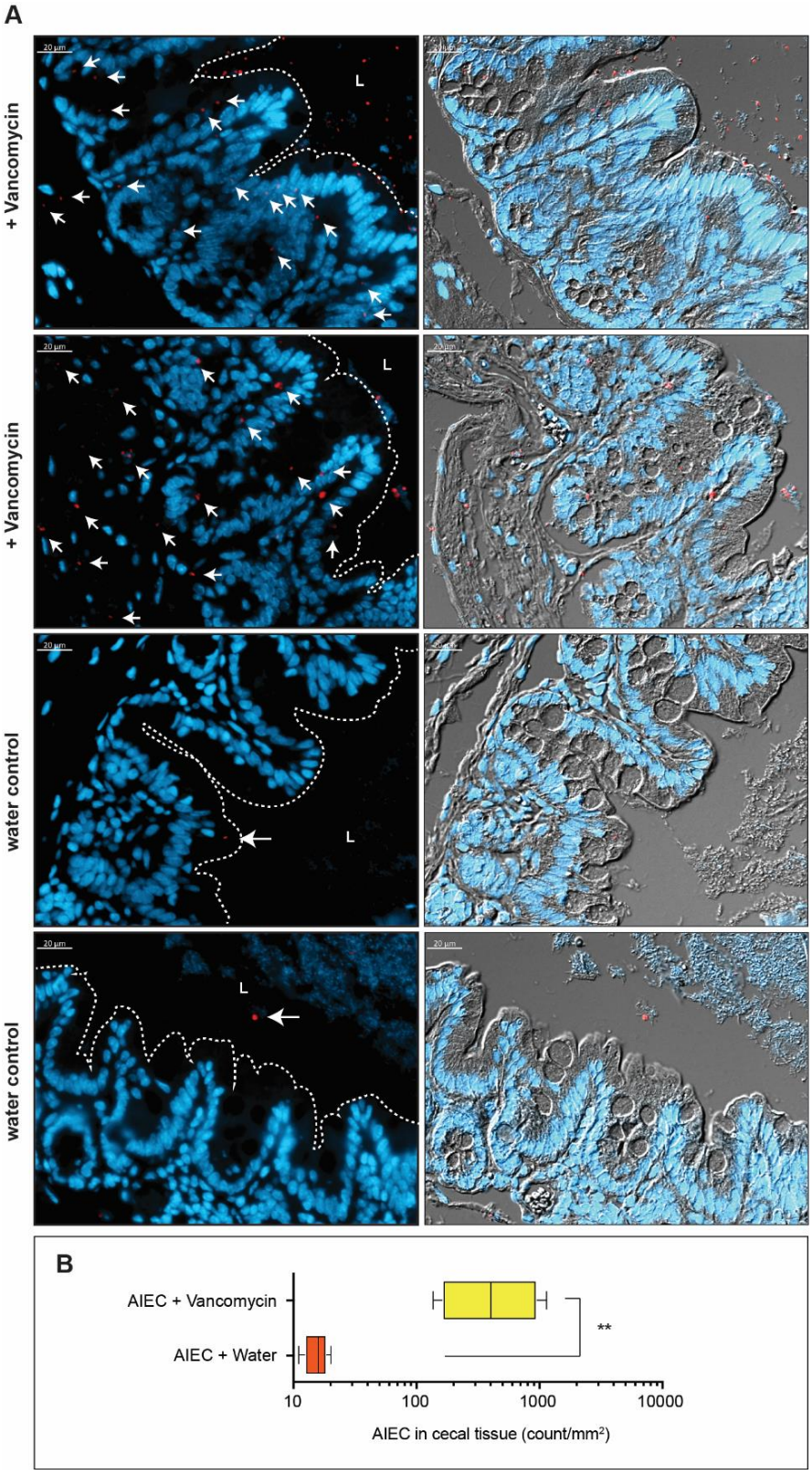

A
+ Vancomycin
+ Vancomycin
water control
water control
L
20 µm
B
AIEC + Vancomycin
AIEC + Water
**
10
100
1000
10000
AIEC in cecal tissue (count/mm²)

Figure 2.5. AIEC mutants lacking nitrate or glucarate/galactarate metabolism show reduced fitness after antibiotic treatments.

A. Competitive indices of Δ*narG/narZ/napA*:WT AIEC from feces of mice treated with PBS or streptomycin (one sample t-test against theoretical CI of 1.0). B. Total AIEC counts from mice were treated with 20 mg streptomycin before infection with 2×10^9 AIEC with a 1:1 ratio of the indicated mutant:wt strain. At day 8, groups were treated with vancomycin in the drinking water. B. Total fecal AIEC (mutant + WT) in days post infection. Data are from 4-10 mice per group. C. Competitive indices of mutant and wt AIEC from indicated tissues taken from mice treated with vancomycin or D, no vancomycin control (one-way ANOVA with Kruskal-Wallis test). Error bars show standard deviation and dotted line shows either limit of detection or a competitive index of 1.0. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

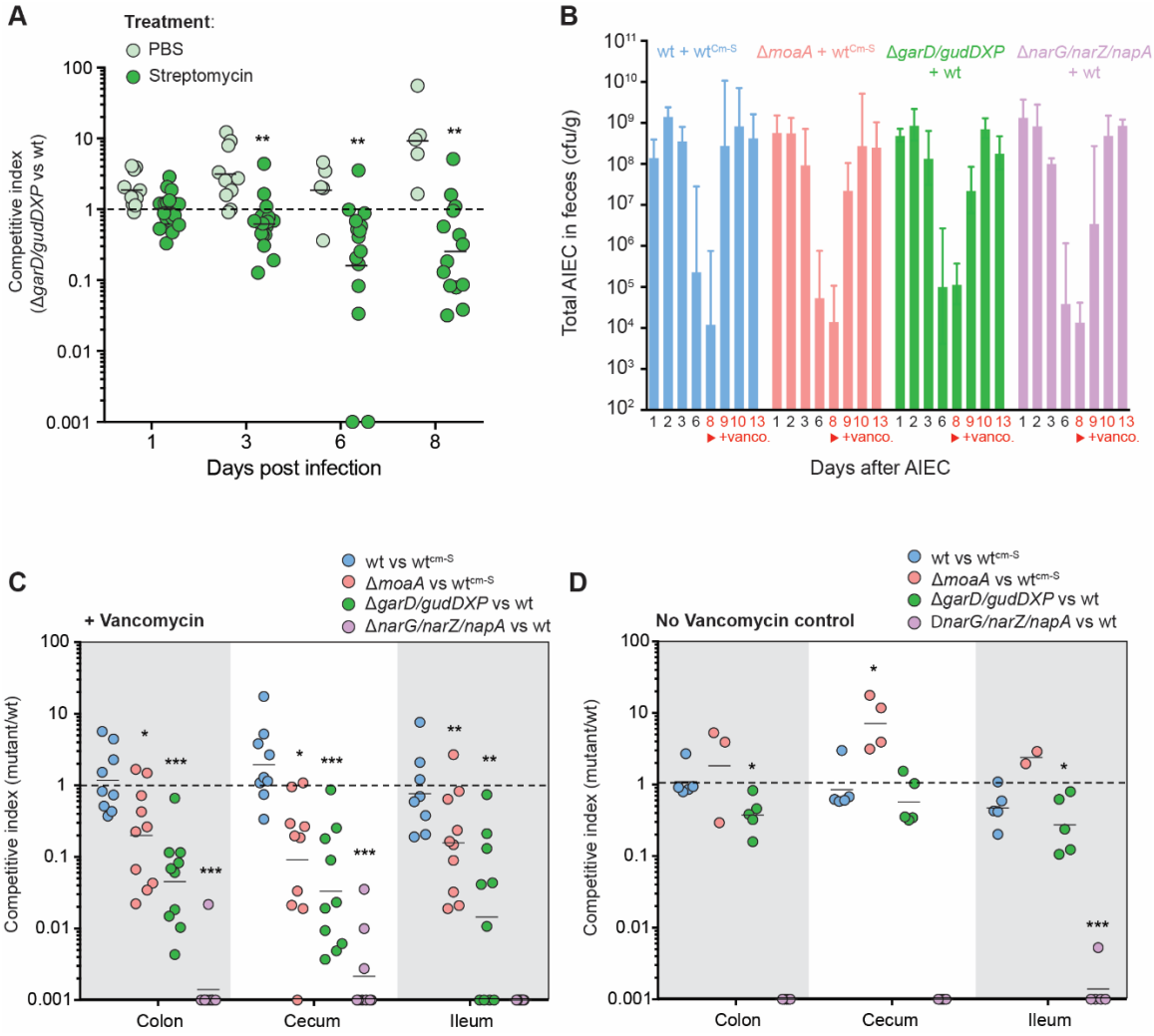

A
Treatment:
PBS
Streptomycin
Competitive index (ΔgarD/gudDXP vs wt)
Days post infection
B
wt + wtCm-S
ΔmoaA + wtCm-S
ΔgarD/gudDXP + wt
ΔnarG/narZ/napA + wt
Total AIEC in feces (cfu/g)
+vanco.
Days after AIEC
C
+ Vancomycin
wt vs wtcm-S
ΔmoaA vs wtcm-S
ΔgarD/gudDXP vs wt
ΔnarG/narZ/napA vs wt
Competitive index (mutant/wt)
Colon
Cecum
Ileum
D
No Vancomycin control
wt vs wtcm-S
ΔmoaA vs wtcm-S
ΔgarD/gudDXP vs wt
DnarG/narZ/napA vs wt
Competitive index (mutant/wt)
Colon
Cecum
Ileum

Figure S2.1. Metronidazole drinking water maintains high levels of AIEC infection. C57BL/6N mice were treated with 20 mg streptomycin and infected with 2×10^9 cfu AIEC. Metronidazole was dissolved in water (750 mg/L) and given to mice from days 0 to 15 post-infection. A, Fecal AIEC cfu counts from mice at various days post infection (Holm-Sidak test). B, Tissue AIEC cfu counts at day 15 post infection (Holm-Sidak test). Dotted line shows limit of detection. ***$p<0.001$.

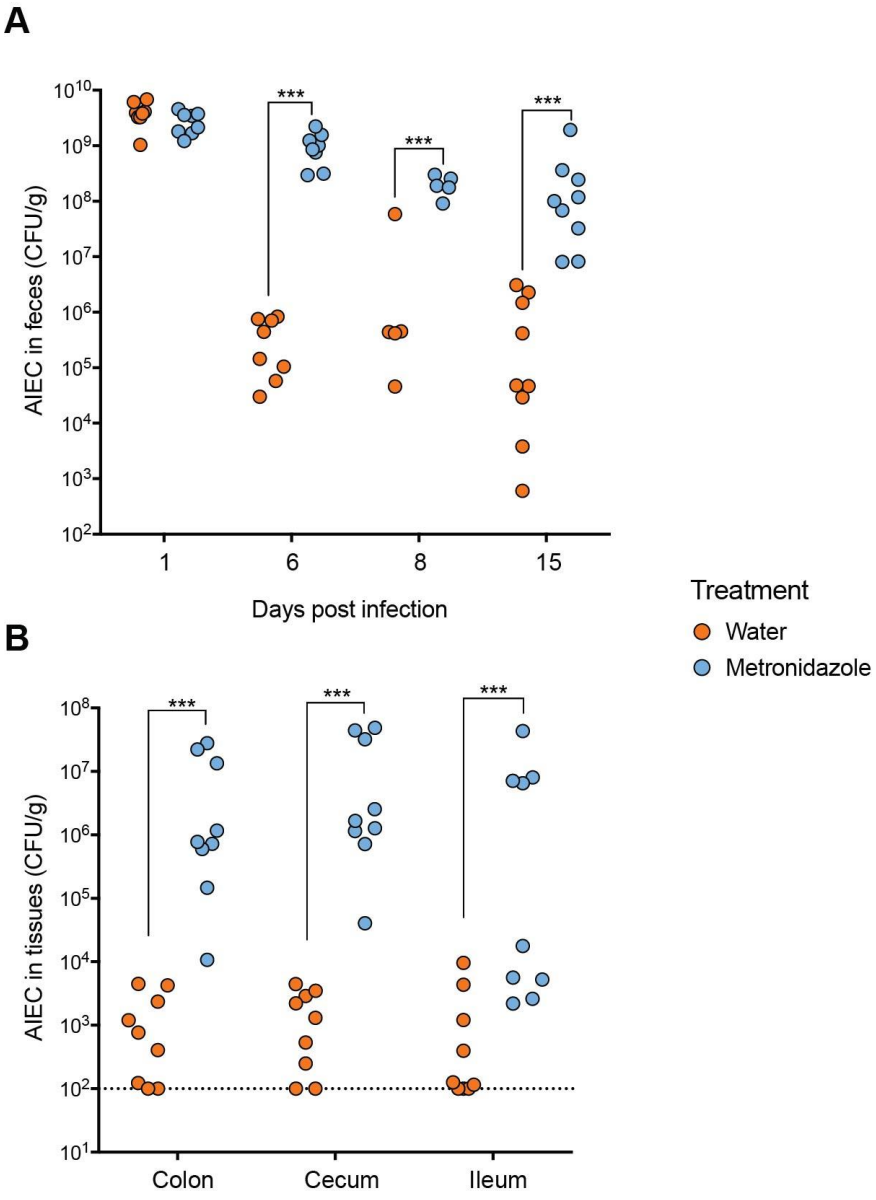

A
AIEC in feces (CFU/g)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
***
***
***
1
6
8
15
Days post infection
B
AIEC in tissues (CFU/g)
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
***
***
***
Colon
Cecum
Ileum
Treatment
Water
Metronidazole

Figure S2.2. AIEC mutants with defects in nitrate or glucarate/galactarate metabolism have reduced growth and fitness in selective media.

A. *In vitro* competitive assay of WT^{cmS} and Δ*moaA* strains in mucin broth with or without added nitrate (Mann–Whitney test). B. *In vitro* competitive assay of WT and Δ*garD/gudDXP* strains in NCE minimal media containing glucose, glucarate, or galactarate as the sole carbon source (One Way ANOVA with Kruskal-Wallis test). C. *In vitro* competitive assay of WT and Δ*narG/narZ/napA* strains in mucin broth with or without added nitrate. D. Oxygen-limited growth curves of strains grown in LB or NCE media with or without nitrate. E. Oxygen-limited growth curves of strains grown in LB or NCE media. Error bars show standard deviation. **p<0.01, ***p<0.001.

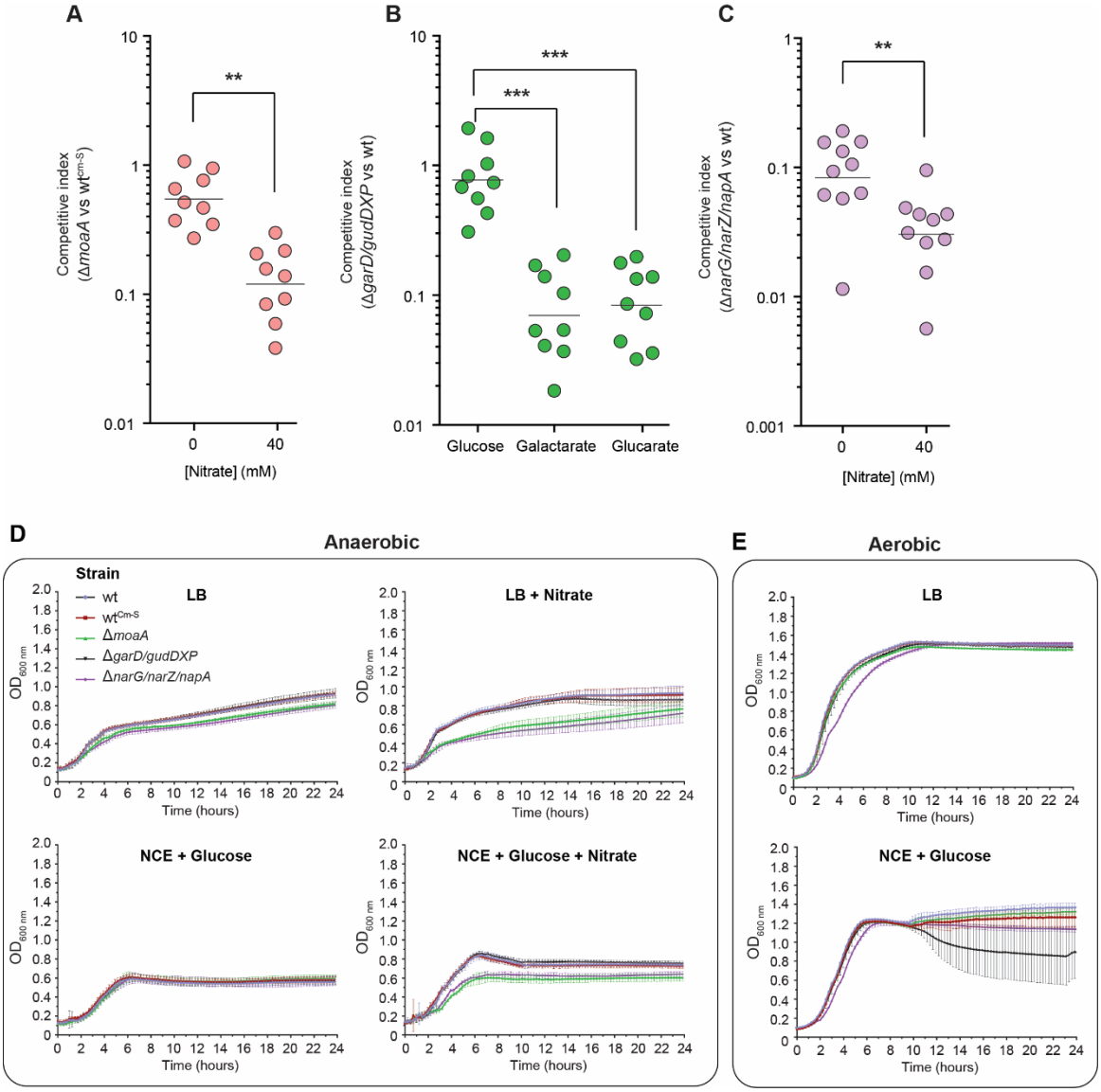

A
B
C
Competitive index (ΔmoaA vs wtCm-S)
Competitive index (ΔgarD/gudDXP vs wt)
Competitive index (ΔnarG/narZ/napA vs wt)
[Nitrate] (mM)
Glucose
Galactarate
Glucarate
D
Anaerobic
E
Aerobic
Strain
wt
wtCm-S
ΔmoaA
ΔgarD/gudDXP
ΔnarG/narZ/napA
LB
LB + Nitrate
NCE + Glucose
NCE + Glucose + Nitrate
OD600 nm
Time (hours)

Figure S2.3. Rarefaction curves of 16S profiling data.

A. Rarefaction curves of observed species before or after treatment with antibiotics. B. Rarefaction curves of Shannon index before or after treatment with antibiotics.

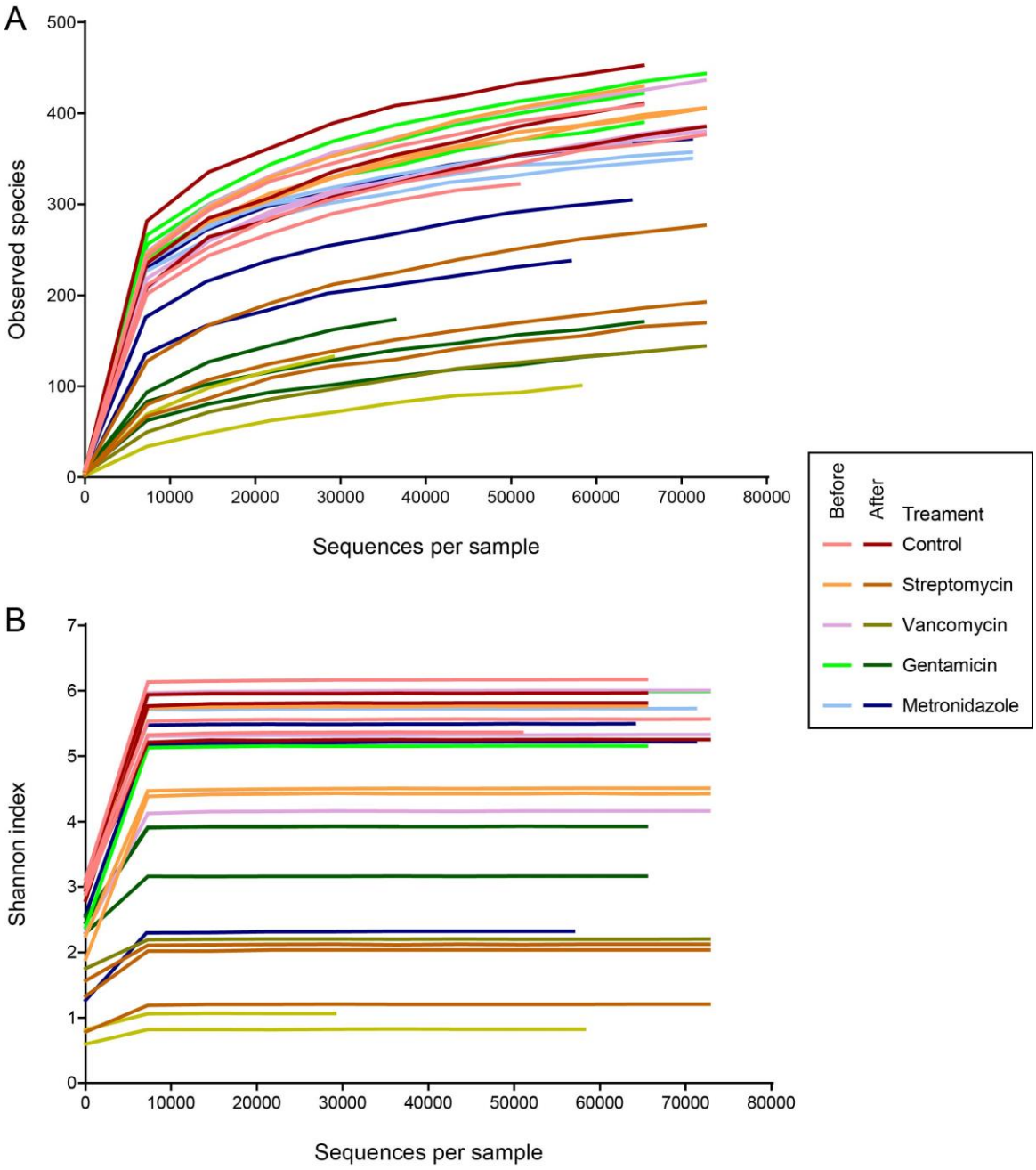

A
Observed species
0
100
200
300
400
500
0
10000
20000
30000
40000
50000
60000
70000
80000
Sequences per sample
B
Shannon index
0
1
2
3
4
5
6
7
0
10000
20000
30000
40000
50000
60000
70000
80000
Sequences per sample
Before
After
Treament
Control
Streptomycin
Vancomycin
Gentamicin
Metronidazole

Table 2.1. List of AIEC mutants used in this study

NRG857c strain	**Genotype or description**	**Antibiotic resistance**
WT	Wildtype	AmpR, CmR, GmS
WT^{cmS}	Δ*catL*	AmpR, CmS, GmS
Δ*moaA*	Δ*moaA*	AmpR, CmR, GmS
Δ*garD*/*gudDXP*	Δ*garD gudDXP::gm*	AmpR, CmR, GmR
Δ*narG*/*narZ*/*napA*	Δ*narG* Δ*narZ napA::gm*	AmpR, CmR, GmR
Plasmid		
pKD3-Gm	pKD3 containing gentamicin resistance instead of chloramphenicol resistance	GmR
pKD46-Kan	pKD46 containing kanamycin resistance instead of ampicillin resistance	KanR
pRE112-Gm	pRE112 plasmid containing gentamicin resistance instead of chloramphenicol resistance	GmR

AmpR, ampicillin resistant; CmR, chloramphenicol resistant; CmS, chloramphenicol sensitive; GmS, gentamicin sensitive; GmR, gentamicin resistant; KanR, kanamycin resistant.

Table 2.2. List of primers used in this study.

Primer	Sequence	Target	Description
neoFwXmnI	AATTGAAAACGTTCAAGATCCCCTCACGCTGCCG	*neoR*	
neoRvXmnI	AATTGAAAACGTTCTCAGAAGAACTCGTCAAGAAGG	*neoR*	
GmFrtFwNcoI	AATTCCATGGGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCTACCTAACAGGTTGGATGATAAGT	*gmR*	
GmFrtRvNcoI	AATTCCATGGGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCTGAGCCTTTCGTTTTATTTGAT	*gmR*	
cp20FwBamHI	AATTGGATCCGGATCCTCTACGCCGGAC	cp20	
cp20RvBamHI	AATTGGATCCCTGTCAGACCAAGTTTACTCA	cp20	
neoKD4FwBamHI	AATTGGATCCAAGATCCCCTCACGCTGCCG	*neoR*	
neoKD4RvBamHI	AATTGGATCCTCAGAAGAACTCGTCAAGAAGG	*neoR*	
catL-1a	ggggtaccAATCGTGTTGAGGCCAACG	*catL*	Allelic exchange
catL-1b	GGTGGTATATCCAGTGATTTTTTTCTCCAT	*catL*	Allelic exchange
catL-2a	ATGGAGAAAAAAATCACTGGATATACCACCCAGGGCGGGGCGTAATTTTTT	*catL*	Allelic exchange
catL-2b	tcccccgggGGCCTGGCATGTTGCAATATTC	*catL*	Allelic exchange
moaA-1a	ggggtaccATGAGAGAACGCGTCCCAGT	*moaA*	Allelic exchange
moaA-1b	TGTAACCATCCGGCAGGC	*moaA*	Allelic exchange
moaA-2a	GCCTGCCGGATGGTTACAGCGCTGCGGGAGAAAAAAC	*moaA*	Allelic exchange
moaA-2b	tcccccgggCGTTTTGTTAGCGACGCCC	*moaA*	Allelic exchange
garD-1a	ggggtaccACAGCGTTGCACGATCGG	*garD*	Allelic exchange
garD-1b	TGCCACATTATCTGTGTCGTGAACT	*garD*	Allelic exchange

garD-2a	AGTTCACGACACAGATAATGT GGCATCAATGGGGACTGCATA ACCAG	*garD*	Allelic exchange
garD-2b	tcccccgggTGAATGTCGAATGGAC GAGTTTTTTGC	*garD*	Allelic exchange
gudDXP-L1	ATGAGTTCTTTAAGTCAGGCTG CGAGCAGTGTGGAAAAACGCA CGAATtaacaattcccctgtagaaataa	*gudDXP*	Lambda red
gudDXP-L2	TTAACGCACCATGCACGGACG CTTGTTATCGAACGTCCAGCCG GGGATatcattcgaaccggtaccca	*gudDXP*	Lambda red
narG-1a	ggggtaccTAATATTTGATTATCCT TTGCGCGGCAT	*narG*	Allelic exchange
narG-1b	GAGAAGCTGGCCATGCCC	*narG*	Allelic exchange
narG-2a	GGGCATGGCCAGCTTCTCGAA GGCAATGACCAGGTACAGG	*narG*	Allelic exchange
narG-2b	tcccccgggCGCCAGCTTGTCAAAC TCAC	*narG*	Allelic exchange
narZ-L1	ATGAGTAAACTTTTGGATCGCT TTCGCTACTTCAAACAAAAGG GCGAAtaacaattcccctgtagaaataa	*narZ*	Lambda red
narZ-L2	TCATTTTTTCGCCTCCTGAACC TGATCGCGACCTTCATCATCCA GCCAatcattcgaaccggtaccca	*narZ*	Lambda red
napA-L1	ATGAAACTCAGTCGTCGTAGCT TTATGAAAGCTAACGCCGTTGC GGCCtaacaattcccctgtagaaataa	*napA*	Lambda red
napA-L2	TTACACCTTCTCCAGTTTGACC GCGCACTTCTTGAAGTCCGTCT CTTTatcattcgaaccggtaccca	*napA*	Lambda red
341F	CCTACGGGAGGCAGCAG	16s rRNA	Illumina sequencing
518R	ATTACCGCGGCTGCTGG	16s rRNA	Illumina sequencing
Tbp-1	ACCGTGAATCTTGGCTGTAAAC	*Tbp*	qPCR
Tbp-2	GCAGCAAATCGCTTGGGATTA	*Tbp*	qPCR
Nos2-3	CATTCAGATCCCGAAACGCTTC	*Nos2*	qPCR
Nos2-4	AGCCTCATGGTAAACACGTTCT	*Nos2*	qPCR
Il1b-1	GCAACTGTTCCTGAACTCAACT	*Il1β*	qPCR
Il1b-2	ATCTTTTGGGGTCCGTCAACT	*Il1β*	qPCR
U16SRT-F	ACTCCTACGGGAGGCAGCAGT	16s rRNA	qPCR
U16SRT-R	TATTACCGCGGCTGCTGGC	16s rRNA	qPCR

Table 2.3. Histopathological scoring for cecal tissue.

LAYER	PATHOLOGICAL CHARACTERISTICS	SCORE	TOTAL
Lumen	empty	(0)	
	Sloughed epithelial cells	scant (1) moderate (2) dense (3)	
	PMNs	< 5 PMNs (0) 5-20 PMNs (1) 21+ PMNs (2)	
Total (lumen)			5
Surface Epithelium	Epithelial Integrity	No Pathological Changes (0) Desquamation (1) Epithelial Erosion (2) Ulceration (3)	
Total (SE)			3
Mucosa	Loss of Crypts	Rare, <15% (1) Moderate, 15-20% (2) Abundant, >50% (3)	
	PMN infiltration	< 5 PMNs (0) 5-10 PMNs (1) 21-60 PMNs (2) >60 PMNs (3)	
	Crypt hyperplasia	Absent (0) Present (1)	
	Crypt distortion	Crypt shortening (1) Mucosal atrophy (shortening and budding of crypts (2)	
	Loss of goblet cells	> 28 goblet cells(0) 11-28 goblet cells (1) 1- 10 goblet cells (2) <1 goblet cell (3)	
	Granulomatous crypt abscesses	(1)	
Total (mucosa)			13
Submucosa	Mononuclear cell infiltrate	1 small aggregate (0) >1 aggregate (1) large aggregates plus single cells (2)	
	PMN infiltrate	No extravascular PMNs (0) Single extravascular PMNs (1) PMN aggregates (2)	
	Lymphocytic infiltrates	No lymphocytes (0) <10 aggregates (1) >50 aggregates (2)	
	Edema	Mild (1) Moderate (2) Severe (3)	
Total (SM)			9

Submucosal edema. (0) no pathological changes. (1) mild edema: 30-49% of the diameter of the entire intestinal wall [tunica muscularis to epithelium]). (2) moderate edema: the submucosa accounts for 50-80% of the diameter of the entire intestinal wall. (3) profound edema: the submucosa accounts for >80% of the diameter of the entire intestinal wall.

PMN infiltration into the lamina propria. Polymorphonuclear granulocytes (PMN) in the lamina propria were enumerated in high-power fields (X400), and the average number of PMN/high-power field was calculated. The scores were defined as follows. (0) 0-5 PMN/high-power field. (1) 5 to 20 PMN/high-power field. (2) 21 to 60/high-power field. (3) 61 to 100/high-power field. (4) 100/high-power field.

Goblet cells. The average number of goblet cells per high-power field (X400). Scoring was as follows: (0) 28 goblet cells/high-power field (1) 11 to 28 goblet cells/high-power field. (2) 1 to 10 goblet cells/high-power field. (3) less than 1 goblet cell/high-power field.

Epithelial integrity. Epithelial integrity was scored as follows: (0) no pathological changes (X400). (1) epithelial desquamation. (2) erosion of the epithelial surface (gaps of 1 to 10 epithelial cells/lesion). (3) epithelial ulceration (gaps of 10 epithelial cells/lesion; at this stage, there is generally granulation tissue below the epithelium).

This table has been modified from Small *et al*., (2016).[162]

CHAPTER 3: STREPTOMYCIN-DEPLETED INTESTINAL MICROBIOTA PROMOTES CHRONIC ADHERENT-INVASIVE *E. COLI* COLONISATION

Author contributions

16s sequencing and the processing of data through the s1p pipeline was performed by the McMaster Genomics Facility. I performed all other experiments and wrote all sections of this chapter.

Abstract

Background: The intestinal microbiota can restrict the ability of bacteria to colonise the gut in a process called colonisation resistance. Adherent-invasive *Escherichia coli* (AIEC), a pathovar of *E. coli* associated with Crohn's disease, lack virulence factors commonly used by other strains of *E. coli* to overcome colonisation resistance. Antibiotics are associated with Crohn's disease incidence and are also known to reduce colonisation resistance towards *E. coli* through the depletion of the intestinal microbiota and the induction of inflammation. We have previously shown that streptomycin reduces colonisation resistance and promotes persistence of AIEC. However, the impact of microbiota perturbation following streptomycin treatment on AIEC persistence has not been studied. We hypothesised that the antibiotic streptomycin would result in a microbiota with reduced diversity and increased susceptibility to long term AIEC persistence.

Methods: We transferred the intestinal microbiota from streptomycin-treated mice to germ-free mice and used increasing doses of AIEC to assess colonisation resistance, and we performed 16s profiling on the fecal microbiota prior to AIEC inoculation.

Results: We found that streptomycin caused a loss of richness and evenness in the intestinal microbiota. While a streptomycin-depleted microbiota did not increase susceptibility to initial AIEC colonisation, once colonisation was established AIEC persisted at higher levels in streptomycin-depleted microbiota mice.

Conclusions: Our results show that the antibiotic streptomycin reduces the diversity of the intestinal microbiota which may impact long-term AIEC colonisation. While an antibiotic depleted microbiota still provides strong colonisation resistance against initial AIEC colonisation when homeostatic conditions return, antibiotics may promote AIEC persistence.

Introduction

The intestine is a diverse environment with substantial changes in the availability of carbon sources and oxygen, the amount of stress from bile salts and acidity, and attachment sites along its length[178]. The variations in physiology between different regions of the intestine creates many niches which are colonised with diverse microbiota. Bacteria within these niches prevent newly acquired bacteria from occupying the intestinal tract in a process called colonisation resistance. Many studies have evaluated the role of colonisation resistance in the intestine towards members of the *Enterobacteriaceae* family such as *Salmonella* Typhimurium (STm) and *Escherichia coli* due to their pathogenic potential and ease of culturing. Colonisation resistance towards

Enterobacteriaceae has been shown to be mediated by a number of interrelated host and microbe mediated factors[168].

One aspect of colonisation resistance is competition from closely related microbes which are thought to compete for the same intestinal niche. Studies from mice have shown that colonisation with a simplified microbiota lacking *Enterobacteriaceae* show poor colonisation resistance towards STm[179], and that the introduction of *Enterobacteriaceae* species and other facultative anaerobes leads to a restoration of colonisation resistance[180]. However, while some commensal *E. coli* are able to prevent colonisation with an isogenic strain, many are not able to prevent infection with pathogenic *E. coli* showing that pathogenic bacteria can occupy or create a different niche to overcome colonisation resistance[181].

Pathogenic STm and *E. coli* can partially overcome colonisation resistance using virulence factors, which can disrupt the inhibitory effects of anaerobic bacteria or their metabolites. In addition, many pathogenic *Enterobacteriaceae* have additional nutrient uptake pathways compared to their commensal relatives that provide a growth advantage during inflammation[151,182]. However, adherent-invasive *E. coli* (AIEC), which are associated with Crohn's disease, do not possess typical virulence factors and the traits that promote their initial colonisation are not well understood[183]. Antibiotics are associated with CD risk and decreased colonisation resistance towards *Enterobacteriaceae*[34,73], and it is possible that antibiotics play a role in promoting colonisation with AIEC. Antibiotics are known to decrease colonisation resistance towards *E. coli* through several well-described mechanisms such as increased availability of nitrate, increased oxygenation,

and decreased inhibition from microbiota-derived metabolites[149,157]. Antibiotics are also known to cause a lasting depletion of the gut microbiota[83], which appears to persist longer than the transient changes in nutrient availability. However, the effects of antibiotic depletion of the gut microbiota on AIEC colonisation resistance have not been studied in isolation from the transient changes in nutrient availability after antibiotic exposure. In this study, we investigate how depleting the gut microbiota using the antibiotic streptomycin influences both initial and chronic AIEC colonisation.

Materials and Methods

Ethics statement

Animal experiments were conducted according to guidelines set by the Canadian Council on Animal Care using protocols approved by the Animal Review Ethics Board at McMaster University under Animal Use Protocol #17-03-10.

Bacterial strains

AIEC strain NRG857c (serotype O83:H1) was isolated from an ileal tissue biopsy from a CD patient in Charite Hospital (Berlin, Germany) and its genome sequence was determined previously[100]. Chloramphenicol (34 μg/mL) and ampicillin (100 μg/mL) were used in LB broth or agar plates to provide selection. Prior to mouse infections or *in vitro* assays, AIEC strains were grown in LB with antibiotics for 16–18 h at 37°C with shaking.

For mouse infections, AIEC were resuspended in phosphate buffered saline (PBS) at the appropriate densities and given to mice as a 100 µL oral gavage.

Animal infections

Six to eight-week-old female SPF C57BL/6N mice were purchased from Charles River Laboratories. Germ-free (GF) mice were acquired from the gnotobiotic facility at McMaster University. Animals were housed in a specific pathogen-free barrier unit under level 2 conditions at the Central Animal Facility at McMaster. For colonisation of germ-free mice, donor SPF mice were treated with streptomycin (20 mg) or PBS by oral gavage. Donor mice were sacrificed 24 hours later and the cecal contents were immediately diluted 1/10 with sterile PBS. GF mice were gavaged with 100 µL of diluted cecal contents within 10 minutes of collection to avoid exposure to oxygen and were housed separately for 4 weeks prior to infection to allow for colonisation. To compare AIEC colonisation in reconstituted GF with conventional mice, SPF mice were treated with PBS or streptomycin (20 mg) one day prior to AIEC inoculation.

Bacterial enumeration

Fecal pellets were weighed, homogenised in 1 mL PBS, serially diluted, and plated onto LB agar plates containing selective antibiotics. Intestinal tissues were flushed with PBS to remove luminal contents, homogenised with a metal bead, and plated in the same manner as feces. Plates were incubated overnight at 37°C and colonies were counted to determine CFU per gram of feces or tissue.

Histopathology

At 28 days post infection, cecal sections were fixed in 10% neutral-buffered formalin overnight and were processed into paraffin blocks. The blocks were cut into two 5 μm sections and stained with H&E, and for each block 9 high power field of view regions were used for histology scoring. The histology scoring system was described previously[162], and has been reproduced as Table 2.3 in detail. The pathology scores within this manuscript reflect the total pathology from all regions (lumen, lamina propria, mucosa, and submucosa).

16s microbiome profiling

DNA was isolated from feces or cecal contents as described in Minas *et al*[165] except the homogenates were not lyophilised but were instead diluted 1:2 into CTAB buffer. Extracted DNA was used to amplify the v3 region of the 16s rRNA gene by PCR. 50 ng of DNA was used as template with 1U of Taq, 1x buffer, 1.5 mM $MgCl_2$, 0.4 mg/mL BSA, 0.2 mM dNTPs, and 5 pmoles of Illumina adapted primers 341F and as described in Bartram *et al*[166]. The following PCR cycling conditions were used: 94°C for 5 minutes; 25 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds; and a final extension of 72°C for 10 minutes. PCR products were visualised on a 1.5% agarose gel. Positive amplicons were normalized using the SequalPrep normalization kit (ThermoFisher#A1051001) and sequenced on the Illumina MiSeq platform at the McMaster Genomics Facility. Resulting sequences were processed with

the sl1p pipeline using the default settings[167]. Briefly, FASTQ reads were trimmed with Cutadapt, aligned with PANDAseq (v2.9), quality trimmed with sickle (v1.33), and chimeric sequences were removed with USEARCH. OTUs were assigned with AbundantOTU+ 0.93b and taxonomic assignments were made using RDP Classifier (v2.2) against the database Greengenes (Feb. 2011). OTU tables, phylogenies, and preliminary analyses are conducted using QIIME and R (v3.3.1).

Rarefaction curves of observed species and Shannon index are shown in Figure S3.1. Reads were rarified at 10754 reads for the Shannon index in Figure 3.3D. Relative abundances of taxa from SPF+strep and GF+strep were compared with SPF samples. Taxa which were >1% abundance in the SPF+strep or GF+strep groups which showed a >2 fold increase or decrease in abundance were screened and several were selected for Figure 3.3 E-G.

Statistical analysis

Data were analyzed using GraphPad Prism 6.0 software (GraphPad Inc., San Diego, CA). Statistical significances were calculated using Mann-Whitney tests, Student T-tests, one-way ANOVAs with Kruskal-Wallis tests, or two-way ANOVAs with Holm-Sidak tests when appropriate.

Results

Streptomycin-depleted intestinal microbiota does not confer increased susceptibility to AIEC infection in germ-free mice

Streptomycin is an antibiotic that is commonly used to lower colonisation resistance towards *E. coli*. In chapter 2, we demonstrated that streptomycin substantially reduces the dose of AIEC needed to establish colonisation and increased persistence. We also showed the streptomycin significantly altered the intestinal microbiota and decreased bacterial richness and evenness. To separate the effects that antibiotics have on bacterial diversity from the transient changes to nutrient availability, we transferred the cecal microbiota from conventional, SPF mice treated with either PBS or streptomycin (SPF donor or SPF+strep donor) mice into germ-free mice (GF+SPF or GF+strep) (Fig 3.1).

Four weeks after transplantation, we treated another cohort of SPF mice with PBS or streptomycin (SPF or SPF+strep). All four groups (GF+SPF, GF+strep, SPF, SPF+strep) were infected with 4×10^2 CFU AIEC on the following day. Fecal burdens following infection indicated that both GF groups were resistant to AIEC infection, but the SPF group was transiently infected (Fig. 3.2A). As expected, the SPF+strep group had high AIEC colonization, which was maintained up to 28 days post infection.

Following clearance of AIEC from the SPF group at 8 days post infection, we re-infected this group and the GF groups with progressively higher doses of AIEC to investigate the minimum dose required for AIEC colonisation. Infection with 4×10^4 CFU of AIEC promoted transient colonisation in all three groups, but all the mice had cleared

by day 21 post-initial infection. These three groups were then infected with 2×10^9 CFU. At this dose, the GF groups maintained AIEC colonisation up to day 28 post-initial infection, while several mice in the SPF group cleared AIEC. Between the two GF groups, those that had received microbiota from streptomycin treated mice (GF+strep) had significantly higher cecal AIEC colonization than the GF+SPF group (Fig. 3.2B).

While we saw differences in the colonisation of AIEC in each of these treatments, bacterial density, measured by fecal DNA content, was significantly reduced only in the SPF+strep group indicating that total bacterial levels recover when transferred to GF mice (Fig. 3.2C). Additionally, we did not observe any significant cecal pathology in any of the groups at day 28 post-infection (Fig. 3.2D).

Streptomycin-depleted intestinal microbiota only partly recapitulates dysbiosis in germ-free mice

While we have previously shown in chapter 2 that streptomycin reduces bacterial richness and evenness, it was unclear if bacterial species were eliminated or were transiently reduced to levels below detection. Furthermore, it was not known if the changes in microbial diversity which occurred with streptomycin would stably colonise untreated GF mice.

To determine the similarity of intestinal microbiota between conventional and colonised GF mice, we performed 16S profiling on feces from the cecal donor samples and all groups of mice at day 0. At the phylum level, we found that SPF, GF+SPF, and GF+strep had similar levels of Bacteroidetes, which were reduced in SPF+strep mice

(Fig. 3.3A). We found that the Shannon index, a measure of species richness and evenness, was significantly decreased in both SPF+strep and GF+strep (Fig. 3.3B). We analysed beta diversity using the Bray-Curtis dissimilarity, which measures the differences between bacterial species diversity. We found that SPF and GF+SPF clustered closely by principal coordinate of analysis (Fig. 3.3C-D), whereas SPF+strep and GF+strep each clustered separately showing that these groups had unique microbial compositions. We also found that the cecal donor samples, which were collected from the ceca lumen rather than feces and were collected four weeks earlier, showed similar clustering to the fecal samples from the SPF and SPF+strep groups. This suggests that inoculum used to colonise GF mice had similar diversity to SPF and SPF+strep intestinal microbiota although it is possible that certain taxa were lost during transfer from donor to recipient mice. Whereas a homeostatic microbiota maintains its community structure when transferred to a healthy GF host, a streptomycin-depleted microbiota adopts a unique structure with altered abundances of bacterial taxa that does not resemble the inoculum. This suggests that streptomycin-induced alterations that occur 24 hours after treatment are transient and a new microbiota composition develops from the remaining taxa in GF mice.

When examining the proportions of individual bacterial groups, we found that several genera within Bacteroidetes such as *Bacteroides* and *Prevotella* were common in SPF and GF+SPF mice but were reduced in SPF+strep and nearly absent in GF+strep (Fig. 3.3E-F). Conversely, we found that *Enterobacteriaceae*, the bacterial family that includes *E. coli*, was elevated only in SPF+strep (Fig. 3.3H). We found similar levels of

Clostridia in all groups (Fig. 3.3H), although the relative proportions of families with Clostridia were altered in SPF+strep (Fig. 3.3I). These results show some taxa which are depleted during streptomycin treatment become undetectable in GF mice, suggesting that these taxa were either eliminated from the microbiota or that an alternative community structure prevents them from returning to homeostatic abundances. However, although *Enterobacteriaceae* was elevated after streptomycin treatment, this family was not elevated after transfer to GF mice. These findings further support the notion that streptomycin can induce transient changes in the microbiota which results in an altered community structure with reduced bacterial richness and evenness.

Discussion

Streptomycin is an antibiotic which is commonly used to lower colonisation resistance prior to colonisation or infection with *E. coli* or STm. However, the role of decreased bacterial diversity following antibiotic treatment has not been studied in isolation. In this study, we have shown that streptomycin causes a loss of bacterial richness and evenness in the fecal microbiome which is maintained when the streptomycin-treated microbiota is transferred to a germ-free host. However, the loss of bacterial diversity is not sufficient to reduce colonisation resistance towards AIEC.

We found that GF+strep mice showed high colonisation resistance against AIEC colonisation compared to SPF+strep. However, GF+strep mice showed increased AIEC persistence. This suggests that the barriers to initial colonisation and persistent

colonisation may be distinct, which can be reflected in the changes in microbiome structure. GF+strep mice had a low species richness and evenness and had undetectable levels of several genera that were similarly decreased in SPF+strep (Fig. 3.3). Our data is consistent with the notion that a loss of bacterial diversity in the microbiota is not an important determinant for initial AIEC colonisation. However, components of the microbiota which are lost during streptomycin treatment may play a role in preventing AIEC persistence within hosts. It is also possible that the transplantation of the microbiota was biased as cecal extracts were exposed to oxygen for a brief period prior to transplantation and it is possible some anaerobic species were lost. Since cecal contents were orally gavaged it is possible that the stomach environment may have also limited the ability of some bacteria to transplant. However, since we found that the GF+SPF microbiota resembled the microbiota of SPF, these bottlenecks do not appear significant.

Endogenous *Enterobacteriaceae* have been shown to contribute to colonisation resistance, but only if the strains are highly related, occupy the same niche, or compete for a common limiting nutrient[179–181]. In our study, levels of endogenous *Enterobacteriaceae* were low and significantly increased only in SPF+strep mice (Fig. 3.3G). Since AIEC were highly infective in SPF+strep mice, this suggests that endogenous *Enterobacteriaceae* did not appear to significantly contribute to colonisation resistance towards AIEC. We have previously found that AIEC do not interfere with STm infection, which also shows that AIEC can occupy a different niche or coexist with pathogenic *Enterobacteriaceae*[162].

The production of SCFAs by the microbiota is another aspect of colonisation resistance which can be altered using antibiotics. The SCFA propionate has been shown to be reduced following streptomycin treatment[184]. Interestingly, we found that the SPF+strep and GF+strep mice had significantly reduced levels of *Bacteroides*, which contain species that are major producers of propionate (Fig. 3.3E). Propionate directly inhibits STm and the ability of the microbiota to produce propionate is a factor that influences the chronic burden of STm in infected mice[36]. While it is not known whether propionate also similarly inhibits AIEC colonisation, decreased propionate levels may be one factor that promotes higher long-term colonisation of AIEC in both SPF+strep and GF+strep mice. However, the high colonisation resistance against AIEC in GF+strep mice suggests that levels of *Bacteroides* may not play a role for initial colonisation.

Previous research has suggested that streptomycin reduces levels of butyrate-producing *Clostridia* which contributes to increased luminal oxygenation[21,32]. However, we did not observe any significant changes in proportions in *Clostridia* either after streptomycin treatment or after transfer to GF mice (Fig 3.3H). This may be due to only measuring relative changes compared to absolute levels of *Clostridia* per gram of feces. We did observe that SPF+strep mice relatively reduced levels of *Lachnospiraceae* and *Ruminococcaceae* (Fig. 3.3I). These families contain most of the butyrate-producing species within *Clostridia*[32], and it is possible that concentrations of butyrate were decreased in SPF+strep mice.

AIEC are hypothesised to evolve uniquely within each individual and it is possible that the isolate we use has evolved for persistence within an individual rather than for

transmission[136]. Pathogenic *E. coli* and STm use virulence factors and induced a controlled inflammatory responses to induce a favourable increase in electron acceptors and to reduce competition from commensal bacteria[21,22]. AIEC do not possess equivalent inflammation-promoting virulence factors, and they may not require them due to the increased inflammatory state of CD. As a result, AIEC may be relatively poorly adapted to initial colonisation in healthy hosts and requires the transient changes in nutrient availability that occur after antibiotic treatment to overcome colonisation resistance. Our results show that streptomycin can reduce bacterial species richness and evenness, and that this can have an impact on AIEC persistence. However, the microbiota, even following streptomycin depletion, can provide strong colonisation resistance to AIEC after homeostatic conditions return. These results suggest that antibiotics can promote both initial colonisation resistance and persistence through different mechanisms.

Figure 3.1. Experimental workflow showing colonisation of germ-free mice with conventional or streptomycin-deplete microbiota.

Donor mice SPF C57BL/6N mice were treated with PBS or streptomycin, and 24 hours later cecal contents were transferred to germ-free mice (GF+SPF or GF+strep respectively) and the microbiota were allowed to establish for 28 days. These groups were first inoculated with AIEC at day 0. As controls, two groups of SPF C57BL/6N mice were treated with PBS or streptomycin 24 hours prior to inoculation with AIEC at day 0.

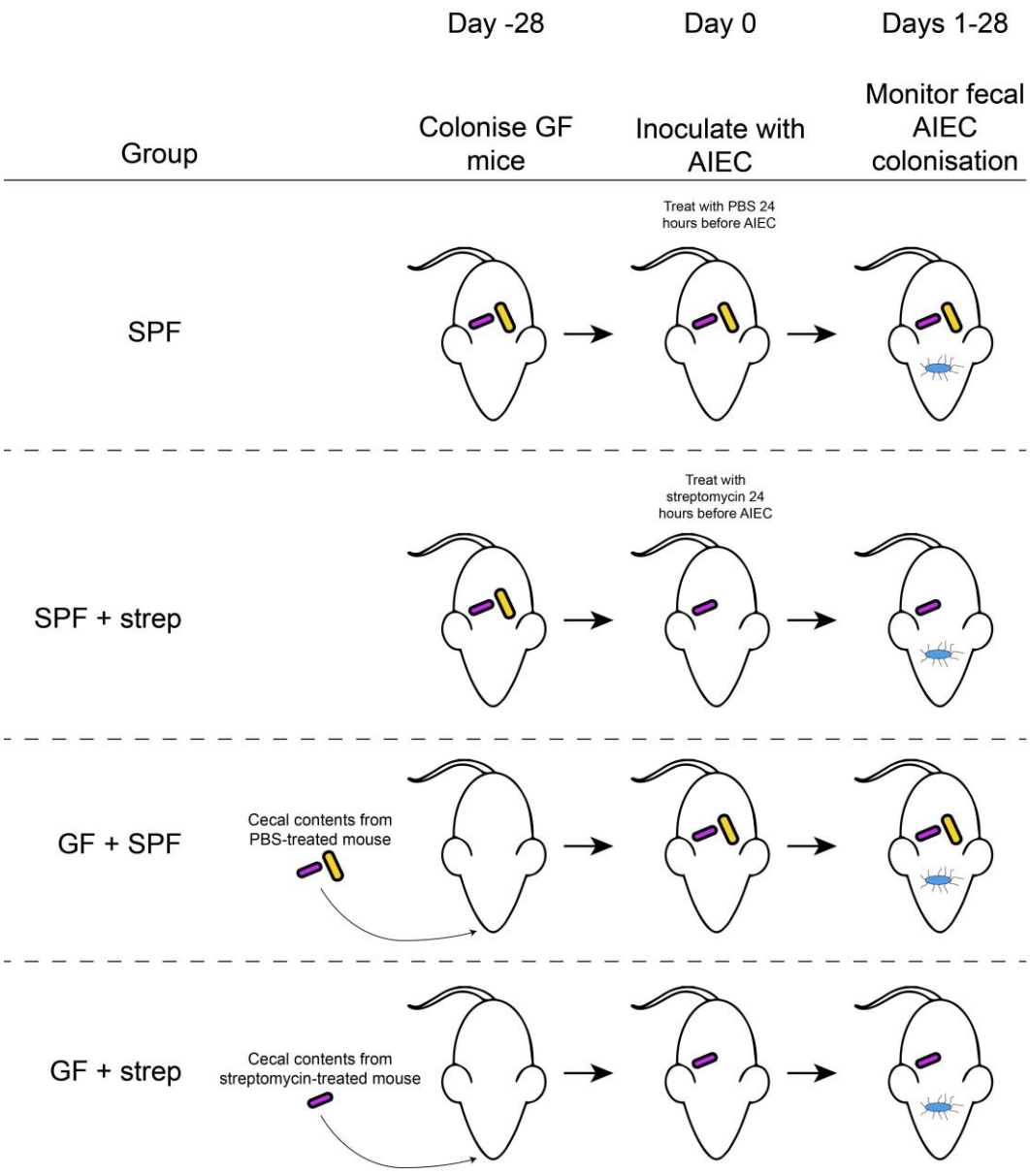

Day -28
Day 0
Days 1-28
Group
Colonise GF mice
Inoculate with AIEC
Monitor fecal AIEC colonisation
Treat with PBS 24 hours before AIEC
SPF
Treat with streptomycin 24 hours before AIEC
SPF + strep
Cecal contents from PBS-treated mouse
GF + SPF
Cecal contents from streptomycin-treated mouse
GF + strep

Figure 3.2. Transferring streptomycin-depleted microbiota to germ-free mice is not sufficient to confer increased susceptibility to AIEC infection.

Both germ free groups (GF+SPF and GF+strep) and SPF control groups (SPF and SPF+strep) were challenged with 4×10^2 CFU AIEC. By day 8, all groups except SPF+strep had cleared AIEC and were challenged with 4×10^4 CFU AIEC. By day 21, these groups had again cleared AIEC and were then challenged with 2×10^9 CFU AIEC. A. Fecal AIEC CFU over days post infection. B. Cecal contents and tissue-associated AIEC CFU at D28 post infection (one-way ANOVA with Kruskal-Wallis test). C. DNA content of fecal pellets at D0 (one-way ANOVA with Kruskal-Wallis test). D. Cecal pathology score at D28 post infection. Dotted line shows limit of detection. *$p<0.05$, **$p<0.01$

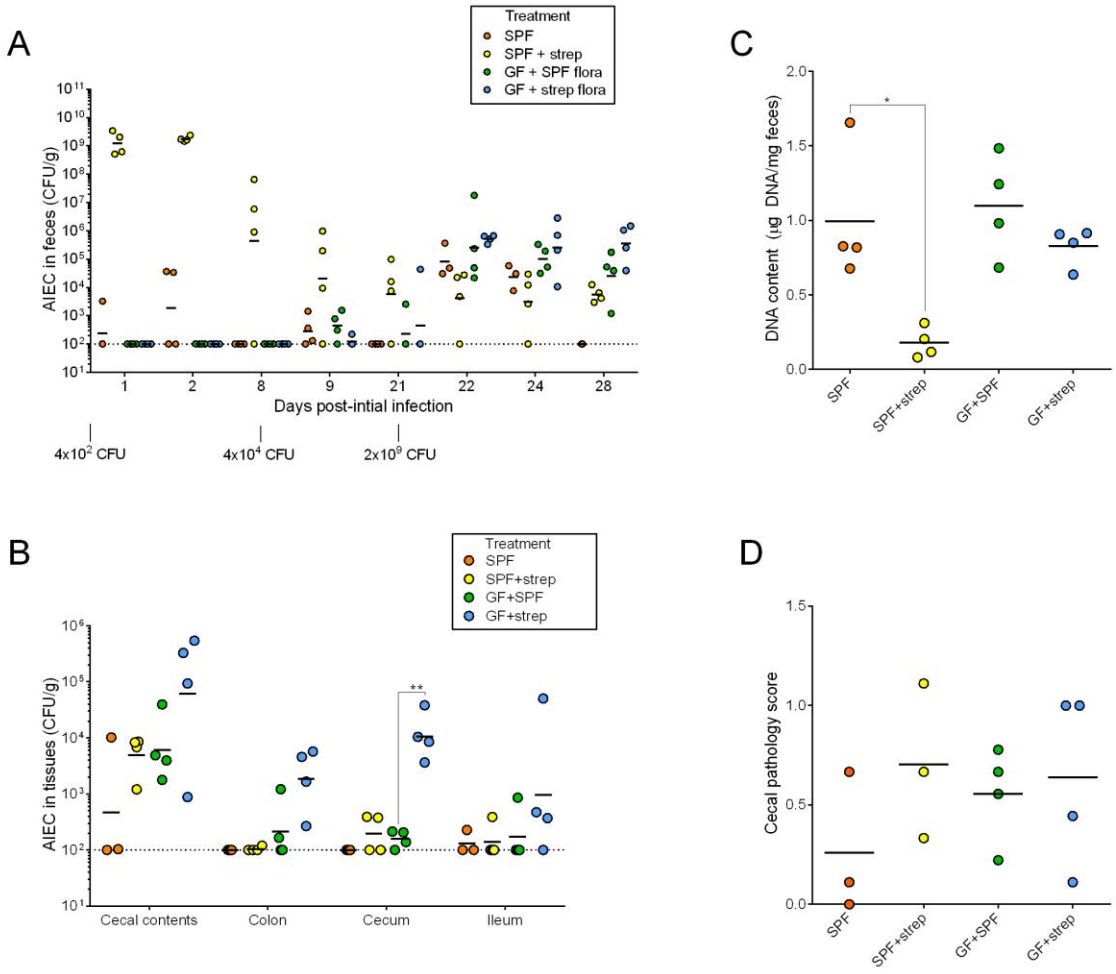

A
Treatment
SPF
SPF + strep
GF + SPF flora
GF + strep flora
AIEC in feces (CFU/g)
Days post-intial infection
4x10^2 CFU
4x10^4 CFU
2x10^9 CFU
B
Treatment
SPF
SPF+strep
GF+SPF
GF+strep
AIEC in tissues (CFU/g)
Cecal contents
Colon
Cecum
Ileum
**
C
DNA content (µg DNA/mg feces)
*
SPF
SPF+strep
GF+SPF
GF+strep
D
Cecal pathology score
SPF
SPF+strep
GF+SPF
GF+strep

Figure 3.3. Transfer of streptomycin-depleted microbiota results in decreased bacterial diversity and a unique microbiome composition.

A. Stacked bar charts of unrooted abundances of bacterial phyla. B. Bray-Curtis principal coordinate of analysis plot of 16S profiling data from feces at day 0, or from cecal contents of donor mice at 28 days before infection. C. Bray-Curtis dissimilarity relative to SPF mice. D. Shannon index of 16S profiling data. E. Relative abundances of *Bacteroides* in fecal or cecal samples. F. Relative abundances of *Prevotella* in fecal or cecal samples. G. Relative abundances of *Enterobacteriaceae* in fecal or cecal samples. H. Relative abundances of Clostridia in fecal or cecal samples. I. Percent abundances of various Clostridia families. Error bars show standard deviation. *$p<0.05$, **$p<0.01$, ***$p<0.001$

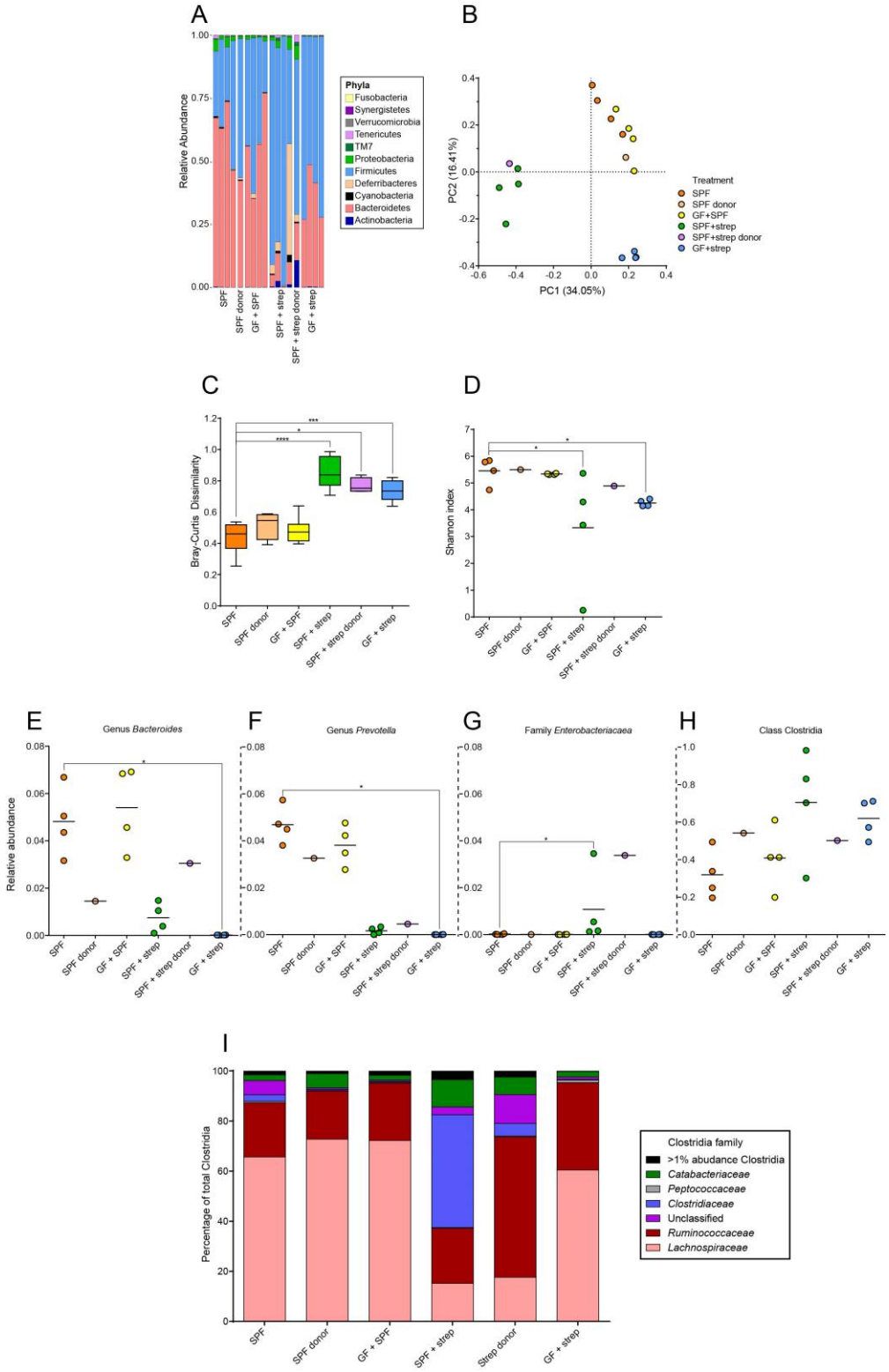
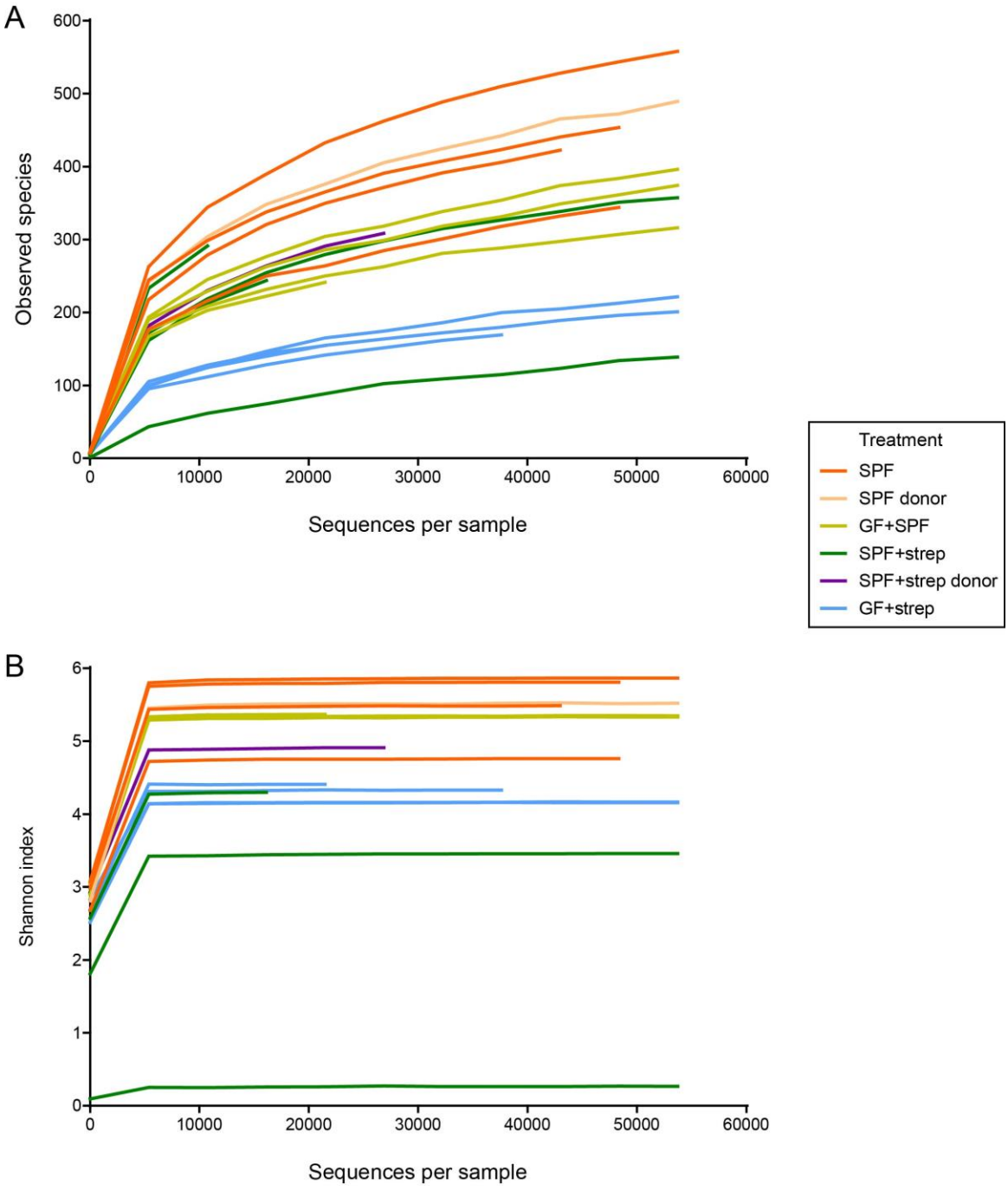

A
Relative Abundance
Phyla
Fusobacteria
Synergistetes
Verrucomicrobia
Tenericutes
TM7
Proteobacteria
Firmicutes
Deferribacteres
Cyanobacteria
Bacteroidetes
Actinobacteria
SPF
SPF donor
GF + SPF
SPF + strep
SPF + strep donor
GF + strep
B
PC2 (16.41%)
PC1 (34.05%)
Treatment
SPF
SPF donor
GF+SPF
SPF+strep
SPF+strep donor
GF+strep
C
Bray-Curtis Dissimilarity
D
Shannon index
E
Genus Bacteroides
Relative abundance
F
Genus Prevotella
G
Family Enterobacteriacaea
H
Class Clostridia
I
Percentage of total Clostridia
Clostridia family
>1% abudance Clostridia
Catabacteriaceae
Peptococcaceae
Clostridiaceae
Unclassified
Ruminococcaceae
Lachnospiraceae
Strep donor

Figure S3.1 Rarefaction curves of 16S profiling data.

A. Rarefaction curves of observed species in SPF or reconstituted germ-free mice. B. Rarefaction curves of Shannon index in SPF or reconstituted germ-free mice.


A
Observed species
600
500
400
300
200
100
0
0
10000
20000
30000
40000
50000
60000
Sequences per sample
Treatment
SPF
SPF donor
GF+SPF
SPF+strep
SPF+strep donor
GF+strep
B
Shannon index
6
5
4
3
2
1
0
0
10000
20000
30000
40000
50000
60000
Sequences per sample

CHAPTER 4: ADHERENT-INVASIVE *E. COLI* EXPANSION DURING DSS COLITIS IS MEDIATED BY NOS2 METABOLITES

Author contributions

I performed all experiments and wrote all sections of this chapter.

Abstract

Background: Crohn's disease (CD) is an inflammatory condition characterised by severe intestinal inflammation. CD is associated with increased colonisation with adherent-invasive *Escherichia coli* (AIEC). In an animal model, AIEC expands following infectious gastroenteritis which causes intestinal inflammation. Intestinal inflammation is associated with increased availability of nitrate, which can be used as an alterative electron acceptor by AIEC to provides a growth advantage. However, the importance of nitrate utilisation on AIEC expansion following an inflammatory insult has not been investigated. We hypothesised that chemically-induced colitis would cause AIEC expansion due to the increased availability of nitrate.

Methods: We infected mice with wild-type and nitrate reductase deficient AIEC. We induced colitis with dextran sodium sulfate (DSS) and inhibited nitrate production with aminoguanidine.

Results: DSS resulted in an expansion of total AIEC levels which was reduced using aminoguanidine, which also reduced intestinal inflammation. However, we found nitrate reductase deficient AIEC showed reduced fitness even in the absence of DSS colitis.

Conclusions: We found that AIEC expands following chemically-induced colitis in a nitrate-dependent manner. These results suggest that AIEC may similarly benefit from the increased availability of nitrate during CD inflammation.

Introduction

Crohn's disease (CD) is an inflammatory bowel disease associated with severe intestinal inflammation causing intestinal strictures, obstructions, and fistulas[42]. CD is characterised by chronic intestinal inflammation which often fails to resolve without the use of anti-inflammatory treatments. CD inflammation is thought to be partially due to inappropriate immune responses against microbial antigens within the intestinal lumen[49], and the intestinal microbiota has been implicated as one of the factors which may drive CD pathogenesis. CD is associated with increased numbers of Proteobacteria, especially *Escherichia coli*[82]. Many *E. coli* isolates from CD patients are part of the adherent-invasive *E. coli* (AIEC) pathovar, which are found in about 50% of CD patients but are uncommon in healthy individuals[94,97].

We have previously found in a mouse model that pre-existing AIEC colonisation results in worsened pathology after infection with the enteric pathogens *Salmonella* or *Citrobacter*[162]. In addition, levels of AIEC expanded during enteric infection suggesting that intestinal inflammation created a favourable environment for AIEC growth. Higher levels of nitrite were found in mice infected with *Salmonella* and AIEC. In the intestine, nitrate is primarily produced from the activation of enterocytes through the expression of NOS2 (iNOS)[32]. NOS2 produces highly microbicidal, locally acting compounds such as peroxynitrite which are thought to be an important for immune clearance of bacterial pathogens[147]. However, these compounds are either unstable or react with other organic molecules to produce several metabolites, including nitrate, which have been shown to be beneficial to the growth of commensal *E. coli*[22]. Nitrate acts as an alternative electron

acceptor which can be reduced to nitrite during anaerobic respiration, and this process produces more energy than fermentative pathways and allows access to additional carbon sources[151,185].

The DSS-colitis model has been commonly used to induce intestinal inflammation in mice and has been previously shown to stimulate the expression of *Nos2*[22]. AIEC strains have been shown to gain a competitive fitness advantage from nitrate utilisation when inoculated into mice with pre-existing DSS-induced colitis[22,186]. However, whether nitrate respiration would also show a similar fitness advantage if AIEC colonisation were established prior to colitis had not been studied. We took advantage of an AIEC genetic mutant (Δ*narG/narZ/napA*) that is unable to metabolise nitrate which was previously used in chapter 2. We also used aminoguanidine, a mechanism-based inhibitor selective for Nos2, to determine the effect of inhibiting Nos2 activity during colitis[157]. The purpose of this study was to determine whether nitrate respiration provides a fitness advantage to AIEC when colonisation is established prior to DSS-colitis.

Materials and Methods

Ethics statement

Animal experiments were conducted according to guidelines set by the Canadian Council on Animal Care using protocols approved by the Animal Review Ethics Board at McMaster University under Animal Use Protocol #17-03-10.

Bacterial strains

AIEC strain NRG857c (serotype O83:H1) was isolated from an ileal tissue biopsy from a CD patient in Charite Hospital (Berlin, Germany) and its genome sequence was determined previously[100]. AIEC mutants Δ*narG*/*narZ*/*napA* were made using allelic exchange or lambda red based recombination systems as described in chapter 2. Chloramphenicol (34 μg/mL), ampicillin (100 μg/mL), and gentamicin (15 μg/mL) were used in LB broth or agar plates to provide selection. Prior to mouse infections or *in vitro* assays, AIEC strains were grown in LB containing all their selective antibiotics for 16–18 h at 37°C with shaking. For mouse infections, AIEC were resuspended in phosphate buffered saline (PBS) at 2×10^{10} CFU/mL.

Growth curves

Growth curves were performed using either LB or minimal media composed of NCE media[22] supplemented with 50 mM glucose and 0.1% yeast extract. Sodium nitrate (40 mM) or aminoguanidine (10 μg/mL) were added media when indicated. Media was inoculated by adding 1 μL of overnight culture to 200 μL of media in a round-bottom 96 well plate. Wells were overlaid with 50 μL of mineral oil and plates were incubated in a Tecan Sunrise. The temperature was maintained at 37°C without shaking and OD_{600} readings were taken every 15 minutes for 24 hours.

Animal infections

Six to eight-week-old female C57BL/6N mice were purchased from Charles River Laboratories. Animals were housed in a specific pathogen-free barrier unit under level 2 conditions at the Central Animal Facility at McMaster University. Mice were infected with 2×10^9 CFU AIEC in a 100 μL suspension by oral gavage. Dextran sodium sulfate (DSS) was dissolved to 5% (w/v) in reverse osmotic water, filter sterilised, and given to mice *ad libitum* in drinking water from days 3-7 post infection. Aminoguanidine (AG) was dissolved at 1 g/L into reverse osmotic water or 5% DSS and was filter sterilised. AG was given to mice in the appropriate groups in either water or DSS from days 3-8. At day 7, DSS was removed and replaced with water or water with AG. Mice were sacrificed at 8 days post infection.

Bacterial enumeration

Fecal pellets were weighed, homogenised in 1 mL PBS, serially diluted, and plated onto LB agar plates containing selective antibiotics. Intestinal tissues were flushed with PBS to remove luminal contents, homogenised with a metal bead, and plated in the same manner as feces. Plates were incubated overnight at 37°C and colonies were counted to determine CFU per gram of tissue. Competitive assays were enumerated by replica plating total isolated bacteria onto selective plates and normalising counts to the input ratio.

Histopathology

At various times following infection, cecal sections were fixed in 10% neutral-buffered formalin overnight and were processed into paraffin blocks. The blocks were cut into two 5 μm sections and stained with H&E, and for each block 9 high power field of view regions were used for histology scoring. The histology scoring system was described previously[162], and has been reproduced as Table 2.3 in detail. The pathology scores within this manuscript reflect the total pathology from all regions (lumen, lamina propria, mucosa, and submucosa).

RT-qPCR

RNA was isolated from tissues by Trizol (Ambion) extraction. Briefly, samples were homogenised in 900 μL Trizol with a metal bead. Samples were mixed with 400 μL chloroform, centrifuged, and the supernatants were added to tubes with 500 μL isopropanol. RNA was precipitated at -20C, dried, and treated with rDNase I. RNA was reprecipitated using LiCl, dissolved in water, and cDNA was synthesized using qScript cDNA Supermix (Quantabio). First strand cDNA was diluted 1:10 and used in qPCR reactions for various genes and the housekeeping gene TATA-binding protein (*Tbp)* (Table 2). Quantitative PCR reactions were carried out in 96-well plates in a LightCycler 480 (Roche) with Quanta Perfecta SYBR Green Supermix (Quantabio).

Nitrite quantification

Nitrite levels were quantified from cecal content homogenates using the Griess assay (Griess reagent (modified), G4410). Cecal contents were centrifuged at 15 000 *g* for 5 min and 500 µL of supernatants were transferred to a fresh tube with 1/10 volumes of zinc sulfate (150 g/L). Samples were vortexed, incubated on ice for 10 minutes, and centrifuged at 15 000 *g* for 5 minutes. Supernatants were mixed 1:1 with Griess reagent and read at 540 nm.

Statistical analysis

Data were analyzed using GraphPad Prism 6.0 software (GraphPad Inc., San Diego, CA). Statistical significances were calculated using Mann-Whitney tests, Student t-tests, one-way ANOVAs with Kruskal-Wallis tests, or two-way ANOVAs with Holm-Sidak tests when appropriate.

Results

Aminoguanidine does not affect AIEC growth *in vitro*

The Nos2 inhibitor aminoguanidine (AG) has been previously used to inhibit the activity of Nos2 in the intestinal tract[22]. To determine if AG has any unintended affects on AIEC growth, we performed oxygen-limited growth curves in the presence of nitrate with both WT and Δ*narG/narZ/napA* AIEC strains in LB media. We found that the

Δ*narG/narZ/napA* strain had relatively slower growth than the WT strain (Fig. 4.1A). This difference was magnified by the addition of nitrate which accelerated the growth of WT AIEC but not Δ*narG/narZ/napA* (Fig. 4.1B). Aminoguanidine did not affect the growth rates of either strain, regardless of the addition of nitrate (Fig. 4.1C-D). Although the Δ*narG/narZ/napA* strain showed reduced growth in LB, the nitrate concentrations within our batch of LB were unknown. We repeated the oxygen-limited growth curves in minimal media and observed no difference in growth rate between WT and Δ*narG/narZ/napA* strains (Fig. 4.1E), but the WT strain again grew more quickly with added nitrate (Fig. 4.1F). These results show that Δ*narG/narZ/napA* does not show increased oxygen-limited growth in the presence of nitrate, and that aminoguanidine does not impact the growth of AIEC regardless of the addition of nitrate.

Aminoguanidine reduces AIEC expansion and inflammation during DSS-colitis

To explore whether AIEC also benefits from metabolites produced during DSS-colitis, we first colonised mice with a 1:1 ratio of WT and Δ*narG/narZ/napA* AIEC without antibiotic pre-treatment. After 3 days, mice were given water or DSS-drinking water with or without AG. We quantified total AIEC levels to identify if expansion occurred and compared the competitive index of Δ*narG/narZ/napA*:WT strains to determine the fitness provided by nitrate respiration. We found that DSS treated mice had significantly increased fecal and tissue AIEC levels, and that total AIEC levels were significantly reduced in DSS+AG mice (Fig. 4.2A-B). While the Δ*narG/narZ/napA*

mutant was outcompeted by WT AIEC in all treatment groups, the fitness of this mutant was not rescued with aminoguanidine treatment (Fig. 4.2C-D).

Il-1β is an inflammatory cytokine which upregulates Nos2 expression in entrocytes[187], and Lcn2 is marker of intestinal inflammation produced by enterocytes[188]. DSS treatment significantly increased the expression of the inflammatory markers *Il1β* and *Lcn2* in the cecum, which were both reduced with aminoguanidine (Fig. 4.3A-B). Similarly, the expression of *Nos2* and nitrite levels were elevated with DSS treatment, although these were not significantly reduced with AG (Fig. 4.3C-D). Finally, DSS significantly reduced body weight and increased cecal pathology compared to mock treated mice, although these changes were not significantly diminished with AG (Fig. 4.3E-F).

Discussion

AIEC are commonly isolated from CD patients and have adaptations which provide increased fitness during intestinal inflammation. Our results showed that Nos2 activity is important for AIEC expansion during DSS-induced inflammation as the inhibition of Nos2 with AG reduced AIEC expansion. These results are similar to previous studies where AIEC is inoculated during DSS-colitis[22,186]. Surprisingly, the Δ*narG/narZ/napA* mutant showed high variability in colonisation and reduced fitness in all treatment groups (Fig.4.2 C-D), although we had previously seen that it had a mean fitness similar to an isogenic WT in mock-treated mice (Fig. S4.1). It is possible that the fitness of Δ*narG/narZ/napA* in mock-treated mice is dependent on the composition of the

gut microbiota. The lack of any rescue in Δ*narG/narZ/napA* fitness, despite a decrease in total AIEC levels, may be due to incomplete inhibition of Nos2 as nitrate levels are still elevated in AG treated mice (Fig. 4.3D). It is also possible that AIEC expansion results from Nos2-derived metabolites other than nitrate such as glucarate and galactarate[149] or N- and S-oxides. A previous study found that AIEC with a deletion in *moaA*, a gene required for all anaerobic respiration pathways, also had reduced fitness during DSS colitis[186]. As a result, AIEC mutants lacking the ability to utilise other Nos2-derived metabolites may be more suitable for follow-up experiments.

Previous work in DSS treated mice colonised with commensal *E. coli* and treated with AG did not show a reduction in cecal inflammatory cytokines or pathology score[22]. This suggests the reduced inflammation we observed in mice treated with DSS and aminoguanidine may be due to a reduction in AIEC burden, although control experiments with DSS and AG without AIEC are needed to confirm this. While AG has not previously shown to reduce inflammation during DSS treatment, sodium tungstate, which competes for molybdenum co-factors needed for anaerobic respiration, has been shown to reduce DSS inflammation even in mice colonised with commensal *E. coli*[186].

The AIEC strain LF82 has previously been shown to worsen colitis in a DSS model, which is dependent on the presence of OmpR, a two-component system, and flagellin[189]. However, LF82 has poor fitness in conventional mice and is rapidly cleared[106,190]. In previous studies with LF82 mice were challenged with 10^8 CFU daily, and colonisation levels of LF82 were not reported[189]. Our data shows that AIEC strain NRG857c, which is substantially more fit when colonising mice[106], is capable of

expansion following DSS treatment once colonisation is established. NRG857c is similarly flagellated and motility is common among AIEC strains[171], and the increased amount of flagellin following AIEC expansion may be responsible for the increased inflammation observed in this study.

While these experiments were performed using chemically induced colitis, it is possible Nos2-derived metabolites are a contributing factor for increased AIEC levels during *Salmonella* co-infection[162]. This could be verified by performing these experiments with AG or using AIEC lacking pathways utilising Nos2-derived metabolites.

NOS2 metabolites likely play an important role in CD pathogenesis. NOS2 expression is increased in IBD patients, and gain-of-function mutations in NOS2 are associated with early onset CD[143,144]. As a result, anaerobic respiration is likely an important metabolic pathway for AIEC in CD patients due to the presence of chronic inflammation and an upregulation of NOS2. It may be possible to use drugs such as aminoguanidine and sodium tungstate, which target the production and utilisation of inflammatory metabolites, to correct for the increased levels of AIEC and other *Enterobacteriaceae* which may contribute to CD.

Figure 4.1. Aminoguanidine does not affect AIEC growth in oxygen-limited media. LB or minimal media was supplemented with nitrate or aminoguanidine (AG) and overlaid with mineral oil. Media was inoculated with WT or *ΔnarG/narZ/napA* and was grown for 24 hours at 37˚C. A. Growth in LB media. B. Growth in LB media with nitrate. C. Growth in LB media with AG. D. Growth in LB media with nitrate and AG. E. Growth in minimal media. F. Growth in minimal media with nitrate. Error bars show standard deviation.

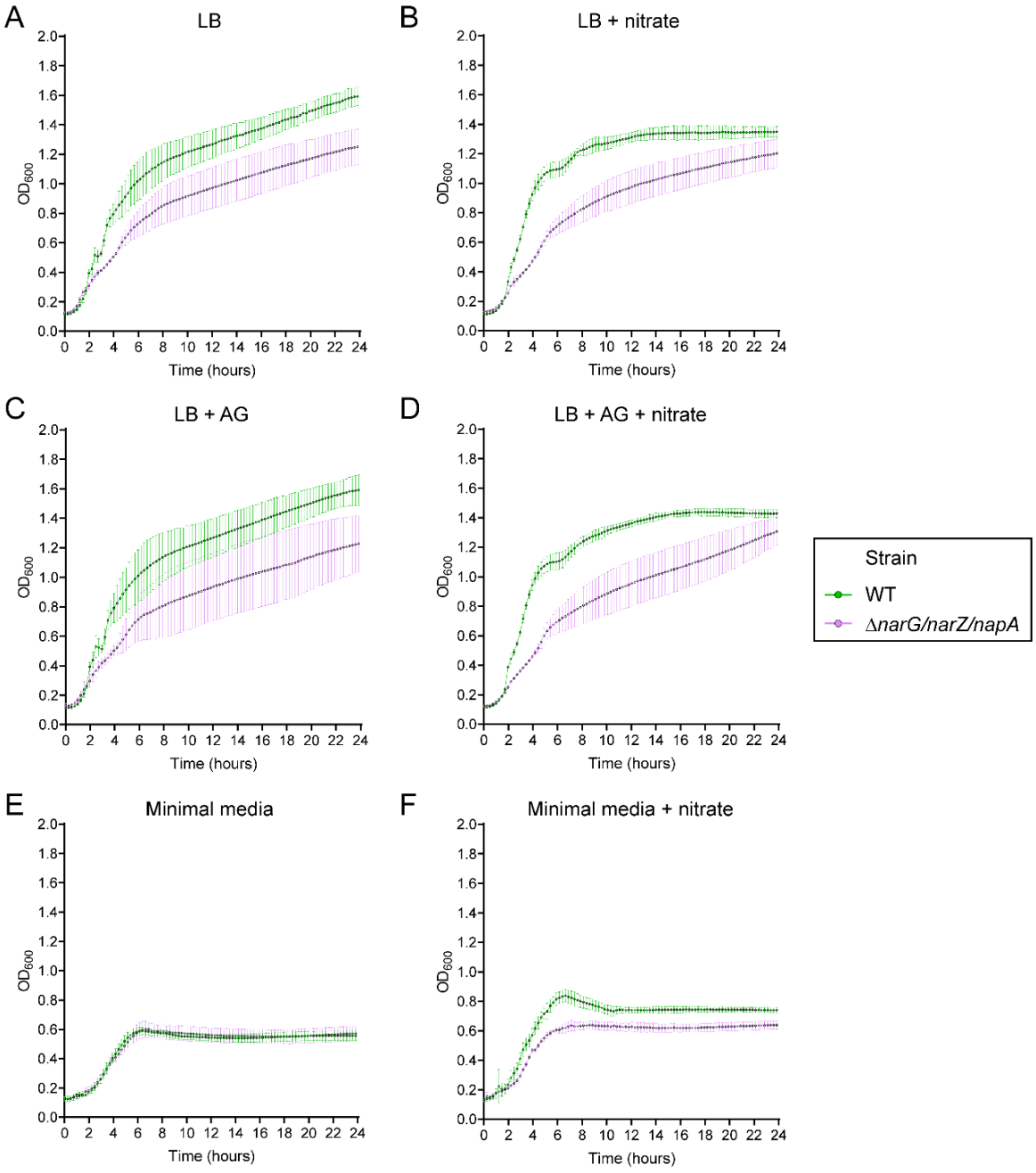

A
LB
B
LB + nitrate
C
LB + AG
D
LB + AG + nitrate
E
Minimal media
F
Minimal media + nitrate
OD600
Time (hours)
Strain
WT
ΔnarG/narZ/napA

Figure 4.2. Aminoguanidine inhibits AIEC expansion following DSS treatment. C57BL/6N mice were infected with 2×10^9 CFU AIEC in a 1:1 ratio of Δ*narG*/*narZ*/napA:WT strains. Mice were treated with 5% dextran sodium sulfate (DSS) in drinking water from days 3-7 post-infection and were sacrificed at day 8 post-infection. A. Total fecal AIEC CFU (WT + Δ*narG*/*narZ*/*napA*) in days post infection (one-way ANOVA with Kruskal-Wallis test). B. Total tissue-associated AIEC CFU at 8 days post-infection (one-way ANOVA with Kruskal-Wallis test). C. Competitive indices of Δ*narG/narZ/napA*:WT AIEC from feces in days post infection. D. Competitive indices of Δ*narG/narZ/napA*:WT AIEC from tissues at 8 days post-infection. Dotted lines show limit of detection or a competitive index of 1.0. *$p<0.05$, **$p<0.01$

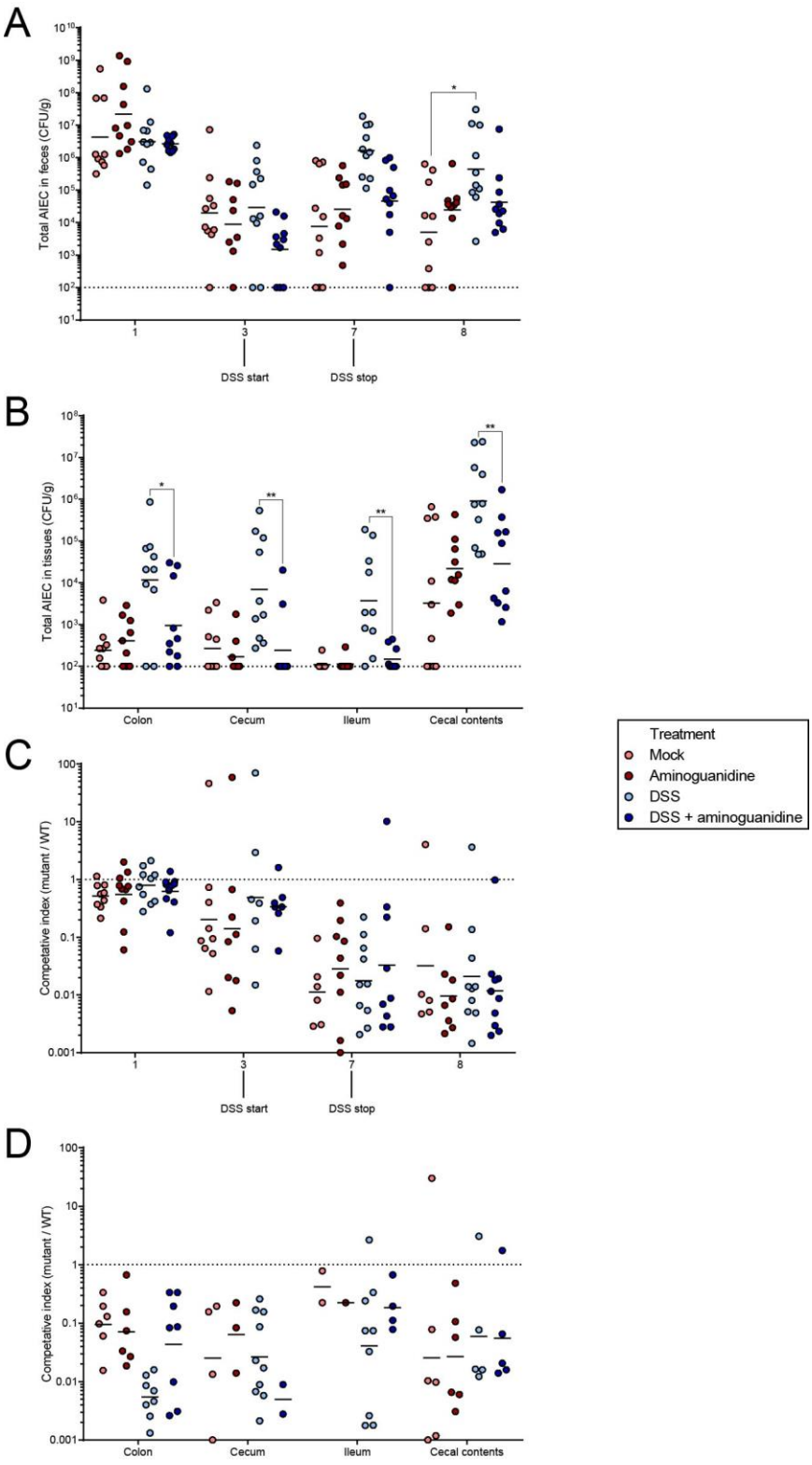

A
Total AIEC in feces (CFU/g)
DSS start
DSS stop
B
Total AIEC in tissues (CFU/g)
Colon
Cecum
Ileum
Cecal contents
C
Competative index (mutant / WT)
DSS start
DSS stop
D
Competative index (mutant / WT)
Colon
Cecum
Ileum
Cecal contents
Treatment
Mock
Aminoguanidine
DSS
DSS + aminoguanidine

Figure 4.3. Aminoguanidine reduces cecal inflammation in AIEC infected mice treated with DSS.

Mice were sacrificed at day 8 post-infection and cecal tissues and luminal contents were collected. Pathology and RT-qPCR quantification were performed on cecal tissue and nitrite levels were measured from cecal luminal contents. A. Expression of *Nos2* relative to mock treated mice (one-way ANOVA with Kruskal-Wallis test). B. Expression of *Il1β* relative to mock treated mice (one-way ANOVA with Kruskal-Wallis test). C. Expression of *Lcn2* relative to mock treated mice (one-way ANOVA with Kruskal-Wallis test). D. Percent of initial bodyweight lost (one-way ANOVA with Kruskal-Wallis test). E, Cecal nitrite levels measured using the Griess assay (one-way ANOVA with Kruskal-Wallis test). F. Cecal pathology score (one-way ANOVA with Kruskal-Wallis test). Dotted line shows no relative change in gene expression or change from initial bodyweight. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$

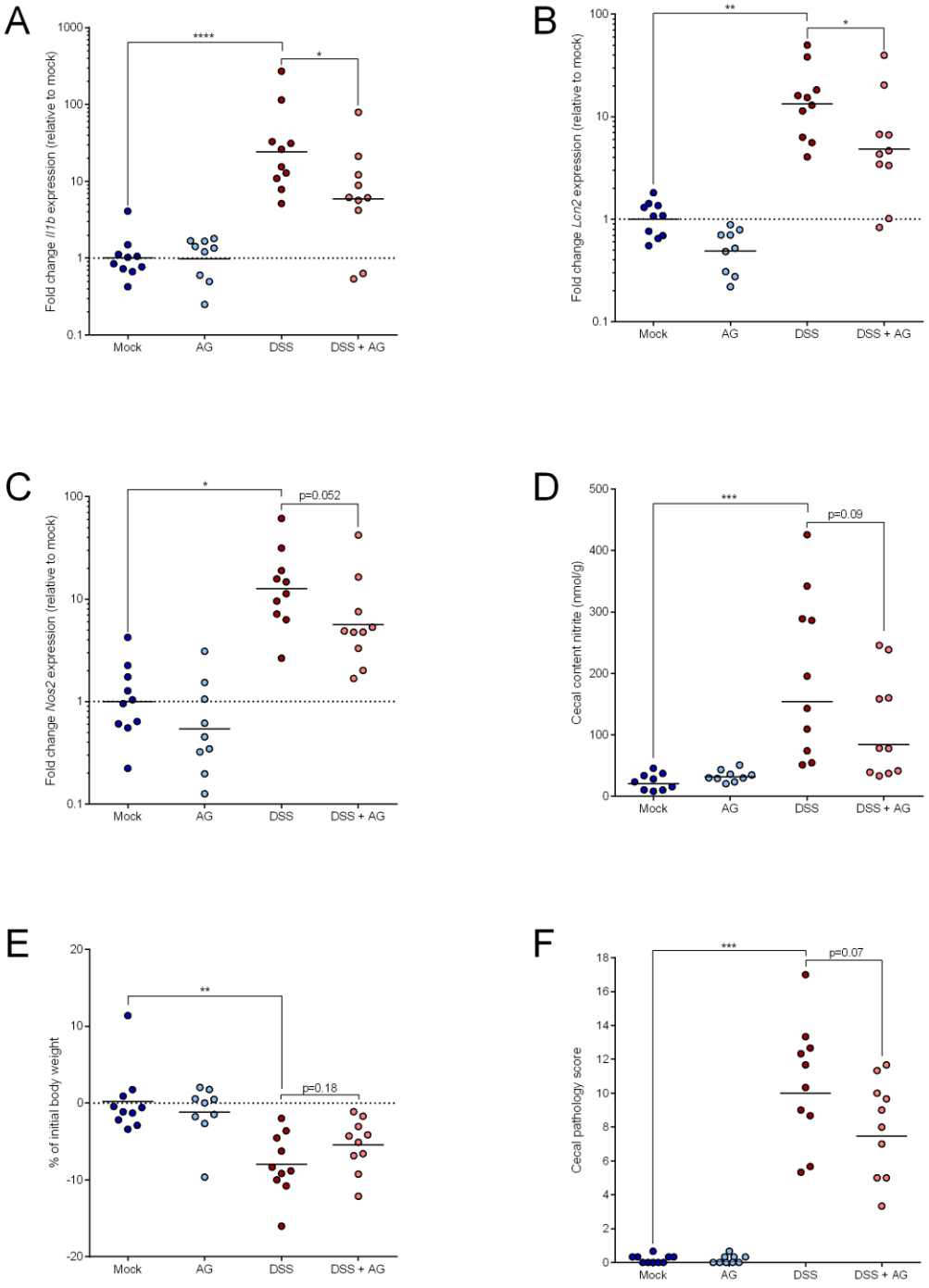
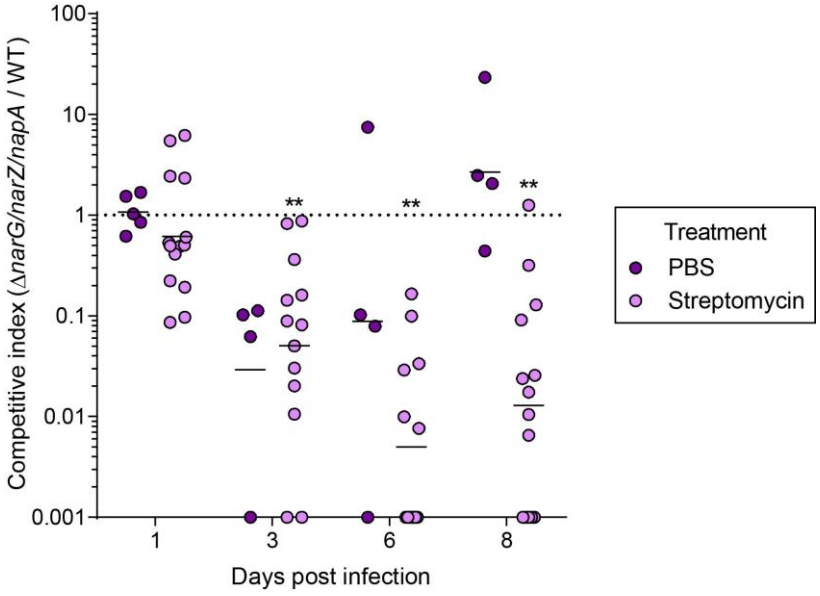

A
Fold change Il1b expression (relative to mock)
1000
100
10
1
0.1
****
*
Mock
AG
DSS
DSS + AG
B
Fold change Lcn2 expression (relative to mock)
100
10
1
0.1
**
*
Mock
AG
DSS
DSS + AG
C
Fold change Nos2 expression (relative to mock)
100
10
1
0.1
*
p=0.052
Mock
AG
DSS
DSS + AG
D
Cecal content nitrite (nmol/g)
500
400
300
200
100
0
***
p=0.09
Mock
AG
DSS
DSS + AG
E
% of initial body weight
20
10
0
-10
-20
**
p=0.18
Mock
AG
DSS
DSS + AG
F
Cecal pathology score
18
16
14
12
10
8
6
4
2
0
***
p=0.07
Mock
AG
DSS
DSS + AG

Figure S4.1. Nitrate-deficient AIEC previously showed similar fitness to WT AIEC in mock-treated mice.

C57BL/6N mice were treated with PBS (mock) or 20 mg streptomycin 24 hours prior to infection with $2x10^9$ cfu AIEC in a 1:1 ratio of Δ*narG/narZ/napA*:WT. Competitive indices were calculated from fecal homogenates using replica plating (one sample t-test against theoretical CI of 1.0). Dotted line shows a CI of 1.0. **$p<0.01$


Competitive index (ΔnarG/narZ/napA / WT)
100
10
1
0.1
0.01
0.001
**
**
**
1
3
6
8
Days post infection
Treatment
PBS
Streptomycin

CHAPTER 5: DISCUSSION

The preceding chapters outlined the mechanisms by which AIEC can both establish initial colonisation within a host and how environmental insults can lead to AIEC expansion. This discussion will frame these findings within the broader field of AIEC biology, Crohn's disease, and intestinal homeostasis.

Unravelling the relationship between AIEC and antibiotics in CD

Chapter 2 discussed how antibiotics, which have been implicated in CD risk in epidemiological studies, can influence initial AIEC infection and expansion of AIEC in previously colonised hosts. We found that AIEC expansion following antibiotic treatment partially resulted from antibiotic-induced inflammation, which has been previously shown for pathogenic *Salmonella* and commensal *E. coli*[149,157].

Antibiotics may play a role in the evolution of AIEC strains within patients. AIEC isolates are enriched for antibiotic resistance genes[135]. This may reflect a selective pressure towards the acquisition of mobile genetic elements with antibiotic resistance markers, as patients with CD are commonly treated with antibiotics. Antibiotics may also play a role in selecting for the adherent and invasive phenotype which characterises AIEC strains. Antibiotics are known to promote the passage of luminal bacterial into the lamina propria through goblet cells[177]. As a result, multiple rounds of antibiotic treatment may select for bacteria which have an increased propensity for intracellular survival, which is another trait found in many AIEC isolates[191]. UPEC often belong to the same phylotype as AIEC and can establish quiescent intracellular reservoirs which are resistant to antibiotic treatment and can lead to chronic infection[120]. It is possible that an intracellular

lifestyle is similarly important for AIEC colonisation and may provide protection from antibiotic treatment and host immunity. Testing CD patients for carriage of AIEC strains can illuminate the effects of antibiotics on AIEC evolution. Genetic and phenotypic comparisons could be made on AIEC isolates before or following antibiotics to determine if antibiotics promote common adaptations. Similar experiments could also be performed using an animal model to infer which selective pressures are acting on AIEC during antibiotic treatment.

While our results found that mice colonised with AIEC following a single antibiotic treatment did not show severe pathology (chapter 2), this model may not recapitulate the same exposure to antibiotics that occur in patients. Many children receive multiple courses of antibiotics of varying antibiotic classes over the course of many years, which have been shown to lead to changes in microbiome composition with increased levels of Proteobacteria[192]. As a result, it is likely that these repeated exposures will produce different changes to the intestinal microbiota and immune system than a single dose of one antibiotic. To test this using a mouse model, mice could be exposed to various classes of antibiotic repeatedly over their lifetime. AIEC could be introduced either prior to or after antibiotic treatment to investigate whether this exposure to antibiotics results in greater pathology.

In addition, it is possible that the conventional mice used in this experiment were not be susceptible to pathology due to an absence of predisposing genetic factors. While the penetrance of CD is relatively low for many CD risk alleles, it is likely that some combination of genetic susceptibility factors is a prerequisite for developing chronic

intestinal inflammation. As a result, it may be necessary to use a mouse model with susceptibility towards intestinal inflammation, such as the $IL10^{-/-}$, $TNF^{\Delta ARE/+}$, or SAMP1/YitFc models that have been previously characterised[193].

Antibiotics might be useful in clearing AIEC in colonised CD patients. However, the high prevalence of antibiotic resistance genes may make this approach difficult. Another approach would be to use bacteriophage therapy, which has been successfully trialed in mice[194]. However, due to the phylogenetic diversity of AIEC strains it may be difficult to develop a phage cocktail that is able to clear diverse AIEC isolate. Since AIEC isolates also lack common canonical virulence factors, anti-virulence strategies would likely have to target pathways that are common broadly among *E. coli*. Anaerobic respiration, which appears to be important to AIEC colonisation of the inflamed intestine, can be inhibiting using aminoguanidine or sodium tungstate in mice[22,186]. Type 1 pilus-mediated adhesion is common among many *E. coli* isolates[195], and anti-adhesives targeting the pilus have been shown to promote clearance of AIEC in a mouse model[196]. If any of these strategies can be shown to reduce or eliminate AIEC in CD patients and provide a clinical benefit, this would show that AIEC play an active role in the pathogenesis of CD.

Antibiotics are known to have a detrimental effect on the intestinal microbiome and may play a role in the development of CD in this way. A better understanding of how antibiotics interact with the microbiome of patients at risk of CD may lead to changes in antibiotic prescribing practices in these patients.

Uncovering factors mediating AIEC colonisation resistance

Chapter 3 focused on the effect of antibiotic depletion on bacterial diversity and colonisation resistance towards AIEC. The results suggested that decreased bacterial diversity, in the absence of exposure to streptomycin, did not reduce colonisation resistance towards AIEC. However, a reduced microbial diversity was correlated with a higher AIEC burden once colonisation was established.

Future studies could investigate whether these effects were due to changes in intestinal metabolites. Previous studies have found that antibiotics reduce levels of SCFA, which are important regulators of intestinal homeostasis and can directly impact *Enterobacteriaceae*[21]. Levels of butyrate should be lowered in streptomycin treated mice, but we did not test whether they were also lowered in germ-free (GF) mice reconstituted with a streptomycin-depleted microbiome. We also observed that *Bacteroides*, which are producers of propionate, were decreased after streptomycin treatment and levels of propionate could be measured from the fecal samples. Decreased SCFA concentrations, resulting from a Western diet, have also been found to correlate with increased AIEC burden and higher tissue association[197]. SCFA are known to inhibit EHEC, but have also been shown to upregulate the expression of virulence factors[198]. To determine if SCFA directly impact AIEC, AIEC could be grown in media with SCFAs at levels found during homeostasis or during intestinal inflammation. AIEC could be tested for changes in phenotypes such as cell invasion, inhibition of growth, or altered expression of virulence factors.

In chapters 2 and 3, we found that the presence and expansion of endogenous *Escherichia* after antibiotic treatment did not confer colonisation resistance towards AIEC. Pathogenic *E. coli* are known to have different carbon source preferences compared to commensal *E. coli*[182]. Our data suggests that AIEC may not compete with endogenous *Escherichia* and that AIEC may not occupy the same niche. To better understand the environment occupied by AIEC within the host, the preferred carbon sources of AIEC strains can be tested *in vivo*. AIEC mutants with defects in various carbon metabolism pathways can be competed against WT AIEC. These experiments could be performed in both healthy mice and in a murine colitis model to determine if and how AIEC changes its nutrient preferences during inflammation and could determine whether the niche AIEC occupies is more similar to commensal *E. coli* or pathogenic *E. coli*.

A reductive approach to understanding the effects of microbe-mediated colonisation resistance against AIEC could be to use a simplified microbiota system. In an unpublished experiment, we found that mice colonised with ASF microbiota and treated with streptomycin showed long-term AIEC colonisation with burdens remaining at 10^8-10^9 CFU/g of feces even after 30 days. This shows that the ASF microbiota is unable to limit chronic AIEC levels to the same degree as a conventional microbiota. Experiments with *Salmonella* have found that the addition of both facultative anaerobes and SCFA-producing anaerobes led to increased colonisation resistance[36,180]. By adding single members of either SCFA producing bacteria or commensal *Enterobacteriaceae*,

the individual contributions of these bacteria towards colonisation resistance can be measured.

While the host microbiota provides one aspect of colonisation resistance, the status of the host can also play an important role. Adhesion to the host epithelium using type 1 pili is an important aspect of AIEC colonisation as *fimH* mutants shows poor colonisation in mice[107]. AIEC is known to adhere to CEACAM6 and Gp96, both of which are glycoproteins which are upregulated during CD inflammation[122,126]. While these glycoproteins are found to be elevated in CD, the regulation of these genes during prodromal CD or during other forms of intestinal inflammation are not well understood. UPEC has similarly been shown to take advantage of altered host glycoprotein expression during inflammation, which promotes chronic infection of the urinary tract[199]. Future studies can investigate how AIEC adhesion sites are altered during antibiotic use or during colitis, and how these changes affect colonisation resistance against AIEC.

A better understanding of the host and microbial factors that mediate colonisation resistance towards AIEC will contribute to a broader understanding of the association between AIEC and CD. These findings could be beneficial towards developing therapeutics which can alter the composition of the intestinal microbiota in CD patients as a means of promoting intestinal homeostasis.

Characterising metabolic adaptations of AIEC to intestinal inflammation

Chapter 4 described how AIEC utilises inflammation-derived metabolites to promote growth during colitis. The particular metabolite investigated in this chapter was

nitrate, which is similarly used for anaerobic respiration in many *Enterobacteriaceae* species[200]. However, some metabolic adaptations are found more commonly in AIEC strains compared to commensal *E. coli* which could be investigated further. The prototypical AIEC strain NRG857c contains the *sit* operon which is also involved in iron uptake[100]. This pathway is found in all clinical *Shigella* isolates but is absent from most commensal *E. coli*, and appears to be important for intracellular iron and manganese acquisition[153]. These findings suggest that the acquisition of *sit* may act as an evolutionary adaptation towards an intracellular lifestyle for this AIEC isolate. As discussed previously, the relative importance of intracellular colonisation of AIEC *in vivo* is not well understood. The acquisition of the *sit* operon suggests that there are evolutionary pressures favouring the acquisition of genes needed for intracellular survival. To determine if *sit* provides an intracellular fitness advantage, the replication and survival of *sit* mutants can be compared to WT AIEC using cell culture models of AIEC infection in macrophages and enterocytes. A *sit*-deficient mutant can also be competed against an isogenic WT in a mouse colonisation model to determine if *sit* provides a fitness advantage, particularly in terms of tissue-association.

Another metabolic adaptation which can be studied is the *pdu* operon, which is enriched in AIEC and is involved in propanediol metabolism[150]. This metabolite has been previously shown to provide a fitness advantage to *Salmonella* during intestinal inflammation[151]. The metabolism of propanediol requires the presence of oxygen or alternative electron acceptors which are similarly more abundant during inflammation. As a result, the presence of the *pdu* operon likely represents another metabolic adaptation

towards an AIEC phenotype. AIEC strains can be tested for their ability to use propanediol as a carbon source and the importance of *pdu* in AIEC can be measured by testing the relative fitness of WT AIEC to a *pdu* mutant in a colitis model.

In addition to knocking out putative metabolic fitness elements from AIEC isolates, another approach to understanding the metabolic adaptations could be to introduce these genetic elements into related, but non-AIEC *E. coli* strains. AIEC are believed to evolve from endogenous *E. coli* from B2 or D clades[116]. It should be possible to introduce some of the genetic elements found in AIEC strains to a commensal *E. coli* from one of these groups, and to test these strains for increased fitness in a colitis model.

AIEC may have undergone regulatory evolution in existing pathways in addition to the acquisition of new genetic elements. *Salmonella* has been shown to have reprogrammed conserved signalling pathways, such as the PhoP-Q two component system, to control the expression of acquired virulence genes[201]. It is possible that similar events have occurred in AIEC isolates, which may explain why AIEC have common phenotypic traits without the acquiring common genetic elements.

To determine if regulatory evolution has occurred in AIEC, several approaches could be used. Bioinformatic comparisons of promoters related to phenotypes observed in AIEC, such as increased motility, can be used to identify potential sites where regulatory inputs have been gained or lost. ChIP-on-chip analysis could be performed using a known transcription factor to compare differences in promoter binding between commensal *E. coli* and AIEC. Finally, the metabolite profile of the intestinal environment is likely an important determinant of virulence. The effect of changing a intestinal environment has

been studied in *Campylobacter*, which is a commensal in chickens but can be pathogenic in humans[202]. To determine the overall effect of luminal metabolites on AIEC expression, RNA-seq could be performed on an AIEC strain and a closely related commensal strain of *E. coli* grown in media supplemented with the fecal supernatants CD or healthy individuals.

Alternatively, fecal samples that have been collected from CD patients over time, or ideally even before CD diagnosis, can be investigated for the evolution of endogenous *E. coli* isolates towards and AIEC phenotype. Similar research has been done using cystic fibrosis-associated *Pseudomonas aeruginosa* which has been shown to continuously undergo genetic changes and clonal expansion during repeated infections[203]. *P. aeruginosa* isolates have been shown to evolve common phenotypic traits, such as increased biofilm formation[204], amino acid auxotrophy[205], loss of motility[206], and antibiotic resistance[207]. The Genetic, Environmental, Microbial Project study may be valuable source of fecal samples as it longitudinally monitors the microbiome of healthy patients with CD risk factors[208]. By comparing the evolution of *E. coli* and AIEC isolates in multiple patients over time, several crucially important details regarding AIEC biology could be elucidated. First, it could conclusively show if AIEC truly evolve uniquely within individuals with CD. Second, it could determine if AIEC evolve prior to CD diagnosis which would indicate that AIEC could be important in the initiation of CD. Third, it could show if there is a sequence of evolutionary events that lead to the AIEC pathotype. Finally, if the host genotype relating to each isolate is known, it may be

possible to determine if there are host-specific determinates that favour the evolution of particular traits.

In summary, there appear to be common evolutionary adaptions in AIEC which include metabolic changes. However, the evolution of AIEC remains poorly understood. Further investigations into AIEC evolution can reveal more about microbial adaption towards inflammation and the pathogenic role of AIEC in CD.

Limitations

While we have shown novel *in vivo* interactions between AIEC and antibiotics, our models have several limitations. First, the antibiotics selected in Chapter 2 were primary selected based on having different spectrums of activity and poor GI absorption (except for metronidazole) rather than clinical use. Streptomycin and gentamicin are rarely given orally and are not associated with CD risk. Metronidazole is associated with early-onset CD[209], but did not affect AIEC colonisation in our model when given as a single dose (Fig. 2.2). Future studies should examine whether antibiotics associated with CD risk also show similar interactions with AIEC.

Additionally, the mice used for most of our experiments were SPF mice, which are tested for an absence of certain virus, bacteria, opportunistic pathogens, and parasites. SPF mice are beneficial for experimental consistency as infections with various pathogens could alter our results. For example, our lab has previously found that *Citrobacter*-induced colitis promotes AIEC expansion and persistence[162]. However, individuals can be colonised with opportunistic pathogens such as *C. difficile*[210], which is

known to cause disease following antibiotic treatment. As a result of the absence of opportunistic pathogens in our model, there may be additional interactions between antibiotics and the microbiota that would not be recapitulated in our model systems.

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