IMPACT OF ADHERENT-INVASIVE *E. COLI* (AIEC) ON THE SMALL INTESTINAL MICROBIOTA AND INFLAMMATION

A Thesis
by
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Submitted to the School of Graduate Studies
at Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

December 2022
Department of Biology

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Abstract

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Resident microbes throughout the gastrointestinal tract (GI) are implicated in host health and progression of diseases such as inflammatory bowel disease (IBD), and more specifically, Crohn's Disease (CD). CD is a chronic inflammatory condition of the entire GI tract. A pathobiont *Escherichia coli (E. coli)* pathotype termed Adherent-Invasive *E. coli* (AIEC) has been associated with increased inflammation in CD patients (1-3). Previous research has studied the impact of AIEC on the microbiota and inflammation in colonic tissues, however, less is known about the effect on small intestinal tissue and its microbiota due to difficulties sampling this region of the GI tract. With increasing morbidity of CD, especially in the United States, it is important to characterize the role of AIEC in the pathogenesis of CD. **The goals of this project were to determine to what extent Crohn's-associated clinical AIEC and non-AIEC strains colonize the small intestine and validate their impact on microbial dysbiosis and small intestinal inflammation.**

Towards this end, we utilized a novel barcoding approach to identify individual sub-strains of *E. coli* from within a complex microbiota. We used a collection of well-characterized AIEC and non-AIEC strains that were isolated from the intestinal mucosa of healthy and IBD patients. The strains were introduced into germ-free mice (either inflammation-susceptible

Il10$^{-/-}$ or inflammation-resistant WT) (4, 5) along with a fecal microbial transplant (FMT) to create relevant conditions for a CD model. Following the 10-week murine model, qPCR was conducted to analyze the proinflammatory response in the SI. In addition, Illumina MiSeq sequencing of 16S amplicons allowed us to assess the relative abundance of individual *E. coli* strains and alterations to the complex microbial community of the small intestine (SI). Our in vivo modeling has indicated that inflammation promotes higher levels of *E. coli* colonizing the SI in inflammation-susceptible mice (i.e., *Il10*$^{-/-}$). This research will further help us identify features of AIEC and non-AIEC *E. coli* important for colonization of the SI.

Acknowledgments

I'd like to thank my PI Dr. Rachel Bleich for mentoring me throughout this journey as well as my committee members: Dr. Cara Fiore, Dr. Michael Opata, and Dr. Mark Robinson; All of whom inspired me to obtain my master's in biology and helped me persevere throughout. Thank you to the Office of Student Research at Appalachian State University for their funding, the UNC-Chapel Hill Microbiome Core Facility for sequencing, and the Arthur Lab at UNC-Chapel Hill for their collaboration throughout this project. A huge thank you to Dr. Chequita Brooks, who's help has been invaluable throughout this process. Thank you to my two amazing undergraduate researchers, Lily Elkin and Sara Parker, without whom I would not be graduating.

Dedication

I want to dedicate my thesis to my mother, MaryAnn Metcalf. She has shown me what determination, hard-work and perseverance look like. From a young age she fostered my curiosity and deductive reasoning skills that have allowed me to grow into the scientist I am today.

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Introduction

One type of inflammatory bowel disease (IBD), Crohn's disease (CD), is a chronic inflammatory condition of the gastrointestinal tract (GIT). Hospitalizations and costs have increased over the past 13 years for those suffering from CD (6). While CD is influenced by host genetic factors, microbial and environmental factors also play a significant role in disease outcome (7-9). With increasing prevalence of CD and no cure currently, it is important to understand the role the microbiome plays in disease pathogenesis. The extent to which microbes influence prognosis is still under investigation; however, there is evidence of a correlation between the presence of a pathobiont group of *Escherichia coli* (*E. coli*), known as adherent-invasive *E. coli* (AIEC) (6), and inflammation of tissues in the GIT (3).

It is well known that resident microbes, especially AIEC, in the GIT can exacerbate inflammation and dysbiosis within the different tissues (10). AIEC are associated with CD due to their significantly higher colonization of inflamed tissues throughout the GIT (2, 5, 11). These organisms also exhibit high genetic diversity indicating that strains evolve separately in different microbial communities (11, 12). AIEC are non-toxigenic, non-pathogenic, and cannot be reliably distinguished from non-AIEC strains by their genomic features (13). Instead, they are differentiated functionally using two *in vitro* assays: adhesion and invasion of epithelial cells, and survival and replication in macrophages (11). Previous research found that AIEC isolated from CD patients exhibited increased motility and acetate metabolization, which helped tissue colonization (12). These results suggest that AIEC colonization of tissues relies on evolution in a host and is affected by the microbial community composition and environment (12). Since each tissue niche and associated microbiota along the GIT are distinct (14), it is important to further characterize the

interactions between AIEC and each GIT tissue to better understand its role in inflammation induction.

In CD, inflammation and dysbiosis go hand in hand (15-17). Dysbiosis occurs when there is an imbalance within the microbiota of the GIT (15). Inflammation is an immunological response to foreign or perceived pathogens that aims to protect the host (15). However, inflammation can exacerbate dysbiosis which can further promote the inflammatory cycle (15). The phenomenon of dysbiosis during inflammation is marked by a decrease in obligate anaerobic bacteria as well as an increase in facultative anaerobic bacteria (15, 17). Classes such as *Gammaproteobacteria* and *Bacilli*, both in the phylum *Firmicute*s, are facultative anaerobic bacteria that are seen in increased amounts in CD. Obligate anaerobic bacterial classes that are commonly seen to be reduced in CD are *Bacteroidia* and *Clostridia* (15). Changes in the community composition of the gut can result in acute intestinal inflammation triggered by the family *Enterobacteriaceae*, which are facultative anaerobic pathogens (15).

The shifts in microbial community structure, specifically the increase and decrease of facultative anaerobes and obligate anaerobes, respectively, influences inflammation (15). In murine colitis models, this microbiota composition shift is commonly seen and is accompanied by inflammation throughout the GIT (15). However, the interaction between host epithelium and gut-associated microbes is usually mutually beneficial (16). Microbes promote the immune system, support host metabolism, and promote cell differentiation (16). In return, epithelial cell metabolism creates an anaerobic environment for the bacteria to colonize the lumen of the GIT (16). Microbial metabolism plays an important role in this symbiotic relationship. In the gut, bacterial communities compete for limited carbon

resources like complex carbohydrates of dietary and/or host origin (17). How microbial metabolism intersects with host epithelium responses also plays a role in gut inflammation (16). When shifts in microbial composition occur, subsequent interactions with epithelial cells elicit a different immune response compared to what occurs in a healthy microbiome. The immune system generates reactive oxygen species (ROS), reactive nitrogen species (RNS), and nitrate which generate electron acceptors, like oxygen, in the large intestine (LI), feeding facultative anaerobic bacteria (15). Release of these molecules increase conversion of hydrogen peroxide to hydroxyl radicals as part of the normal inflammatory response (16). In addition, oral antibiotics can also increase oxygen availability, promoting the growth of *Enterbacteriaceae* like *Escherichia coli* (*E. coli*) (16). Increased electron acceptors in the LI further nourishes bacteria such as *E. coli* and allows them to bloom in this environment (15, 16). Findings such as these, support that dysbiosis is not solely an effect of colitis, but that it can also facilitate worsening CD symptoms (16).

I. *Enterobacteriaceae* in a Healthy Gut

In a healthy gut, the lumen maintains a mainly anaerobic environment (15). Since there is little oxygen present, families like *Enterobacteriaceae* readily consume any traces of oxygen present in the lumen (15). This leaves *Enterobacteriaceae* unable to compete with obligate anaerobic bacteria for high-energy nutrients, such as complex polysaccharides, to support fermentation under the reduced oxygen conditions (16); thus, contributing to their growth limitation in the distal lumen of the gut (16). The communities that inhabit the distal gut are densely packed and compete for the limited nutrients (16). So, diet has an impact on microbial composition in the GIT (16). Changes in diet can alter the community at a species level, however, obligate anaerobic *Clostridia* and *Bacteroidia* still persist over

Enterobacteriaceae (15, 16). They utilize strategies that allow them to outcompete *Enterobacteriaceae* during anaerobic growth (15, 16).

One strategy they utilize is carbohydrate acquisition (15). *Clostridia* use carbohydrate acquisition to their benefit by using glycoside hydrolases to degrade complex carbohydrates to produce oligosaccharides. In turn, the oligosaccharides are transported across the cytoplasmic membrane by an ATP-binding cassette transporter (15). *Bacteroides* employ a different system; the method they use is encoded by the sucrose synthase (*sus*) gene cluster of *Bacteroides thetaiotaomicron*. This is a glycan acquisition strategy that works to degrade starch into malto-oligosaccharides, then actively transport them against their concentration gradient so they can be further degraded into glucose (15). The glucose produced can then be actively transported and used to support growth due to fermentation (15). Both *Clostridia* and *Bacteroidia* use glycoside hydrolases to concentrate carbohydrates at their surfaces then use an active transport system to import substances through lipid bilayer into the cell (15). By contrast, *E. coli* rely on the presence of oligosaccharides passively transported across the outer membrane through diffusion channels in order to accomplish degradation of complex carbohydrates (15).

II. Shifts in bacterial taxonomy during inflammation

Several factors contribute to changes that cause shifts in the bacterial composition of the microbiota (15-17). Again, these shifts in the bacterial taxonomy are characterized by an increase in facultative anaerobic bacteria like *Enterobacteriaceae* and decrease in obligate anaerobic bacteria like the Firmicutes phyla. One factor that can lead to this change is diet and it can lead to a shift in species level taxonomy (17). However, the immune system's

response to pathogens can also play a role in the taxonomy shift observed in the LI and small intestine (SI) microbial species composition (15, 16).

It is important to note that diet can impact microbial diversity of the microbial population and subsequently, the immune response (16, 17). As mentioned above changes in diet, such as high carbohydrate intake with little fiber, can shift the species level taxonomy and favor facultative anaerobic species that are found at increased levels in CD patients (17). The microbiota composition and inflammatory conditions established through poor diet can have consequences such as poor epithelial integrity and increased inflammation (15-17). One serious consequence of intestinal inflammation is diarrhea, which limits the availability of fermentable carbon sources to host mucus-derived carbohydrates and triggers an inflammatory response (15, 16).

As part of the host immune response, antimicrobial peptides are released and can play a role in intestinal inflammation (15). The antimicrobial molecules can interfere with microbial acquisition of iron or zinc, influencing species-level shifts in the microbial community (15). Another group of antimicrobials produced during inflammation are ROS and RNS (15). When proinflammatory cytokines, such as IFN-$\boldsymbol{\gamma}$, are released by intestinal epithelial cells, hydrogen peroxide can be produced which initiates a cascade resulting in production of superoxide radicals (15). The recruitment of proinflammatory cytokines to intestinal epithelium can be accompanied by transmigration of neutrophils, a type of white blood cell, into intestinal lumen which further exacerbates the inflammatory response (15). Thus, the production of RNS and ROS are made as part of the immune response to gut microbes (15). Altogether, these processes convert bactericidal RNS and ROS into non-toxic products like S-oxides, N-oxides, and nitrate, the presence of which causes a noticeable

change in the growth conditions of the distal gut (15). As mentioned earlier, the LI is devoid of exogenous electron acceptors and, as a result, fermentation of carbohydrates is the main strategy that microbial communities in healthy LI's use to support anaerobic growth. Thus, generation of S-oxides, N-oxides, and nitrate as by-products of the inflammatory response gives rise to alternative nutrient sources for facultative anaerobic microbe growth (15).

Production of such products can be beneficial for *E. coli* because they can use them as terminal electron acceptors in anaerobic respiration (15). In contrast, *Clostridia* and *Bacteroidia* have primitive electron transport chains and lack a terminal oxidoreductase that is needed to use the exogenous electron acceptors generated through inflammation (15). Moreover, *E. coli* encodes dehydrogenases that are crucial catalysts for electron transfer reactions. They function by coupling respiratory electron acceptors to the electron donor formate, a fermentation end-product present in the LI. The presence of exogenous electron acceptors in the inflamed gut could play a role in *E. coli*'s ability to confer a fitness advantage over obligate anaerobic bacteria (15).

III. Shifts in bacterial taxonomy in SI & LI in chronic inflammation

Any shifts in the taxonomic composition of the GIT microbiota are impacted by diet and the immune response. Thus, it is important to understand how the immune response and dysbiosis are impacted by chronic inflammation (15-17). In the last section we discussed the importance of electron acceptors in the growth of microbes in the intestines. It is important to acknowledge that host-derived electron acceptors play a role in the enhanced growth of commensal *Enterobacteriaceae* in the inflamed gut of individuals with IBD (15). The bloom of *Enterobacteriaceae* creates a powerful, selective force that is most likely the culprit for the evolution of pathogenic species within this family (15).

A type of pathogenic *E. coli* termed Adherent-Invasive *Escherichia coli* (AIEC) are commonly associated with chronic intestinal inflammation in CD (6). *E. coli* are closely related to the *Salmonella* lineage and share a common ancestor from which they diverged. The *Salmonella* lineage acquired virulence factors either through plasmids or horizontal gene transfer. *Salmonella* encodes a type-3-secretion-system (T3SS), which is a virulence factor that helps them evade the host immune system (15). *Salmonella* are linked to acute intestinal inflammation and while they are closely related to AIEC, the latter is associated with chronic intestinal inflammation. More recently it has been demonstrated that some *AIEC* use a type-4-secretion-system (T4SS) to invade intestinal epithelial cells and survive in macrophages (4, 15). The T4SS helps AIEC colonization through biofilm formation, DNA transfer, and protein secretion (4). These factors play a role in pathobiont colonization and can trigger the intestinal inflammation seen in CD patients (15). The use of virulence factors gives bacteria a competitive advantage within the intestinal microbial community, especially when it comes to exacerbating inflammation in the GIT (4, 15, 18). Acquisition of virulence factors, whether by gene transfer or plasmid, allows these pathogenic bacteria to create a new niche within the inflamed intestinal environment of the gut (15) thus exacerbating both inflammation and dysbiosis.

The LI is much more well-characterized compared to the SI in terms of microbial taxonomy shifts which is largely due to its location *in vivo* (19). However, understanding taxonomic shifts in microbial community composition is important to create a more well-rounded understanding of the host-microbe interaction in CD. The SI functions to absorb nutrients from digested food and thus the immune response is slightly different compared to the LI. Paneth cells (PCs) play a big role in the immune response in the SI (20, 21). PCs line

the crypts, invaginations along the mucosal surface, of the SI and secrete antimicrobial proteins which mediate host-microbe interactions (21). During inflammation PC homeostasis can be disrupted which can lead to apoptosis or dysfunction which contribute to inflammation and dysbiosis (20). There are several mechanisms that allow for these species level shifts (15-17).

IV. What are the known mechanisms?

Mechanisms that employ products like ROS and RNS in addition to other immune system derived products contribute to the chronic inflammatory response seen in CD (15, 16). The degradation of ROS to oxygen by catalases serves as the terminal electron acceptor for aerobic bacteria growth in the intestines. ROS are antimicrobial and thus contribute to the shift in microbial species of the microbiota (16). In addition, the host immune response releases the antimicrobial proteins that produce ROS and RNS that facilitate the growth of *Enterobacteriaceae*, like *E. coli* (16). In addition, formate can serve as an electron donor in conjunction with oxygen as the terminal electron acceptor (17). Moreover, the recruitment of neutrophils can influence oxygen levels in the intestines (16). Research has identified bacterial formate oxidation and oxygen respiration as metabolic signatures in inflammation-associated dysbiosis (17).

Formate is a microbial fermentation end-product that microbes need for respiration (17). A study tested whether microbes that do not produce formate were able to grow in a murine DSS-induced inflammatory model (17). The study was replicated three times and the result were the same; bacteria deficient in formate production were outcompeted by their formate-producing counterparts (17). Thus, indicating its importance in contributing to chronic inflammation in IBD patients (17). In addition, formate concentration levels were

increased during inflammation in mice treated with the pathogen rather than the control mice which received no pathogenic microbes (17). Furthermore, metagenomic studies were conducted that analyzed genes encoding for the electron transport chain, such as respiratory dehydrogenases, terminal oxidases, and terminal reductases. The data revealed a high relative abundance of these genes in association with the DSS-induced inflammation model, indicating that formate dehydrogenase plays an important role (17).

Diet, genetics, and microbes all play a role in disease etiology and morbidity. The host immune response and changes in diet can impact bacterial taxonomy in the microbiota and, in turn, influence inflammatory responses (15-17). Most shifts observed due to these factors occur at the species level, which has an important impact on the microbiota and subsequent immune response. These species level shifts can cause induction of the proinflammatory response and upregulate production of antimicrobial peptides that increase oxygen production in the intestinal epithelium. This provides a terminal electron acceptor for microbes such as *E. coli* which utilize fitness advantages to bloom in the otherwise anaerobic environment (15). The molecule formate is also produced as a by-product of bacterial fermentation and provides an electron acceptor for the family *Enterobacteriaceae*; further supporting their growth and helping them confer a fitness advantage (17). As these mechanisms contribute to dysbiosis, they further exacerbate inflammation as well through supporting the growth of facultative anaerobic microbes in the intestinal epithelium. Ultimately, the shift in taxonomy of the microbiota creates an environment that supports a bloom of typically aerobic bacteria and plays a role in chronic inflammation of intestinal tissues (15-18).

V. Microbiota and Host Immune Response

Crohn's disease is a chronic inflammatory condition of the gastrointestinal tract (GIT) and a type of inflammatory bowel disease (IBD). Crohn's disease is unique in that it is an autoimmune disorder; however, it also has a multifactorial etiology. This means that there are genetic, environmental, and microbial components that all impact CD. Autoimmune disorders occur when the host immune system recognizes self-peptides as foreign invaders and attacks. In CD, this occurs due to an inappropriate response toward the commensal microbiota and leads to chronic inflammation. Both the innate and adaptive immune responses play a role in the chronic inflammation and, there are key players that enhance the inflammatory response in IBD (22, 23).

There are more than several trillion microbes that live symbiotically in the human microbiome (23). While we know CD is multifactorial, genetic factors alone do not increase CD pathogenicity (23). This is where the microbiome comes in. Environmental and microbial factors contribute to the dysbiosis experienced within the GIT microbiome and impact disease outcome (23). Dysbiosis occurs when there is an imbalance of microbes within the microbiota. The phyla found in the highest abundance in the gut are *Firmicutes, Bacteroidetes, Proteobacteria,* and *Actinobacteria.* Compared to healthy guts, microbiomes in a state of dysbiosis are characterized by an increase in facultative anaerobic bacteria and a decrease in obligate anaerobic bacterial communities. These communities compete for limiting energy sources, such as carbon and, mostly, complex polysaccharides derived from diet or host (23). Bacteria also help protect the intestinal epithelium from pathogens through production of antimicrobial compounds via the host immune response (23).

The host immune system responds to pathogens by producing antimicrobial agents and other proteins. This process is activated by intestinal cells (22). Cytokines, arachidonic acid metabolites, reactive oxygen species (ROS) intermediates, and growth factors all have important regulatory and effector functions relevant to the inflammation we see in the intestines (22). Cytokines are proteins, activated in the immune response, that play a role in inflammatory response cascades (22, 24, 25). Cytokines function in autocrine, paracrine, and endocrine activities which impact both local and systemic intestinal inflammation, as seen in CD (22). In addition, cytokines regulate and amplify the immune response which can cause tissue damage and induction of other inflammatory pathways through the recruitment of effector molecules (22). However, not all cytokines cause inflammation; they also play an important role in suppressing inflammation and facilitating tissue repair (22). Cytokines alone are not the main mediators of intestinal mucosa inflammation in the immune response of CD patients (22, 26).

The adaptive and innate components of the immune system influence these important molecules (ROS, RNS, and cytokines) and, ultimately, impact the role they play in intestinal inflammation in IBD (26). To understand how these molecules function in the immune response, we must first understand the distinction between the two branches of the immune system. The innate immune system immediately recognizes pathogen presence and aims to control infection through induction of pro-inflammatory cytokines that recruit phagocytic and antigen presenting cells (APCs), like neutrophils and macrophages. The adaptive immune system orchestrates an antigen-specific response through the use of T and B cells to create a more sophisticated and specialized response tailored to the pathogen (26). Activation of the immune response is important to protect the GIT from pathogens. In a normal healthy gut,

there is a state of controlled inflammation. This means downregulation of inflammatory signals by the mucosal immune system so commensal organisms can exist symbiotically with the host (26). However, any defects in the immune system will have an impact on the immune response and resulting inflammation (26). This is especially relevant in the context of Crohn's Disease.

VI. Mucosal Immune Response in GIT of CD Patients

Some mechanisms utilized by the host immune system are the mucosal and epithelial barrier, pro-inflammatory signaling pathways and intestinal innate and adaptive immune components (23). These mechanisms help limit colonization of pathogenic bacteria and microbiota-driven inflammation. The epithelium provides a physical barrier between luminal contents, everything that resides in the opening of the intestines, and the underlying lamina propria (23). For reference, the mucosal layer is the innermost layer of the GIT and is made up of the epithelium, lamina propria, and muscularis mucosae (thin layer of smooth muscle). The lamina propria separates the epithelium lining the GIT and the smooth muscle which connects to the circulatory system (23). Specialized secretory cells, like goblet cells, plasma cells and Paneth cells, within the GIT produce and secrete mucus, plasma and antimicrobial peptides, respectively, that function as a line of defense to decrease the microbial burden of epithelium (23). Expression of some antimicrobial proteins, like α-defensins, are readily expressed in intestinal epithelial cells; however, other antimicrobial proteins, such as β-defensins, C-type lectin regenerating islet-derived protein 3γ, and a subset of α-defensins are regulated by bacteria-activated toll-like receptors (TLRs) or nucleotide-binding oligomerization domain-containing protein 2 (NOD2) signaling (23). Another defense the gut utilizes are microbiome-derived metabolites, small molecules with

antimicrobial properties that promote resistance to colonization of pathogenic species (17). One example of this is the microbial derived molecule, polysaccharide A (PSA) isolated from *B. fragilis*, an important commensal microbe of the human gut (23, 27). In an experiment, Mazmanian et al. found decreased colonization of *H. pylori*, a common GIT pathogen, upon introduction of PSA through downregulation of *Interleukin-17* (*Il17*) and upregulation of *Interleukin-10* (*Il10*), the genes encoding for the pro-inflammatory cytokine *Il17* and anti-inflammatory cytokine *Il10*, respectively, through $CD4^+$ T cells (23, 27, 28). In addition, anaerobic bacteria aid in digestion by breaking down indigestible complex polysaccharides to produce short chain fatty acids (SCFAs) (23). Production of these metabolites feed the bacteria that encourage anti-inflammatory activity because they are an important energy source in the colonic epithelium (23). While the host immune response and commensal bacteria have defenses that aim to protect and maintain the lamina propria of the GIT, they do not always succeed in stopping microbial pathogens (22, 23, 26).

When looking at the microbiota composition in CD patients, an increase in facultative anaerobic bacteria and a decrease in obligate anaerobic bacterial communities are commonly seen (22, 26). This imbalance in the microbiome facilitates a marked decrease in community homeostasis compared to the microbiota community found within a healthy gut (23). Furthermore, a reduction in obligate anaerobic bacteria is associated with a decrease in microbial diversity and increase in bacteria with pro-inflammatory properties (23). Bacteria like adherent-invasive *Escherichia coli* (AIEC) are mucosa-associated pathobionts strongly linked with CD pathogenesis (23). They are able to replicate in macrophages and penetrate the epithelial barrier, further contributing to IBD pathogenesis and chronic inflammation (23). Previous studies have shown that an abundance of mucosal microbiota in IBD patients

is positively correlated with disease severity and increased concentrations of AIEC have been linked to CD (23, 29). These findings reiterate the importance of the microbiota composition and how dysbiosis within the GIT can impact inflammation (3). The increase in bacteria, coupled with their ability to adhere to and invade epithelial cells, affects intestinal integrity; thus, further contributing to the cycle of dysbiosis and chronic inflammation within the GIT through the induction of pro-inflammatory immune response (23). Imbalances within the microbiota and immune system evasion techniques utilized by bacteria are ways in which the microbial component of CD enhances disease pathogenicity.

The environmental aspect of CD is strongly impacted by diet. One diet that has been strongly linked to IBD is the western diet. It consists of high sugar and fat, with low dietary fiber, fruits, and vegetables (23). The western diet is also associated with altered structure and function of the commensal gut flora. Anaerobic bacteria convert dietary fiber to SCFAs through fermentation in the intestine, which can affect gene expression and composition of the intestinal microbiota community (23). A decrease in SCFA production can lead to a decrease in SCFA-producing bacteria (23). In addition, since SCFAs feed bacteria that increase anti-inflammatory activity, a decrease in that population could cause the opposite effect (23). While diet can negatively impact IBD, it can also have a positive effect (23). For example, the aryl hydrocarbon receptor (AhR) is a transcription factor (TF) found in the cytoplasm of macrophages, B cells, T cells, dendritic cells (DCs), and intestinal epithelial cells. Diet derived AhR ligands, like kynurenine made from the amino acid tryptophan, are able to help regulate intraepithelial lymphocytes, lymphoid cells, immune and inflammatory reactions and maintain homeostatic mucosal function in the GIT (23). This is accomplished because the AhR ligand promotes local IL22 production which stimulates the production of

antimicrobial peptides and mucin, contributing to pathobiont resistance and mucosal preservation (23). In the GIT, epithelial cells are in direct contact with many molecules and organisms that contribute to the host-immune response. Factors such as pathogenic microorganisms, dietary antigens, drugs, or toxic components, can induce the host immune response (23). Common over-the-counter drugs like non-steroidal anti-inflammatory drugs (NSAIDs) have been associated with flares of CD because they can disrupt the epithelial barrier (26). Although not all studies have associated NSAIDs with worsening colitis, disruption of the epithelial barrier would increase intestinal permeability (26).

The genetic component of CD can also impact intestinal barrier and immune system function (23). Various proteins and defects in proteins, epithelium, and the immune system contribute to chronic inflammation (23, 26). Defects in the epithelial lining of the GIT are commonly seen in CD (26). The epithelial lining of the GIT, surface mucus, peristalsis and secretion of host protective factors are ways normal permeability in the gut is maintained (26). The epithelium itself is composed of cells and paracellular space that contain tight junctions (26). Tight junctions allow for selective entry of nutrients, fluids, and microorganisms past the epithelial barrier (26). Recent research has identified DCs within the paracellular space and alluded to their importance in forming tight junctions (26). There are many ways in which the epithelial barrier is naturally protected, however, if there are alterations in any of these cells, molecules, etc. the epithelial barrier can expose the mucosal immune system to potential pathobionts from the intestinal lumen (26). Interactions between luminal contents and the epithelium can stimulate the production of Th1 cells in addition to proinflammatory cytokines such as TNFα and IL1β (26). Changes to the epithelial barrier

can result in increased permeability, decreased epithelial integrity, and abnormal mucus composition (23, 26).

SCFA production is important to maintain a diverse microbial population. However, when a decrease in SCFA production occurs, bacteria are able to utilize glycan, a complex carbohydrate in the mucus, as a metabolite. The digestion of mucus decreases the protective functionality it provides for the epithelium, allowing more opportunity for bacterial adherence to the protective epithelial layer and increasing epithelium permeability (26). Defects in the permeability of the intestinal epithelium are linked to IBD. Maintenance of epithelial integrity is influenced by presence and sensing of bacteria (26). The role of Toll-like receptors (TLRs) in regulating the intestinal response to bacteria and secretion of protective factors, such as defensins, also play a role in this signaling response (26). In the small intestine, there is a smaller bacterial load, and the epithelial cells are more responsive to lipopolysaccharide (LPS) than in the colon (26).

The SI maintains tissue specific homeostasis through TLR expression in cellular compartments (26). TLRs are important regulators in the induction of the immune and inflammatory responses (26). They protect against inappropriate activation of the inflammatory reaction in response to commensal bacteria and defend the host against pathobionts (26). In response to non-commensal pathogen associated molecular patterns (PAMPs), the TLRs induce antimicrobial peptide expression, barrier reinforcement, and proliferation of epithelial cells (26). Regional variations in the type of cells that express TLRs allows for more regulation of TLR expression in the inflammatory response (26). In healthy colonic epithelium, two toll-like receptors (TLR2 and TLR4) are expressed at the cell surface and are known to be poorly responsive to LPS (26). In conditions of prolonged LPS

or lipoteichoic acid (LTA) exposure, the cells can develop tolerance, which causes a muted response to LPS with each subsequent exposure to the respective PAMPs (26). This, in turn, leads to decreased TLR surface expression and *Il1* receptor-kinase activity (26). The goal for TLRs exposed to commensal microbes is to downregulate the TLR-dependent response, while tolerance is not the appropriate regulatory response for pathogens (26). TLR5 is the receptor for monomeric flagellin which is a bacterial protein that composes flagella and a virulence factor of Gram-negative and Gram-positive bacteria (26). Typically, pathogenic organisms release monomeric flagellin that activate TLR5 although some commensal bacteria are also flagellated (26).

In murine and human colitis models, antibody responses to commensal bacteria with flagellin and pathogenic bacteria with flagellin were detected. This is another example of one way the immune system may act inappropriately in response to microbes in CD (26). TLR signaling is important for healing injured intestinal epithelial cells and clearing out intramucosal bacteria (26). After epithelium injury, like with administration of dextran sodium sulfate (DSS), animals deficient in TLR signaling have decreased intestinal cell proliferation (26). Therefore, it is hypothesized that there is a certain amount of TLR signaling needed in the intestine to protect the host/maintain homeostasis (26).

The mutations in the gene encoding for Nucleotide-binding oligomerization domain 2 (NOD2) also plays a role in the genetic component of CD pathogenesis (26). NOD2 is a critical regulator of inflammation, and mutations within the protein have been associated with decreased SCFA-producing bacterial strains and increased *Actinobacteria and Proteobacteria* which result in intestinal flora shifts in patients with IBDs (23). SCFA production is important for maintaining proper function of G-coupled protein receptors

(GPCRs) (23). Thus, mutations in the NOD2 protein increase a patient's susceptibility to inflammation due to dysbiosis (23). Proinflammatory mediators IFNγ and Tnfα, secreted by Th1 cells, can upregulate NOD2 expression in intestinal epithelial cells, further contributing to dysbiosis within the intestinal environment (23). The genetic, environmental, and microbial aspects of CD each have a unique role that work synergistically to create the dysbiosis and inflammation seen in CD (23, 26). Moreover, all three factors initiate an immune response which enhances disease pathogenesis (23).

VII. Immune Response & Major Players

The innate and adaptive immune responses play important roles in the chronic inflammation observed in CD (23). Cells of the innate immune system include dendritic cells, macrophages, natural killer cells (NKCs), neutrophils, basophils, natural killer T cells (NKTCs) and T lymphocytes. Macrophages and Dendritic cells are APCs and help activate the adaptive immune response (22). Once triggered, a cascade of various proteins and immune cells are activated (22). The innate immune system functions to recognize highly conserved structures found on pathogenic microorganisms (26). Some of the conserved structures are lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acids (LTAs), mannans and bacterial DNA; these are referred to as PAMPs. Effector cells like macrophages, dendritic cells (DCs) and B cells have pattern recognition receptors (PRRs) on their surfaces. Once stimulated, these antigen presenting cells (APCs) begin contributing to the immune response immediately. One job of the innate immune system is to recognize the presence of a pathogen, in addition to general defense, and provide the necessary signals to kick-start the adaptive immune response to the pathogen (26). TLR signaling plays an important role in maturation of APCs which are necessary for T cell selection. In this

system, PRRs recognize PAMPs which initiates the development of regulatory T cells (Tregs) in the gut and suppression of chronic inflammation to commensal bacteria (26). Disfunction in this narrow distinction between a commensal and pathogenic organism could influence the chronic inflammatory response seen in CD (26).

IL1β is a key immunoregulatory cytokine that amplifies the inflammatory response through activation of an immune cell cascade (22). It is predominantly secreted by macrophages in the lamina propria, rather than epithelial cells, and stimulates the production of cytokines, arachidonic acid metabolites, and proteases by intestinal macrophages, neutrophils, and epithelial cells (22). IL1 also has the ability to induce IL2 and IL2 receptors on T lymphocytes. In turn, the T lymphocytes (TH1 helper cells) secrete IL2 and interferon gamma (*IFNγ*), which, together, stimulate an immune response mediated by cytotoxic T lymphocytes, macrophages, and NK cells (22). In actively inflamed tissues, IL1 activity is dramatically increased compared to inactive IBD patients (22).

Studies have found increased production of *IFNγ* and Tnfα in mucosa of CD patients in addition to increased expression of Th1 cytokines (26). One possible explanation for the increase in expression of mucosal Tnfα could be due to polymorphisms in the *Tnfα* gene which have been found in certain populations (24, 26). Specifically, polymorphisms within the promoter region could result in the different levels of Tnfα expression (26). However, there is a prevalence of cytokine expression abnormalities found in CD (26). Transfer of CD4+ T cells transduced with T-bet (the transcription factor for Th1-type differentiation of T cells) results in colitis in animal models (26). T-bet expression is increased in the lamina propria T cells in CD (26). This increase in T-bet expression is paralleled by an increase of STAT1 expression, an *IFNγ* dependent transcription factor, and increased sensitivity to Il12

stimulated production of *IFNγ (26)*. There is an increase of CD4+ T cells in the mucosa of CD patients. Increased levels of CD4+ T cells can promote or repress the inflammatory response based on what subtype they differentiate into. In CD lamina propria T cells are resistant to activation-induced apoptosis, whereas lamina propria T cells in healthy patients have been found to undergo apoptosis, thus contributing to the abnormal immune response experienced in CD (26).

The abnormal immune response can also be influenced by the cytokine Interferon-γ (IFN*γ*). *IFNγ* can increase mucosal permeability through modification of epithelial tight junctions that then induces intracellular adhesion molecules which enhance neutrophil transmigration across epithelial monolayers (22). IFN*γ* enhances expression of major histocompatibility complex II (MHC II) molecules on macrophages, B lymphocytes, and dendritic, endothelial, mesenchymal, and epithelial cells (22). MHCII molecules present the antigen to T cells in the adaptive immune system so they can differentiate into the appropriate cells to destroy the identified pathogen (22, 26). This increases the antigen-presenting function of these cells, thus increasing mRNA expression of *IFNγ* (22).

Cytokines induce epithelial expression of many proteins, including other cytokines like IL6 (22). IL1 and Tnfα stimulate the proliferation of smooth muscle cells and fibroblasts which induce synthesis of IL1, IL6, Tnfα and other cytokines (22). IL6 is produced by activated macrophages and is reproducibly increased in actively inflamed tissues (22). In healthy patients, DCs in the mucosa help to protect against pathogens. DCs generate IL6 and additional factors to remove any suppressive effects of Tregs (26). Cytokines produced by APCs polarize the immune response in CD. Production of Il12 by DCs or macrophages is needed for expression of *IFNγ* and is essential for Th1-type CD4+

T-cell differentiation in CD (26). Il12 is produced by DCs in the terminal ileum and is involved in activation of APCs that induce T cell polarization to Th1 subtype (26).

Finally, IL17 is another cytokine that is found with increased mRNA expression in CD (23). Several studies (23, 28) have shown IL17 plays an integral role in IBD inflammation development (28). In the adaptive immune response, macrophages, and dendritic cells (DCs) stimulate naïve CD4+ T-cells to differentiate into Th17 cells. IL17 is the critical cytokine produced by Th17 cells, however, both Tnfα and *IFNγ* are produced as well. In the inflamed intestine, cytokines and growth factors can alter the activation state of mesenchymal cells which amplifies the inflammatory response, that probably plays a role in fibrosis; an important complication of CD (22). At the mucosal level, Tnfα, Il1b, Il12, Il17, *IFNγ*, and Il6 have been associated with inflammation. Through looking at the relative abundances of these cytokines we can infer which of them play key roles in inflammation of intestinal tissues.

The objectives of this thesis were to: determine to what extent Crohn's-associated clinical AIEC and non-AIEC strains colonize the SI, validate their impact on microbial dysbiosis and inflammation, and characterize the impact of patient-derived *E. coli* strains on the SI microbiota.

Materials and Methods

E. coli Strains

We utilized seven clinical *E. coli* strains that were isolated from ileal tissue of Crohn's disease and non-Crohn's disease patients (2, 23, 30). Strains were classified as AIEC or non-AIEC using standard in vitro assays to evaluate adhesion/invasion to Caco2 colonic epithelial cells and uptake/survival in J774 macrophages (1, 2). These strains were referenced

by their blinded laboratory designation: JA0018, JA0019, JA0022, JA0036, JA0044, JA0048, and JA0091 (30). Five of these strains were originally isolated by KW Simpson at Cornell University as the strains CU39ES-1, CU532-9, CU568-3, CU37RT-2, and CU42ET-1. HM670 was isolated and gifted by Barry Campbell from Liverpool University (31), and LF82 was gifted to KW Simpson by Arlette Darfeuille-Michaud (1).

Barcoding Technique

We are using a novel barcoding technique developed by Dr. Bleich while working in the Arthur Lab at UNC Chapel Hill. Our seven isolates have been molecularly barcoded with an approximately 60 bp unique identifier inserted 25 bp downstream of the *glmS* gene, a neutral site of the *E. coli* genome, via Tn7 transposon insertion (5). This technology is used to identify an individual barcoded strain from within a larger complex community like the microbiome. The Tn7 transposon demonstrates remarkably less insertion bias, compared to other transposon sites, within a target DNA (32). This technique was utilized for tagging the patient-derived AIEC and non-AIEC strains for easier identification, as these strains cannot be distinguished through 16S analysis. Previous work from the Bleich and Arthur labs have utilized this technique on the lower GI tract and found some clinical *E. coli* isolates enhanced inflammation which supported an *E. coli* bloom in the lumen and intestinal tissue of the colon. These findings support the validity of this approach and its feasibility and utility for this project. A kanamycin resistance gene was simultaneously inserted with the barcode, giving the seven *E. coli* strains kanamycin resistance for easier selection.

Mice

The germ-free mice (129Sv/Ev background) were bred and raised in the National Gnotobiotic Rodent Resource Center at UNC Chapel Hill. The experiments and procedures were approved by UNC's IACUC.

Murine FMT Preparation

Fecal microbial transplant 1 (FMT 1) was prepared anaerobically from 7 C57BL/6 WT specific pathogen free (SPF) mice that were *Helicobacter spp.* free. Briefly, colonic and cecal content were removed in an anaerobic chamber and resuspended in sterile, reduced PBS to make a slurry. The slurry was homogenized by vortexing and physical disruption with a filter pipette. Glycerol was added, for easier streaking later, to 15% final concentration before aliquoting and storing at -80 ^{o}C. Fecal microbial transplant 2 (FMT2) was prepared in the same manner from 7 gnotobiotic C57BL/6 WT male mice that were gavaged with 100 uL of FMT1.

Mouse Model

We tested the colonization of our pool of *E. coli* isolates in the established interleukin-10 (*Il10* $^{-/-}$) mouse model that mimics chronic inflammatory conditions like CD (3, 33). *Il10* $^{-/-}$ (inflammation-susceptible) and wild-type (inflammation-resistant) 129S6/SvEv mice were reared germ-free to adulthood (8-10 weeks) in the cohorts listed in Table 2. Mice were colonized with an even mixture of a total of 10^8 CFU of the barcoded *E. coli* strains and maintained in SPF housing. One week after colonization, mice were given 100 uL of thawed FMT for colonization competition with a normal, murine microbiota. Two weeks post-FMT, we gave kanamycin water ad libetum for 2 weeks to suppress the microbiota and ensure that some of the barcoded *E. coli* strains could persist to the end of our

10-week model (30). Mice were harvested 6 weeks later by CO_2 asphyxiation for a total of 10 weeks colonization post-FMT. Stool samples were removed from the lumen and 1 cm of jejunal and ileal samples were taken for sequencing analysis. Mice that received just FMT were treated the same as above without the one week of colonization with barcoded *E. coli* strains. Mice that received just barcoded *E. coli* were treated the same as above just without the FMT gavage after 1 week of colonization.

Internal Cohort Designation	**Condition**	**Mouse Genotype**	**Number of Mice**	**Number of Cages**	**Gender**
JA123	7 Barcoded *E. coli* + FMT1	*Il10*$^{-/-}$	17	5	7F/10M
JA134	FMT1 only	*Il10*$^{-/-}$	13	4	5F/8M
JA137	7 Barcoded *E. coli* only	*Il10*$^{-/-}$	9	4	3F/6M
JA143	7 Barcoded *E. coli* + FMT1	WT	17	4	8F/9M
JA207	NC101 *E. coli* + FMT2	*Il10*$^{-/-}$	13	4	8F/5M
JA216	3 Barcoded E. coli + FMT2	*Il10*$^{-/-}$	8	3	4F/4M
JA218	7 Barcoded *E. coli* + FMT2	*Il10*$^{-/-}$	13	4	6F/7M
JA226	1 Barcoded *E. coli* + FMT2	*Il10*$^{-/-}$	7	2	4F/3M
JA240	FMT2 only	*Il10*$^{-/-}$	9	5	5F/4M

Table 1: Mouse cohorts used in study.

DNA Extraction

Jejunal tissue samples from the mice were previously collected in the Arthur Lab at UNC Chapel Hill. DNA was extracted from the samples in the Bleich Lab using the Qiagen DNeasy Blood and Tissue kit to clean up the DNA. Tissue samples were placed in a tube with zirconium beads and incubated at 37˚C with a lysis buffer and lysozyme (20mg/mL of buffer). Then 10% SDS solution and proteinase K (15 mg/ml) were added, and the samples were incubated at 60˚C. Phenol:Chloroform:Isoamyl alcohol (25:24:1) was added to the

samples and the cells were physically disrupted using a bead beater to ensure all cells were lysed. A phenol/Chloroform extraction and ethanol precipitation was performed. Samples were pelleted and resuspended in nuclease free water. Buffer AL (Qiagen kit) and ethanol were added to the samples and placed in a spin column. Using AW1 and AW2 from the Qiagen kit the samples were washed, and the DNA was eluted using nuclease-free water.

Illumina Sequencing Library Preparation

The jejunal and ileal tissue samples were prepared for Illumina MiSeq sequencing by amplifying the 16S V4 region or molecular barcode and attaching the Illumina adaptors and indices. The initial amplification of the V4 regions for both tissues used the KAPA2G Robust PCR kit. We used standard MiSeq multiplexing forward and reverse primers for library preparation. For clean-up HighPrep PCR reagent magnetic beads bind the DNA while on a magnet and samples undergo two ethanol washes before being resuspended with nuclease-free water and the eluent with DNA collected. The second amplification adds Illumina indices and the Illumina adapter sequence using the KAPA HiFi HotStart ReadyMix. Following the final PCR step the HighPrep PCR reagent magnetic beads are used again in the DNA clean-up. Equal amounts of each sample were pooled and subjected to paired end Illumina sequencing. Illumina MiSeq sequencing was completed at the University of Chapel Hill Microbiome Core Facility.

Sequencing Analysis

Raw sequencing reads were processed in conjunction with the Fodor Lab at UNC Charlotte using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline. Taxonomy was assigned using SILVA databases. Bray-Curtis distances between samples were calculated

using genera abundance. P-values were adjusted with the Banjamini-Hochberg method and significance was assessed with a threshold of False Discovery Rate (FDR).

qRT-PCR

To quantify jejunal tissue cytokine expression, RNA was extracted using Trizol reagent following manufacturer's protocol and subjected to DNaseI treatment. Up to 500 ng of RNA was reverse transcribed into cDNA using random hexamer primers. Amplification was performed in triplicate with SYBR green qPCR chemistry using primers for *Tnfα* (F-5'-ACCTCACACTCAGATCATCTTCTC-3', R-5'-TGAGATCCATGCCGTTGG-3'), *Il1b* (F-5'-ACAGAATATCAACCAACAAGTGATATTCTC-3', R-5'-GATTCTTTCCTTTGAGGCCCA-3'), *Ifnγ*, *Il12b(p40)* (F-5'-CGCAAGAAAGAAAAGATGAAGGAG-3', R-5'-TTGCATTGGACTTCGGTAGATG-3'), *Il6* (F-5'-GAAATGATGGATGCTACCAAACTG-3', R-5'-CTCTCTGAAGGACTCTGGCTTG-3'), *Il17a* (F-5'-AACCGTTCCACGTCACCCTGGA-3', R-5'-TGGTCCAGCTTTCCCTCCGCA-3') and *Gapdh*(F-5'-GGTGAAGGTCGGAGTCAACGGA-3', R-5'-GAGGGATCTCGCTCCTGGAAGA-3'). *Ct* values were normalized to *Gapdh* to generate Δ*Ct* values, and fold changes were calculated by ΔΔ*Ct* to the Δ*Ct* of uninflamed controls. A two-way unpaired ANOVA was run to establish significance. PCR was performed in technical duplicates or triplicates.

Results

To investigate how AIEC are impacting the SI microbiome in an *Il10*$^{-/-}$ model, mice stool collected at various points throughout the experiment was analyzed. To resemble CD more closely, it is important that the AIEC and non-AIEC strains were able to colonize the GIT of the mice. The fecal *E. coli* loads were measured periodically throughout the

experiment (Fig. 1A) which resulted in *Il10*$^{-/-}$ mice having higher fecal loads compared to WT treatments. Relative abundances of *E. coli* sequences were analyzed in Figure 1b, which showed that *Il10*$^{-/-}$ treatments had significantly more *E. coli* in their stool as the experiment continued compared to WT. A comparison of WT and *Il10*$^{-/-}$ stool microbiota (Fig. 1C) was done to further explore the shifts in bacterial composition. WT and *Il10*$^{-/-}$ mice had different stool microbial compositions at the genus level compared to one another. To determine whether AIEC were able to colonize the jejunum, PCR was conducted to analyze *E. coli* specific 16S band presence in the different treatments (Fig. 1D). WT mice that received FMT1 and the *E. coli* slurry had 100% *E. coli* 16S band presence across all samples with that treatment (Fig. 1D). There was 88% 16S band presence in the treatment group that received *Il10*$^{-/-}$ + FMT and 78% 16S band presence for the *Il10*$^{-/-}$ + *E. coli* groups. Finally, the treatment group that received *Il10*$^{-/-}$ + FMT2 had the lowest 16S band presence at 70% across the samples.

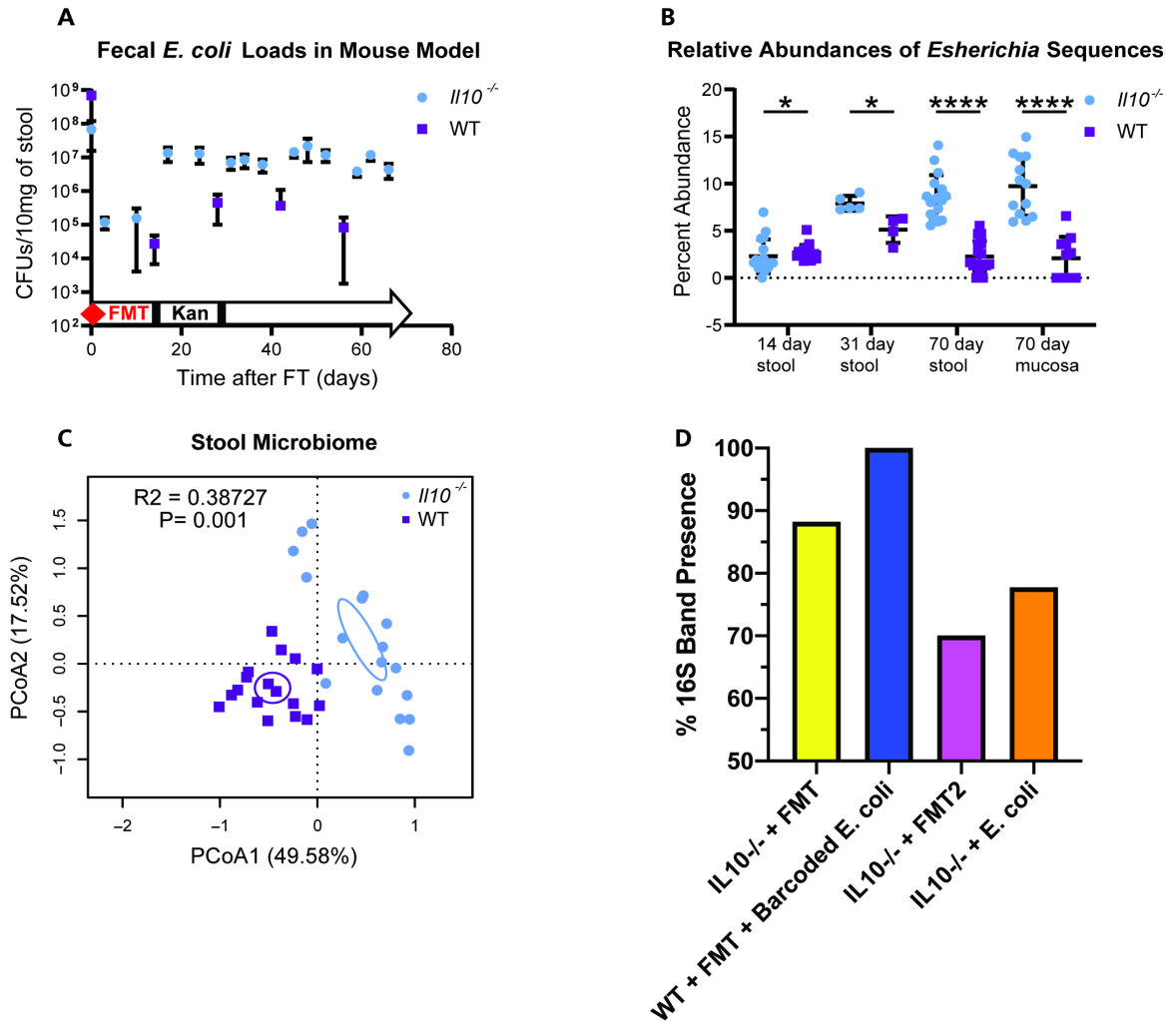

Figure 1. *E. coli* colonize *Il10*$^{-/-}$ mice. (A) Fecal *E. coli* loads in murine model over time. (B) Relative abundances of *Escherichia* sequences at 14 days (pre-kanamycin), 31 days (post-kanamycin), and 70 days. (C) Stool microbiome PCoA analysis of *E. coli* + FMT1 colonization between *Il10*$^{-/-}$ and WT mice. (D) % Presence of *E. coli* specific16S band across mice within that treatment.

Following investigation of AIEC ability to colonize the GIT, their impact on microbial composition was examined. The FMT2 used was created by passaging FMT1 through germ-free mice, and the microbiota across both FMTs started out very similar in composition aside from a slight increase in *Akkermansia* in FMT2 (Fig. 2A). Further analysis of the microbiota revealed that JA207, *Il10*$^{-/-}$ + NC101 + FMT2, and JA226, *Il10*$^{-/-}$ + 1 *E.* coli isolate + FMT2, changed the most compared to the *Il10*$^{-/-}$ control group that only

received FMT2. NC101 is a well-known, murine AIEC isolate. The JA216 (*Il10*$^{-/-}$ + 3 *E.* coli isolates + FMT2), JA240 (*Il10*$^{-/-}$ + FMT2) and JA218 (*Il10*$^{-/-}$ + 7 *E.* coli isolates + FMT2) treatments had similar microbial composition, seen in Figure 2B. The alpha diversity of the microbiota, calculated by Shannon index, was determined at in the fecal content as well as the ileum (Fig. 2C). The fecal content had a higher species diversity compared to the ileum across all treatments. The control JA240, *Il10*$^{-/-}$ + FMT2, had the highest diversity in fecal content while the other cohorts were similar. The ileum had similar diversity indexes for all four treatments (Fig. 2C).

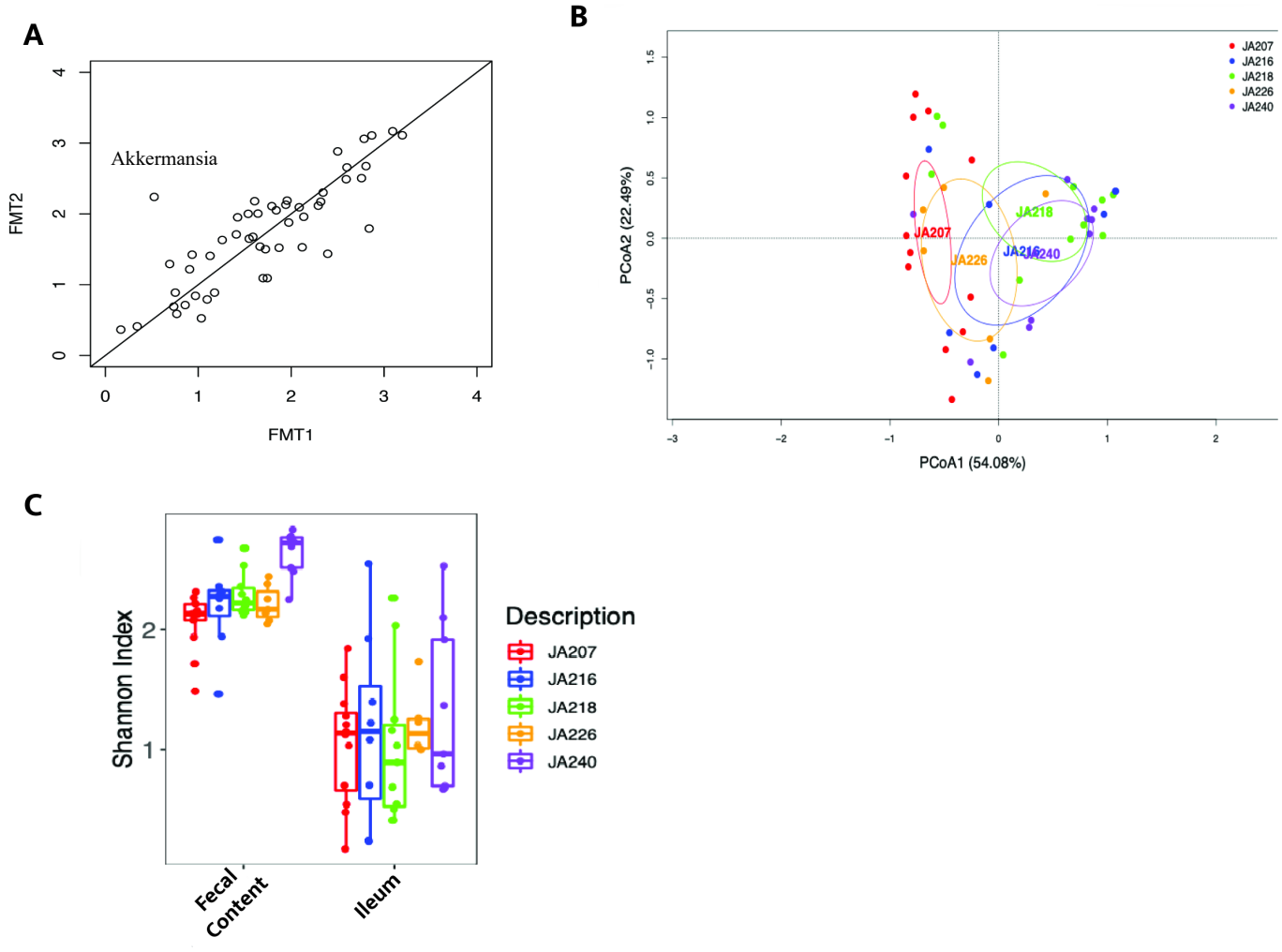

Figure 2. Microbial composition in the SI is impacted less by AIEC colonization. (A) The microbial composition of FMT1 and FMT2 is similar. (B) Microbial composition in the ileum in *Il10*$^{-/-}$ mice. (C) Shannon Diversity Index of fecal content and ileum in *Il10*$^{-/-}$ mice.

Proinflammatory cytokine expression in the jejunum of Il10*$^{-/-}$ *mice is Impacted by AIEC

Proinflammatory cytokine expression was investigated to determine which cytokine genes were upregulated under the inflammatory conditions. We hypothesized that the relative abundance of cytokine mRNA would be increased in this pro-inflammatory murine model, similar to how the proximal colon responded (Supplemental Fig. 1A). The jejunum samples were obtained from mice that received one of the following treatments: Adherent invasive *E. coli* (AIEC) + Fecal Microbial Transplant (FMT), FMT only, FMT2 only, *E. coli* + FMT2, or AIEC only. The relative abundance of cytokine mRNA expression was measured in the jejunal samples across each treatment type. Expression of *Tnfa*, in Figure 3A, was higher in the treatment that received *E. coli* + FMT1 than the mice that received the *E. coli* + FMT2. FMT2 was a fecal microbial transplant slurry derived from a culture of the original FMT1. Mice with the inflammatory microbiota colonization type, FMT1 only, had a higher relative abundance of *Tnfa* compared to mice that only received *E. coli*. The relative abundance of *Tnfa* in the *E. coli* + FMT1 and FMT2 only were similar.

Expression of *Il1β* was investigated as well and as observed in Figure 3B, the relative mRNA abundance of *Il1β* was similar in the *E. coli* + FMT and *E. coli* + FMT2 treatments. The FMT2 only treatment also had a similar relative abundance of *Il1β* mRNA compared to the FMT1 only group. Finally, the *E. coli* only treatment group had higher relative expression than the FMT1 and FMT2 groups and it was lower than the inflammatory microbiota + *E. coli* treatments.

To further understand how the immune response was impacted by this study, *Il17* was next investigated (Fig. 3C). Both groups that received the FMT2 had a lower relative abundance of *Il17* mRNA relative to their counterparts that received the initial FMT. The *E. coli* + FMT2 group had half the amount of *Il17* expression as seen in the *E. coli* + FMT1 treatment group. In addition, the FMT2 only group had about half the abundance of *Il17* expression as the FMT only group.

Next, *Il6* was investigated. The relative abundance of *Il6* mRNA was lowest compared to all the cytokines investigated (Fig. 3D). The *E. coli* + FMT2 and FMT2 treatments had the lowest relative abundance of *Il6* expression while the *E. coli* + FMT1 and *E. coli* only groups had similar levels. The FMT1 only group had the highest relative abundance of *Il6* mRNA. The next cytokine investigated was *Il12β(p40)* (Figure 3E). The *E. coli* + FMT1 treatment had the highest relative abundance compared to the other four groups.

Finally, the relative abundance of *IFNγ* expression was investigated. The FMT only group had the highest relative mRNA abundance across all the treatment groups (Figure 3F). The expression levels exhibited by the FMT only treatment was significantly higher than the *E. coli* + FMT, *E. coli* + FMT2, and *E. coli* only groups.

In addition to looking at cytokine expression across the various treatments, analyzing the relative abundance of the cytokines for each treatment helped provide a more complete picture of what the mRNA expressions could indicate. In Figure 4A, the relative abundance of *IFNγ* was significantly higher compared to all other cytokines observed in the FMT only treatment group. The other cytokines, *Il6, Tnfα, Il12, Il1β, Il1b(p40),* and *Il17* all had relatively similar expression levels (Figure 4A). The treatment in Figure 4B also had a similar outcome to Figure 4A with *IFNγ* having a significantly higher relative abundance compared to *Il6, Tnfα, Il12,*

and *Il17* in the FMT2 only treatment group. *IFNγ* relative mRNA expression was higher than *Il1b(p40)*, however, it was not significantly different. In Figure 4C *Il12b(p40)* had the highest abundance closely followed by *Il1β* which had the second highest relative abundance in the WT mouse group. The other four cytokines, *Il6, Tnfα,* and *IFNγ* had a relative abundance of five or lower. Compared to the other treatments and relative mRNA abundances, the mRNA expression levels in Figure 4C are lower than the relative abundances seen in Figures A, B, D, E, and F. Figure 4D shows *Il6* had the lowest relative abundance in *Il10*$^{-/-}$ + FMT + *E. coli* treatment. Interestingly, *IFNγ* is seen in slightly lower abundance compared to *Il17* and *Il1β* (Figure 4D). In addition, *Tnfα* and *Il12b(p40)* have similar relative mRNA abundances around 10 and are lower than *Il17, IFNγ*, and *Il1β*.

The *IFNγ* levels are highest in the treatments that received just FMT, FMT2 or AIEC + FMT2 in addition to having the *Il10*$^{-/-}$ genotype. Figure 4E shows *IFNγ* and *Il1β* have similar relative abundances and both have the highest expression out of the cytokines investigated for this group. *Il17* had the third lowest relative abundance in the *Il10*$^{-/-}$ + FMT2 + *E. coli* treatment (Fig. 4E). Both *Tnfα* and *Il12b(p40)* had similar relative abundances. The last treatment investigated for relative cytokine mRNA abundance was the *Il10*$^{-/-}$ + *E. coli* group (Fig. 4F). Expression of *Il1β* was the highest out of all cytokines investigated. *IFNγ* had the second highest relative abundance. The other cytokines, *Il6, Il17*, *Tnfα* and *Il12b(p40)* all had the lowest relative mRNA abundances detected for the treatment group (Fig. 4F).

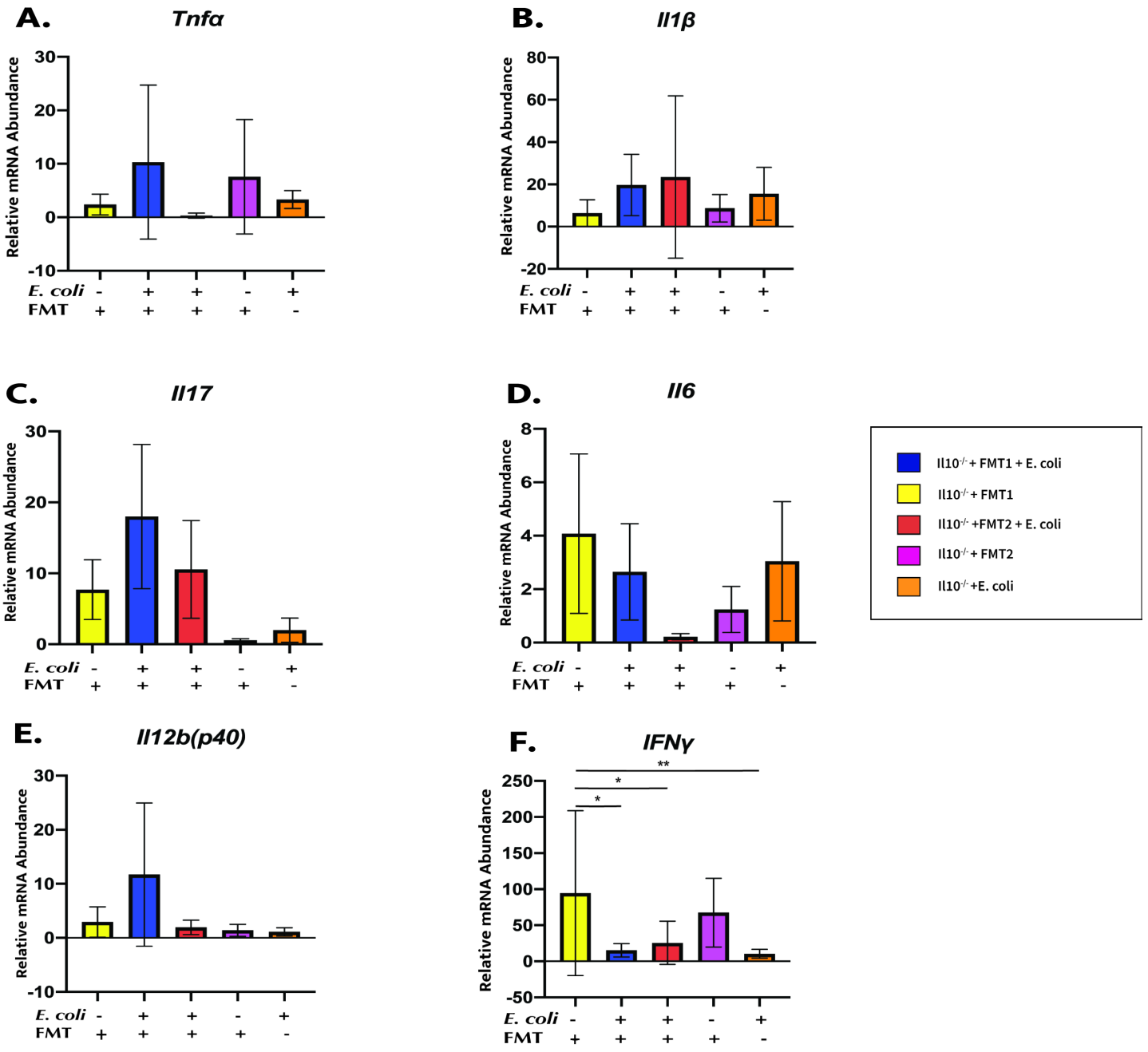

Figure 3. Cytokine Expression in Jejunal Tissue is Impacted by AIEC (A) Relative mRNA abundance of *Tnfα* in *Il10*$^{-/-}$ mice that received *E. coli* only, FMT only, FMT2 only, FMT2 + *E.coli*, or FMT + *E. coli*. (B) Relative mRNA abundance of *Il1β* in *Il10*$^{-/-}$ mice that received *E. coli* only, FMT only FMT2 only, FMT2 + *E.coli*, or FMT + *E. coli*. (C) Relative mRNA abundance of *Il17* in *Il10*$^{-/-}$ mice that received *E. coli* only, FMT only FMT2 only, FMT2 + *E.coli*, or FMT + *E. coli*. (D) Relative mRNA abundance of *Il6* in *Il10*$^{-/-}$ mice that received *E. coli* only, FMT only FMT2 only, FMT2 + *E.coli*, or FMT + *E. coli*. (E) Relative mRNA abundance of *Il12b(p40)* in *Il10*$^{-/-}$ mice that

received *E. coli* only, FMT only, FMT2 only, FMT2 + *E.coli*, or FMT + *E. coli*. (F) Relative mRNA abundance of IFNγ in *Il10*$^{-/-}$ mice that received *E. coli* only, FMT only, FMT2 only, FMT2 + *E.coli*, or FMT + *E. coli*.

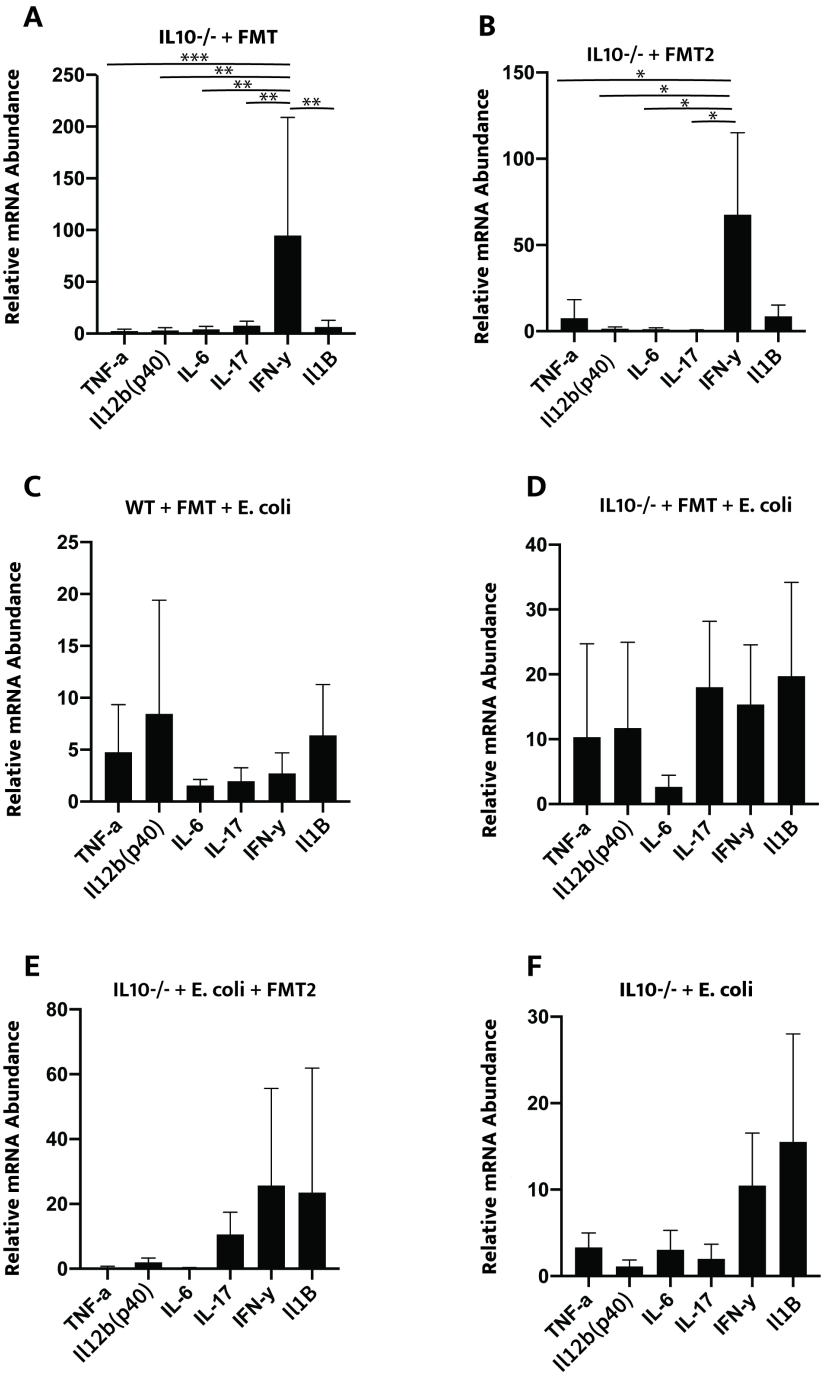

Figure 4. Cytokine Expression in Jejunal Tissue is Impacted by AIEC. (A) Relative mRNA abundance of various cytokines in the *Il10*$^{-/-}$ + FMT treatment. (B) Relative mRNA abundance of various cytokines in the *Il10*$^{-/-}$ + FMT2 treatment. (C) Relative mRNA abundance of various cytokines in the WT + FMT + *E. coli* treatment. (D) Relative mRNA abundance of various cytokines in the *Il10*$^{-/-}$ + FMT + *E. coli* treatment. (E) Relative mRNA abundance of various cytokines in the

Il10$^{-/-}$ + FMT2 + *E. coli* treatment. (F) Relative mRNA abundance of various cytokines in the *Il10*$^{-/-}$ + *E. coli* treatment.

Discussion

Chronic inflammation takes place over a long period of time and its establishment in our murine model is supported by the data seen in Supplemental Figure 1. The *Il10*$^{-/-}$ model, in germ-free mice, was used for this experiment because of its well-documented use in previous IBD models of infection (33, 34). While Dextran Sulfate Sodium (DSS) models of IBD have been shown to establish a chronic inflammation, they do not fully capture the genetic component of Crohn's Disease (CD). DSS models of CD use a chemical agent to trigger inflammation, while the *Il10*$^{-/-}$ murine model supports inflammation through a genetic mutation that inhibits the mice from producing the anti-inflammatory cytokine IL10. In the absence of IL10, a chronic inflammatory model can be established. This model also utilizes germ-free mice, mice that have been bred in an environment devoid of microbes and given a fecal microbial transplant to reproduce physiologically relevant conditions with consistent microbiota composition. This type of murine model is favored over the DSS model because the inflammation is produced as a result of the genetic mutation rather than chemical induction. As a result, increased proinflammatory cytokine expression in the immune response is indicative of inflammation and is a common aspect of CD (22, 26, 33). The *Il10*$^{-/-}$ *genotypic* model of infection allows for a closer recapitulation of this compared to a DSS model. Although, lack of the anti-inflammatory cytokine Il10 in the model means the response is not as similar to the normal immune response seen in CD. Regardless, tracking the increasing inflammation allowed us to observe the microbial community composition change as the environment of the gut changed over time (33, 34).

In our murine model, the initial stool samples collected at day 14 pre-antibiotic treatment were similar between the WT and *Il10*$^{-/-}$ mice. The next fecal samples were collected at day 28 post-antibiotic administration, the plot points begin to diverge from one another, indicating a shift in microbial composition between the WT and *Il10*$^{-/-}$ mice at day 28. By day 70, the stool microbiome samples had distinct microbial composition between WT and *Il10*$^{-/-}$ mice. These findings indicate that the longer mice were colonized with *E. coli* and the fecal transplant, the more the dissimilar the microbial community composition became between WT and *Il10*$^{-/-}$ mice. Indicating that genotype and levels of inflammation in the gut impact microbiome composition.

There are niche-specific differences between the microbial communities and immune response in the large and small intestinal tissues. It is important to study the tissue-associated microbiota because this is where the microbes interact most directly with the host, exerting their potentially proinflammatory effects. The mucosal microbiome is generally regarded as more relevant to diseases such as IBD and cancer. One example of this is *pks*+ *E. coli* that require cell: cell contact between bacterial and mammalian cells to exert genotoxicity (33). Accordingly, a *pks*+ strain that does not colonize the mucosa is unlikely to cause cancer, whereas one that colonizes the mucosa is now in a prime location to exert this carcinogenic activity (33).

Using this murine model, it is important to establish that the human-associated clinical strains of *E. coli* were able to colonize and persist in the SI. Fecal *E. coli* loads were taken to monitor the levels of *E. coli* present throughout timecourse of the experiment (Fig. 1A). This analysis revealed that the *Il10*$^{-/-}$ genotypic mice had higher fecal *E. coli* loads compared to WT treatments. In addition, the relative abundances of *Escherichia* sequences

in stool and at colonic tissue were looked at which supported the initial hypothesis that *Il10*$^{-/-}$ mice would have more *E. coli* colonization compared to WT mice (Fig. 1B). It is important to remember that increased colonization of a specific microbe can cause dysbiosis which is an imbalance in the gut microbial community. To visualize whether or not the microbiota of the *Il10*$^{-/-}$ mice had changed compared to the WT, we used principal coordinate analysis of the stool microbial community, which supported that dysbiosis had occurred and the two microbiotas had changed overtime (Fig 1C). Finally, to confirm that the pool of *E. coli* were present in the jejunum, we looked for presence of an *E. coli* 16S gene using PCR (Fig. 1D). We detected this gene across a majority of samples. All of the WT mice had presence of the gene while the treatments with the *Il10*$^{-/-}$ genotype had over 50% detection of the band in samples. This showed that we were able to detect these *E. coli* strains in the SI. Interestingly, in Figure 1D the group that received the FMT2 had least number of samples with the *E. coli* 16S gene present. This indicates that the *E. coli* were not able to colonize as well.

Since FMT2 was derived from a culture of FMT1 the community composition was analyzed to ensure there were not significant differences between the two that would skew the results (Fig. 2A). This then allowed for comparison of the various treatment types in the ileum that received the FMT2. We wanted to test whether changes to the microbial community were a general response to the presence of *E. coli* or if it was specific to the strains of the *E. coli* that were present. The samples received varying numbers of *E. coli*, anywhere from 0-7 of the AIEC/non-AIEC strains in addition to FMT2. In the ileum, the community composition of the samples that received clinical *E. coli* isolates changed compared to the samples that received the murine *E. coli* strain NC101 + FMT2. However, there was overlap between cohorts that received 0, 1, 3, or 7 clinical strains of *E. coli* +

FMT2. This indicated that the microbiota in the SI was impacted less by the clinical isolates than was previously seen in the LI (data not shown). Taxonomic diversity was then investigated (Fig. 2C). The Shannon diversity index revealed there was greater alpha diversity in the fecal content of the mice compared to the ileum. This is expected because there is a larger number of bacteria present in the LI microbiota compared to the SI. Overall microbial increases as you move through the GIT. However, there was no difference in diversity when 0, 1, 3, or 7 clinical strains of *E. coli* were present with FMT2.

At this point it is important to note that the FMT2 and FMT1, albeit quite similar, produced slightly different results in their respective treatments. The community compositions were similar enough that we hypothesized FMT2 should have produced similar results. However, the change in results between treatments that received FMT2 compared to FMT1 could be due to small changes in the microbial population, alteration in function of the community, or production of different metabolites. Although microbiota composition was similar overall, the minor changes in the community taxonomy and possible difference in small molecule production can impact the immune response. Thus, the relative mRNA abundance of several cytokines was investigated.

Analysis of cytokine expression supports that chronic inflammation was established within the murine model because of their increased relative abundance. Il1β, Tnfα, Il12β(p40), Il6, and Il17 are proinflammatory cytokines that have been previously found to be associated with inflammation in Crohn's Disease (CD) (23, 26). *Il1β* is a key immunoregulatory cytokine that amplifies the inflammatory response by activating an immune cell cascade, most likely the Il1β and Tnfα cascade(22). In chronic inflammation models, *Il1β* has also been shown to cause epithelial cell necrosis in the small intestine (SI)

(22). In addition, apoptosis of epithelial cells in the SI would increase the release of pro-inflammatory cytokines, thus, exacerbating inflammation. In addition, upregulation of *Il1* can stimulate anion secretion by epithelial cells through induction of prostaglandins (26). Epithelial cell necrosis and increased inflammation due to prostaglandin recruitment play a role in the production of Tnfα (26). It can work synergistically with Tnfα to stimulate proliferation of fibroblasts and intestinal smooth muscle cells to enhance production of Il1, Il6, and Tnfα in these cells (26). There was an increased relative abundance of *Il1β* expression suggesting that pro-inflammatory cascades were activated as evidenced by presence of the other investigated cytokines. Il1β plays a significant role in induction of the inflammatory response through various pathways and presence of this cytokine at the highest relative abundance indicates its upregulation (Figures 3 A, B, D). Furthermore, Il1b had the highest relative mRNA abundance in the *Il10*$^{-/-}$ + *E. coli* and *Il10*$^{-/-}$ + *E. coli* + FMT treatments (Fig. 4 D, F) thus, further supporting a pro-inflammatory cascade involving this cytokine was activated.

The cytokine *Tnfa* is well-characterized in its role as an inflammatory protein. As previously mentioned, it can stimulate secretion of prostaglandins alone or in concert with *Il1β*, contributing to inflammatory activity (22). In CD patients, one-way *Tnfa* can be produced by epithelial cells is when acted upon by the Il23/Il17 pathway (28). Tnfα tends to work synergistically with Il1β and Il17, as part of different pathways, to enhance inflammatory gene expression (28); for example, Il1 and Tnfα play a role in stimulating the pathway that produces the proinflammatory cytokines, Il6, Il17 and Tnfα, which were also measured (Figure 3 A, C, D) (22). Expression of *Tnfa* was detected, although, in a lower relative abundance (Fig. 3A; Fig. 4). However, in *Il10*$^{-/-}$ mice that received a FMT + *E.*

coli, Tnfa levels were elevated compared to other treatments (Fig. 4 D). Knowing that Tnfα commonly acts with other cytokines to enhance inflammation (24), the lower mRNA expression of this cytokine could indicate its role as a co-regulatory cytokine in the induction of inflammatory pathways.

Il6 is produced by activated macrophages and in inflamed tissues, macrophage reproducibility increases thus stimulating heightened production of this cytokine (22). It has been shown that Il1β and Tnfα work together to stimulate a pathway that produces Il6 (26). In addition, activity of Il6 is notably increased during times of active inflammation in IBD patients (22). This increased production of Il6 occurs when CD4+ T-cells differentiate into pathogenic Th17 cells (26); the increased production of Th17 cells leads to enhanced production of the pro-inflammatory cytokines Il6, Il17 and Il12 (26). Futhermore, Il6 is produced by macrophages in the inflammatory response. There was an elevated level of relative *Il6* mRNA expression detected in the jejunum of the mice (Figure 3D). However, the relative mRNA abundance of *Il6* was lower compared *Tnfa* and *Il1b* levels when looking across the treatments (Fig. 4 A-F). The increased abundance of *Il1b* and presence of *Tnfa*, support that the synthesis of *Il6* is occurring and further contributing to inflammation and dysbiosis (Fig. 3; Fig. 4).

The cytokine Il12 signals through a heterodimeric receptor complex composed of two subunits: Il12rβ1 and Il12 rβ2. Il12β(p40) plays a role in activation of antigen presenting cells (APCs) that prime Th1 cells for induction of T-cell proliferation (26). Th1-dependent T-cell differentiation leads to the production of both IFNγ and Tnfα (26). Based on the results, *Il12β(p40)* and *Tnfa* were both upregulated (Figure 1 E, A). In the normal immune response regulatory T cells (Treg's) help to suppress chronic inflammation in response to

commensal bacteria (26). However, patients with CD commonly have an abnormal immune response to their own commensal microbiota (26, 28). Differentiation of CD4+ T-cells into pathogenic T-helper type 17 cells (Th17) is enhanced by the Il12 cytokine family (28). Moreover, pathogenic Th17 cells increase production of Il17, another inflammatory cytokine, that is able to initiate a cascade of more pro-inflammatory cytokines such as Tnfα, IFNγ, and Il1β (28). Figure 3 E, 4 D, E, and F show that the Il12b(p40) subunit of *Il12* was expressed in the jejunum and relative abundance was increased in the presence of *E. coli*. In addition, IFNγ relative mRNA abundance was significantly higher upon introduction of FMT1 (Fig. 4 A).

Association of the proinflammatory cytokine Il17 with ileal and colonic CD has been investigated and increasingly well-characterized (28). In the adaptive immune response, macrophages, and dendritic cells (DCs) stimulate CD4+ naive T-cells to differentiate into Th17 cells. Il17 is the critical cytokine produced by Th17 cells, however, both IFNγ and Tnfα are also produced (23, 28). Th17 cells are common in the submucosa of CD patients (5), so, when Th17 cell proliferation is increased, so is the production of Il17 and induction of the pro-inflammatory cascade it activates (28). Furthermore, the presence of Il17 is crucial for inflammation to develop, as demonstrated by Zhang et al. (23, 28). Our findings showed there was an increased relative abundance of *Il17* found in the jejunum of the mice (Fig. 3 C), especially in for *Il10-/-* mice that received *E. coli* + FMTs (Fig. 4 D). This suggests that its presence was important for establishing the chronic inflammation seen in *Il10*$^{-/-}$ murine model. Altogether, these results indicate that there is increased inflammation in the jejunum.

Although the experiment was set up so that we could create the best possible conditions for a CD model, there are still many limitations that could have impacted the results. Kanamycin was used to select for the transformed *E. coli* however, addition of the kanamycin could have skewed the results. While using kanamycin for selection should have altered the microbiome in the same way across cohorts, this still could limit the study's findings seen in the PCoA plots. The murine model used also presents limitations because the mice are genetically altered to be *Il10* deficient and mature in a germ-free environment. While these controls do help create a physiological relevant condition, the genetically altered mice and introduction of a microbiome also impact the results of the study. Another limitation would be that the *E. coli* strains utilized were human-associated strains and this was conducted in a mouse model. Finally, the use of jejunal tissue rather than other regions of the GI tract could also have played a role in skewing the obtained results.

Conclusions and Future Directions

Our investigation showed that AIEC were able to colonize the SI and that chronic inflammation occurred. Both of which indicate that a CD model was established. In addition, the microbial composition shifts in the fecal content of *Il10*$^{-/-}$ mice, seen in Figures 1 and 2, indicate that dysbiosis was occurring in the fecal content. However, AIEC had less of an impact in the SI. The upregulation of cytokines witnessed in Figures 3 and 4 supports establishment of chronic inflammation and dysbiosis within the SI of the mice. While inflammation and dysbiosis did occur, the extent to which specific AIEC or non-AIEC strains were impacting this were unclear and need to be further investigated.

This research was conducted as a follow up to the proximal colon data obtained from previous data from the lab to better understand the impact AIEC has on dysbiosis and inflammation in CD pathogenesis. While this study was the first step towards investigating inflammatory cytokine expression in the jejunum, more research is needed to investigate what immunological cascades are activated and play a role in the inflammation we saw in the jejunal tissue. The SI microbiome as a whole is still poorly characterized, largely due to its location making it difficult to investigate *in vivo*. However, to create a more complete picture of the role these microbes play in CD pathogenesis, more research examining the inflammatory response at the jejunal tissue level would help add another piece to the puzzle. In future experiments, investigating the relative abundance of colony stimulating factor 2 (*csf2*), a glycoprotein which acts as a cytokine and can impact Treg's function, could shed some light on what other pathways are involved in the inflammatory response in CD. Again, this would allow for a more complete look at which cytokines, and their subunits, are more abundant in this model. To determine what pro-inflammatory cascades play a role in the inflammatory reaction seen in CD, inhibition of immune cells, like macrophages, or inflammatory cytokines, like Il17, Tnfα, or Il1, could help us better understand the immune response in the SI. Furthermore, use of ELISAs to quantify protein expression of these cytokines could provide more information rather than looking only at transcription. Flow cytometry could also be used to pinpoint which immune cells are producing the proinflammatory cytokines investigated.

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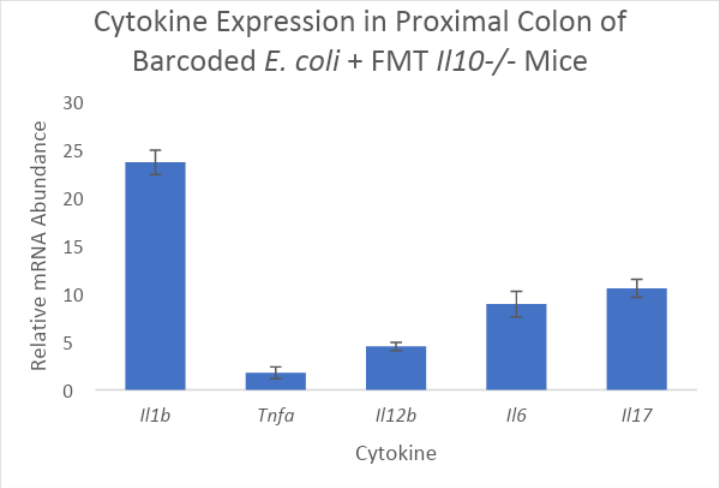
Supplemental Figures

A



Supplemental Figure 1. Relative mRNA abundance of *Tnfα, Il1b*, *Ifnγ*, *Il12b(p40)*, *Il6*, and *Il17* in the proximal colon (Bleich et al.).

Vita

Emma Metcalf was born in Wilmington, North Carolina and raised in Raleigh, North Carolina. She attended Appalachian State University for her Bachelor of Science in Biology in 2020 and her Master of Science in Biology in 2022.

While attaining her Masters' at Appalachian State University she was elected Secretary of the Biology Graduate Student Association, was a teaching assistant for Introductory Biology II lab and Molecular Biology lab, and conducted research in the Bleich Lab. In addition, she was obtained a grant from the Office of Student Research at Appalachian State University and presented at the Association for Microbiology Conference in 2022.

Emma has accepted a Visiting Professor position at Lees McRae College in Banner Elk, NC and aspires to attend medical school in the near future.