ELUCIDATING THE POTENTIAL ROLE OF ARYL HYDROCARBON RECEPTOR IN THE PATHOGENESIS OF *CAMPYLOBACTER JEJUNI*.

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Comparative Medicine and Integrative Biology—Doctor of Philosophy

2022

ABSTRACT

ELUCIDATING THE POTENTIAL ROLE OF ARYL HYDROCARBON RECEPTOR IN THE PATHOGENESIS OF *CAMPYLOBACTER JEJUNI*.

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Campylobacter jejuni is a leading cause of human foodborne gastroenteritis in the US, with an incidence rate of 13.6 diagnosed cases per 100,000 individuals. The most frequent cause of *C. jejuni* infection in the US is the consumption of chicken contaminated during processing. Macrolide antibiotics such as azithromycin and ciprofloxacin are the drug of choice to treat *C. jejuni* infection in human populations. However, the over-use of antibiotics has led to the emergence of antimicrobial-resistant *C. jejuni* strains and reduced treatment efficacy. The development of antimicrobial resistance traits in *C. jejuni* isolates has augmented the need to develop innovative strategies to treat drug-resistant *C. jejuni* infections in human and animal populations.

Members of the genus *Lactobacillus* are commonly used as probiotics, however the mechanisms by which they provide protective health effects remain elusive. In the first study, we described a novel mechanism by which *L. murinus* attenuates pro-inflammatory responses in the human intestinal epithelial cells. The results showed that *L. murinus* activates aryl hydrocarbon receptor (AHR) to decrease the secretion of IL-8 in response to exogenous stimulation by TNF-alpha in the human intestinal epithelial cells. Furthermore, activating the AHR with its defined ligand also reduced the secretion of IL-8 upon TNF-alpha stimulation. These results suggest that AHR can a novel target for

inflammatory bowel disease (IBD) treatment. Furthermore, these results suggest that *L. murinus* can be a novel probiotic for treating IBD.

In the 2nd study, we determined the effect of prophylactic inoculation of *L. muirnus* on the pathogenesis of *C. jejuni* in the BALB/c IL-$10^{-/-}$ mice. A total of 41 BALB/c IL-$10^{-/-}$ mice were used in this study. 11 mice were sham inoculated, 10 mice received only *L. murinus*, 10 mice received only *C. jejuni,* and 10 mice in the test group received both *L. murinus* and *C. jejuni* such that *L. murinus* was inoculated 32 days before *C. jejuni* infection. In addition, 30 days post-C. *jejuni* challenge mice were sacrificed and assessed for gut pathology. Fecal samples were also collected to access bacterial colonization levels in the gut through routine culture techniques and 16S sequence analysis. Both positive control group for *C. jejuni* and test groups mice developed severe colitis. 16S analysis of fecal DNA revealed that bacterial diversity in the test and positive control group for *C. jejuni* was significantly less ($P<0.001$) than in the *Lactobacillus* only and negative control group. These results suggest that prophylactic administration of *L. murinus* does not protect BALB/c IL-$10^{-/-}$ mice from developing disease following *C. jejuni* infection.

Overall, this dissertation contains identification of a novel mechanism of action of *L. murinus*. The results provide insights for the identification of novel targets to treat *C. jejuni* disease without using antibiotics. This dissertation provides a basis for the future studies to further dissect the role of the AHR in the pathogenesis of *C. jejuni*.

This thesis is dedicated to my grandmother (late) and my daughters, Khadija and Aima.

ACKNOWLEDGMENTS

I am grateful to my advisor Dr. Linda Mansfield for her support throughout my academic career. I am thankful to all my graduate committee members, including Dr. James Pestka, Dr. John LaPres and Dr. Norbert Kaminski. Each member provided me guidance throughout this study.

I am eternally grateful to Dr. Norbert Kaminski for allowing me to use his laboratory equipment to generate data for this project. I also extend my thanks to the members of the Kaminski Lab, including Robert Crawford, for his technical assistance.

I am obliged to Dr. Srinand Sreevatsan and Dr. Colleen Hegg for their consistent support throughout my graduate studies.

My sincere thanks also go to Dr. Julia Bell for her assistance in multiple aspects of this study. Furthermore, I am thankful to the graduate and undergraduate students of Mansfield's Lab for their support.

I am indebted to my wife and to my daughters for their love and support. Furthermore, I am eternally grateful to my father and mother for their love, support, and belief in me to make this work possible.

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Campylobacter species disease burden in animals

Campylobacter spp. are ubiquitous and colonize the gut of many animals and birds such as cattle, pigeons, water-fowls, and crows [1]. The *Campylobacter* spp. constitute a diverse group of bacteria, some of which cause severe diseases in humans and animals; others reside in the gut as commensals or lack clear association with overt disease manifestation [2]. A cross-sectional study described the prevalence of *Campylobacter* spp. in various animal host species. The prevalence in cattle, sheep, ducks, and pigeons ranged from 21.9% to 27.8% [3]. *Campylobacter* spp. causes disease in cattle, goats, and sheep. The two main *Campylobacter spp.* associated with disease in ruminants are *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* [4]. In sheep and goats, *C. fetus* subsp. *fetus* causes abortion, whereas *C. fetus* subsp. *venerealis* causes infertility and embryonic mortality in cattle [4]. Chickens were found to have the highest prevalence (41%) of *Campylobacter* spp. [3]. In chickens *Campylobacter* spp. (such as *C. jejuni*) are thought to reside as a commensal [5]; however, recent literature has challenged this paradigm by reporting that in susceptible breeds of chicken *C. jejuni* damaged gut mucosa and induced a prolonged inflammatory response in the gut [6]. Moreover, *C. jejuni* infection caused diarrhea in the susceptible breeds of chicken [6].

Campylobacter jejuni in chickens: a commensal or a pathogen?

Early studies reported that *C. jejuni* infection does not lead to the development of clinical disease in chickens [7, 8]. This observation led scientists to hypothesize that *C. jejuni* resides as a commensal in the chicken gut [9]. When chickens were experimentally infected with clinical *C. jejuni* strains isolated from patients who developed enteritis, none

of the infected chickens developed enteritis, nor did they manifest any clinical signs of disease [8]. Similarly, in another study, 9-day old chickens were inoculated with *C. jejuni* by crop gavage. No clinical signs of illness or gross pathological lesions were found in any of the infected groups at necropsy despite successful colonization of *C. jejuni* in the chicken gut [10]. Since then, various studies have reported the ability of *C. jejuni* to colonize the chicken gut, but the persistent lack of clinical disease manifestation in chickens, i.e., enteritis and bloody diarrhea, led to development of the paradigm that *C. jejuni* acts as a commensal in chickens [7, 11].

Recent studies have challenged this long-standing paradigm of *C. jejuni* commensalism in the chicken. *Suzanne* et al. [6] infected different commercial breeds of chicken having varying abilities to gain weight. It was found that breeds having a faster weight gain ability developed diarrhea and inflammatory response when challenged with *C. jejuni*. Whereas the breed having slower weight gain ability had no clinical signs of the disease and developed a less pronounced inflammatory response as compared to the fast-growing breed. Furthermore, they found that the slow-growing breed had a significantly higher induction of IL-10 (50-fold higher) as compared to the fast-growing breed.

Xi et al. [12] also reported that the genetics of the chicken plays an important role in determining the resistance and susceptibility of different chicken breeds to *C. jejuni* infection. Day-old broilers from two different parental lines (Line A and Line B) were infected with *C. jejuni*. On days 7 and 14 post-infection, the cecal contents from both lines

were cultured for *C. jejuni*. It was found that Line A (resistant breed) had significantly fewer *C. jejuni* colony forming units in their ceca as compared to Line B (susceptible breed). Also, gene expression profiling of Line A (resistant breed) and Line B (susceptible breed) indicated that genes responsible for host defense responses were upregulated in Line A as compared to Line B. Interestingly, circadian rhythm genes were also significantly upregulated in Line A (resistant breed) birds as compared to Line B (susceptible breed) birds [13]. These results indicate that *C. jejuni* does not merely reside as a commensal in the chicken gut, rather is fully capable of causing enteritis in a susceptible host.

Chickens: a leading cause of *C. jejuni* transmission to humans

Over the past decades, advances in genetics and nutrition have enabled large-scale poultry production. Per capita availability of chicken meat has significantly increased with the advent of large-scale poultry production [14]. Unfortunately, some pathogens, such as *C. jejuni,* can also spread more easily between birds in large-scale poultry production because more birds are housed in these intensive poultry farming units [15]. Furthermore, during processing, chicken meat can get contaminated with *C. jejuni* present in intestinal contents [16]. Scalding, evisceration, washing, and chilling are the major cross-contamination points during chicken slaughter processing [17]. Chicken contaminated during processing is the principal source of *C. jejuni* transmission to humans [18, 19]. However, *C. jejuni* can also infect humans through contaminated water, milk, other dairy products and by direct contact with infected animals [5].

C. jejuni: a human pathogen

C. jejuni is a leading cause of foodborne gastroenteritis in the US, with an incidence rate of 13.6 diagnosed cases per 100,000 individuals [20]. Because many sporadic human infections go undiagnosed, it is predicted that the actual incidence is much higher, with estimates of approximately 1.5 million cases of campylobacteriosis in the US per annum [20]. *C. jejuni* is a broad host range pathogen residing as a commensal in the gastrointestinal tract of many agricultural animals [5]. Thus, human infections most commonly occur from the consumption of contaminated water or animal products [21, 22]. The most frequent cause of *Campylobacter* infection in the US is the consumption of chicken contaminated during processing [21]. Campylobacteriosis (a disease in humans) is characterized by mild to severe bloody diarrhea, abdominal pain, fever, and severe intestinal inflammation that lasts from 7-10 days followed by resolution [5].

C. jejuni infection can also lead to the autoimmune disease Guillain- Barré syndrome (GBS), a paralytic illness resulting from an immune system attack on the peripheral nervous system, which results in flaccid paralysis [23]. GBS has been documented to occur due to molecular mimicry between oligosaccharide motifs on the outer surface of *C. jejuni* and gangliosides on the surface of peripheral nerves [24]. Antibodies especially of the IgG1 subclass are generated against the lipooligosaccharides of certain *C. jejuni* strains that also bind to GM1 and GD1a gangliosides on peripheral nerves which precipitates complement-mediated damage [25, 26]. Other autoimmune sequelae to *C. jejuni* infection include reactive arthritis, and Reiter's syndrome [27, 28].

C. jejuni pathogenesis in humans

In the intestinal epithelial tract, the mucus layer is considered the first line of defense against enteric pathogens. The average thickness of mucus in the gut is 700 μm and it consists of mucin glycoproteins, lysozymes, defensins, and antibodies that function in the clearance of enteric pathogens [29, 30]. However, certain gut pathogens and *C. jejuni,* in particular, have adapted sophisticated mechanisms to breach the protective mucus barrier and establish colonization. For instance, *C. jejuni* has shown chemotactic motility towards mucins [31]. Once it encounters this layer, the fluid nature of the mucus aids in *C. jejuni* motility towards intestinal epithelial cells powered by the flagella [32, 33]. All of these factors facilitate *C. jejuni* colonization in the mucus of the intestinal epithelial tract where it can persist for long periods of time.

After establishing itself in the mucus, *C. jejuni* then invades the intestinal epithelial cells (IECs). IECs serve as a barrier between the mucus layer and intestinal immune cell populations residing in the lamina propria [34]. This barrier property of the intestinal epithelial cells is mainly due to the presence of junctional complexes between the epithelial cells. These junctional complexes consist of specialized intercellular structures, formed by tight junctions, adherens junctions, and desmosomes. Tight junctions between cells consist of proteins including claudins, occludin, and junctional adhesion molecules, whereas adherens junctions mainly consist of cadherin molecules [35]. Thus, tight junctions between neighboring intestinal epithelial cells are formed by the assembly of multiple proteins that controls the permeability of ions, nutrients and water [36].

“Leaky gut” syndrome is often considered a hallmark of human campylobacteriosis, facilitating the translocation of *C. jejuni* from inflamed intestines to deeper body tissues [37]. *C. jejuni* has multiple molecular mechanisms to breach the intestinal tight junction barrier. Harrer *et al*. [38] reported that *C. jejuni* secretes a serine protease (*htr*A) that cleaves occludin, thereby facilitating pathogen translocation across the intestinal epithelial barrier. The role of *htrA* in translocation across the intestinal epithelial barrier was further confirmed by using the *htrA* knockout mutant (Δ*htr*A). Δ*htr*A showed a significantly reduced ability to cross the tight junction barrier *in-vitro*. These results indicate that *C. jejuni* possesses intricate mechanisms to breach tight junctions in order to invade deeper body tissues [38].

Once *C. jejuni* reaches the basolateral layer of the IEC’s, it expresses the adhesin CadF to bind to the fibronectin complex to invade IEC’s [39]. Thereafter, *C. jejuni* expresses a type three secretion system (T3SS) virulence protein CiaD that is required for the invasion of host cells [40]. IEC’s respond to *C. jejuni* invasion by producing proinflammatory cytokines such as IL-8, also known as a “neutrophil chemotactic factor” as it specifically attracts neutrophils [41-43]. Neutrophils kill bacterial pathogens by phagocytosis, secretion of anti-bacterial proteins, or by releasing NETs (Neutrophil Extracellular Traps) [44]. S. Callahan *et al.* [45] recently showed that *C. jejuni* was internalized by neutrophils derived from healthy donors. Moreover, neutrophil-derived proteins such as myeloperoxidase, neutrophil elastase, and lipocalin-2 negatively impacted *C. jejuni* growth *in-vitro*. Furthermore, ferrets infected with *C. jejuni* showed increased neutrophil activation levels in the colon that directly correlated with intestinal

inflammation and pathology [45]. These results indicate that where neutrophils function to kill *C. jejuni,* their increased activation also results in intestinal tissue damage [45].

C. jejuni also invades the antigen-presenting cells residing in lamina propria, such as macrophages and dendritic cells [46]. It was shown that *C. jejuni* can replicate in human mononuclear cells and induce cytokine secretion including IL-1β and tumor necrosis factor-alpha [47, 48]. *C. jejuni* can also induce apoptosis in these mononuclear cells by secreting cytolethal distending toxin (CDT) [49]. CDT has DNAase activity and is one of the major virulence factors of *C. jejuni* [50, 51]. The inflammatory reaction initiated in response to *C. jejuni* infection is generally considered host protective and helps clear the infection. However, excessive inflammation that is not downregulated can be detrimental to the host [52-54]. Therefore, mice deficient in anti-inflammatory cytokines such as IL-10 are a robust *C. jejuni* disease model and display clinical features of human campylobacteriosis upon infection [55]. These findings indicate that excessive host inflammation contributes to the pathogenesis of *C. jejuni*-mediated colitis.

The current treatment regimen for *C. jejuni* and its challenges

Treatment options for *Campylobacter* infection primarily include the use of antibiotics such as azithromycin and ciprofloxacin. However, the over-use of antibiotics has led to the emergence of antimicrobial-resistant *C. jejuni* strains and has reduced treatment efficacy [56, 57]. In 2015, the National Antimicrobial Resistance Monitoring System (NARMS) reported that 47% of *C. jejuni* isolates were resistant to tetracycline, whereas 25% of the isolates were resistant to ciprofloxacin [58]. More than 35,000 people

die each year in the United States from antibiotic-resistant infections [59]. The Centers for Disease Control and Prevention (CDC) has categorized drug-resistant *C. jejuni* as a serious public health threat [57, 59]. The development of antimicrobial resistance traits in *C. jejuni* isolates has augmented the need to develop innovative strategies to treat drug-resistant *C. jejuni* infections in human populations.

Alternatives to antibiotics to reduce *C. jejuni* prevalence

Amid increasing concerns about antibiotic-resistant *C. jejuni* isolates, there is a dire need to develop alternatives to antibiotics to reduce the *C. jejuni* bacterial burden in all of its hosts. Probiotics are commonly used in poultry husbandry and human medicine to maintain gut health [60]. A large body of work has been performed in the last two decades to validate probiotics as a means to reduce *C. jejuni* prevalence in chickens. *Lactobacillus* spp. (LAB) are the most commonly used commensal bacterial genera to be used as probiotics in poultry and humans [61-63].

Several studies have confirmed that LAB can significantly decrease the growth of *C. jejuni in-vitro*. Chaveerach *et al*. [64] described that the growth of *C. jejuni* was negatively affected by the chicken LAB isolate (P93) *in-vitro*. It was found that the production of volatile fatty acids (VFA) from the P93 LAB strain was a major mechanism in inhibiting *C. jejuni* growth *in-vitro*. Several other studies have confirmed the role of VFA in inhibiting the growth of *C. jejuni in-vitro* [65-67]. Other mechanisms by which LAB spp. can inhibit the growth of *C. jejuni in-vitro*, includes the production of hydrogen peroxide [65] and bacteriocins [68].

LAB strains have also been shown to attenuate the ability of *C. jejuni* to adhere to or invade human intestinal epithelial cells cultured *in-vitro*. In a recent study by Taha-Abdelaziz *et al*., [69] the LAB strains (*L. salivarius*, *L. johnsonii*, *L. reuteri*, *L. crispatus*, and *L. gasseri*) alone or in combination reduced the ability of *C. jejuni* to adhere to and invade Caco-2 cells cultured as a confluent monolayer. Moreover, co-incubation of LAB strains alone (except *L. reuteri*) or in combination reduced the expression of *C. jejuni* virulence genes, including motility associated genes (*flaA, flaB*, and *flhA*) and the gene responsible for invasion (*CiaB*). These findings indicate that LAB strains can attenuate *C. jejuni* virulence properties. Similarly, Wang *et al*. [70] screened seventy-eight LAB strains for anti-*Campylobacter* activity. They found that *L. casei* ZL4 was able to reduce the adhesion and invasion of *C. jejuni* to HT-29 cells. The high concentration of lactic and acetic acid produced by *L. casei* ZL4 in these adherence invasion assays was responsible for inhibiting *C. jejuni* growth *in-vitro.* These results demonstrate that LAB strains possess multiple mechanisms to affect *C. jejuni* growth, adherence, and invasion capabilities *in-vitro*.

In-vivo effects of LAB strains on reducing *Campylobacter spp.* colonization in the gut has also been reported [71]. Smialek *et al.,* [72] reported that the addition of selected LAB strains (*L. lactis*, *L. casei*, *L. plantarum*) into chicken feed resulted in decreased *Campylobacter spp.* colonization in the gut. Furthermore, less *Campylobacter spp.* carcass contamination was observed in the experimental group given probiotic (LAB strains) as compared to the sham-fed control group. Saint-Cyr [61] also reported similar findings when 10^7 CFU of *L. salivarius* SMXD51 were given by oral gavage to broiler

chickens followed by oral challenge with *C. jejuni*. At 35 days of age, the group that was given *L. salivarius* SMXD51 had a 2.81 log reduced load of *C. jejuni* in the cecal contents as compared to the control group. Furthermore, the 16S sequence analysis of the chicken gut microbiota revealed that certain bacterial genera were more abundant in the *L. salivarius* treated group as compared to the control group. A higher abundance of *Anaerotruncus*, *Escherichia* and *Flavonifractor* species were found in the *L. salivarius* treated group as compared to the control group [61].

In the developed world, chicken contaminated during processing is the principal source of *C. jejuni* infection in humans [5]. Decreasing the bacterial load of *Campylobacter spp.* in the chicken can significantly reduce human exposure to *C. jejuni*. Rosenquist *et al.* [73] developed a mathematical risk model to assess the human exposure to *C. jejuni* from processed chicken meat. They found that the incidence of human campylobacteriosis could be reduced 30 times if one obtains a 2 -log reduction of *C. jejuni* contamination in the chicken carcass. The results of this quantitative risk assessment model are important for effective implementation of prevention methods because probiotic supplementation to chickens has been shown to result in a significant reduction in the *C. jejuni* bacterial load in the chicken gut and carcass contamination [61, 71, 72].

Probiotics possess multiple mechanisms that function to reduce *C. jejuni* invasion. For example, LAB strains (*L. salivarius*, *L. johnsonii*, *L. reuteri*, *L. crispatus*, and *L. gasseri*) upregulated the phagocytic activity of chicken macrophage-like cells (MQ-

NCSU) [69]. LAB strains were also able to upregulate the expression of proinflammatory cytokines (IFN-gamma, IL-1beta, and IL-20p40) in chicken macrophages. Moreover, a combination of *L. salivarius, L. reuteri,* and *L. crispatus* was able to upregulate the expression of co-stimulatory molecules CD40, CD80, and CD86 in chicken macrophages [69]. However, the immunomodulatory mechanisms by which probiotics manifest anti-*Campylobacter* mechanisms are not yet completely understood.

Recently Lamas *et al.* [74] reported a novel mechanism of action for *Lactobacillus*-based probiotics. They reported that three LAB strains (*L. murinus* CNCM I-5020, *L. reuteri* CNCM I-5022, and *L. taiwanensis* CNCM I-5019) were able to metabolize tryptophan into ligands that activated the aryl hydrocarbon receptor (AHR) to protect mice from colitis. These results suggested a novel mechanism of action of such probiotics in maintaining gut homeostasis by activating AHR. AHR was initially defined for its role in xenobiotic metabolism [75], however, recent literature has identified the emerging immuno-modulatory roles of AHR [76]. Identification of AHR in certain immune cell types has posed the possibility of AHR as a drug target for novel therapeutics against bacterial pathogens [77-79].

<u>The classical role of AHR in xenobiotic metabolism</u>

The AHR was first identified due to its binding with toxicant 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) [75]. Dioxins are a group of chemically related compounds that are environmental pollutants. Dioxins are generated as by-products in the industrial manufacturing of various products such as chlorophenols, herbicides, and

pesticides [80]. Apart from dioxin release as industrial by-products, occasional industrial accidents also lead to environmental contamination with dioxins which can cause lethal toxicity in humans and animals. One such incident occurred in Seveso, Italy, where due to an industrial accident, large amounts of TCDD was released into the environment, and thousands of people were exposed to the toxic levels [81]. Epidemiological studies confirmed that this cohort of people living in the TCDD contaminated zones were more susceptible to cancer, diabetes, and chronic obstructive pulmonary disease (COPD) as compared to people living in TCDD free zones [82].

In mammalian cells, TCDD specifically binds to the AHR, which is a ligand-activated transcription factor. In the cytoplasm, the AHR is retained in its inactive form as a complex with heat-shock protein 90 (hsp90) and AHR-interacting protein (AIP/XAP) [83]. Upon binding to its ligand, the AHR translocate to the nucleus and dimerizes with the AHR nuclear translocator (ARNT) and regulates the transcription of a battery of genes, including cytochrome P450 superfamily members, i.e., CYP1A1, CYP1A2, and CYP1B1 [84]. Cytochrome P450 (CYPs) are enzymes that can metabolize xenobiotics by oxidizing them into harmless and excretable metabolites. The CYP enzymes are the most abundant of all the xenobiotic-metabolizing enzymes [85]. Therefore, CYP1A1 is considered a biomarker for AHR activation [86].

The emerging role of AHR in the gut immune system

With the discovery of AHR expression in the cells of the immune system, this receptor has received significant attention from immunologists regarding its potential as

a therapeutic target for immunomodulation [76]. AHR is heavily expressed at mucosal barrier sites such as lungs, skin, and the gastrointestinal (GI) tract. In the GI tract, AHR is expressed by certain innate and adaptive immune cell types. In innate immune cells, AHR is highly expressed by dendritic cells [87]. Among innate lymphoid cells (ILCs), AHR is expressed by ILC2 [88] and ILC3 [89]. AHR is also expressed by cells of the adaptive immune system, such as T and B cells. AHR is highly expressed by T-helper 17 cells (Th17) [90] and marginally expressed by T-regulatory cells [91]. Furthermore, AHR is also expressed by innate TCR$\gamma\delta$ T-cells [76]. However, AHR is not expressed by naïve T-cells, Th1 and Th2 cells [92]. It appears that all B cells express AHR, but certain types of B cells, such as marginal B cells and B1 B cell subsets, have higher AHR expression. The presence of AHR in the vast majority of gut resident immune cells suggest that it may function in a regulatory role and makes it an ideal target for immunomodulation against gut pathogens.

Sources of AHR ligands in the gut

Aside from synthetic ligands such as TCDD and FICZ, many naturally occurring ligands in the gut also activate AHR. The two most important sources for the production of AHR ligands in the gut arise from diet and the members of the gut microbiome [93]. In the diet, cruciferous vegetables contain high amounts of AHR ligands. Broccoli is a rich source of glucobrassicin, which is cleaved into indole-3-carbinol (I3C) by digestive enzymes. It has been found that I3C activates AHR [94]. Flavonoids are another group of compounds that can interact with AHR. Flavonoids are found in fruits and tea. Most of the flavonoids are AHR antagonists, but some of them can activate AHR, such as diosmin,

tamarixetin, tangeritin, and quercetin [95-97]. Curcumin which is found in turmeric can also activate AHR [98]. Furthermore, several dietary plant compounds, such as carotenoids and tryptophan, can induce AHR activation [99].

Gut microbiota is another important source for the production of AHR ligands. This is because some micro-organisms can produce metabolites that can act as AHR ligands [93]. Members of the gut microbiota documented to produce AHR ligands include *Enterococcus faecalis* [100], *Bacteroides spp.* [101] and *Citrobacter spp.* [102]. However, certain probiotic *Lactobacillus* spp. can also produce AHR ligands, such as *L. reuteri* [74, 103], *L. murinus* [74], *L. taiwanensis* [74] and *L. bulgaricus* OLL1181 [104]. Zelante *et al.* [103] described that *L. reuteri* was able to produce indole-3-aldehyde (I3A) when mice were given an unrestricted supply of tryptophan in the diet. I3A was produced as a by-product of the tryptophan-indole pathway. However, *L. johnsonii* was not able to metabolize tryptophan to I3A under carbohydrate starvation conditions. These results indicate that not all members of the genus *Lactobacillus* are able to produce AHR ligands. In another study, supplementation of *L. murinus*, *L. reuteri,* and *L. taiwanensis* were able to protect mice from developing colitis by the activation of AHR [74]. These results suggest that activation of AHR by probiotics can be a potential mechanism to protect against intestinal inflammatory insults.

AHR promotes host resistance against bacterial pathogens

The role of AHR in promoting host resistance against several bacterial pathogens has been reported. Vorderstrasse *et al.* [77] described the TCDD (potent AHR agonist)

induced protection of C57BL/6 mice against lethal *Streptococcus pneumoniae* challenge. Mice were given TCDD or vehicle one day prior to the *S. pneumoniae* challenge. Only 35% of the vehicle-treated mice survived, whereas TCDD treatment increased mouse survival rate to 75%. Furthermore, TCDD treatment significantly reduced the *S. pneumoniae* bacterial count in the lungs. It was found that the protective effect of AHR activation was not associated with increased inflammatory response in the lungs as proinflammatory cytokines such as IFN-gamma, TNF-alpha, and IL-1β were significantly reduced in the TCDD treated group as compared to the control group. However, the mechanistic basis for AHR-induced protection against a lethal *S. pneumoniae* challenge remained elusive.

The mechanistic basis for AHR-induced protection against bacterial infections [89, 105] began to be revealed when cytokine production was compared between immune cells (isolated from lamina propria) of $AHR^{-/-}$ and $AHR^{+/+}$ mice. In $AHR^{+/+}$ mice, the ILC3 produced more IL-22 as compared to ILC3 isolated from $AHR^{-/-}$ [89]. These results indicated that AHR plays an important role in intestinal IL-22 expression. Also, the antimicrobial peptide RegIIIγ produced by mammalian cells was significantly reduced in $AHR^{-/-}$ mice compared to AHR sufficient mice. There was a 100% survival rate in $AHR^{+/+}$ mice (n=6) when they were given a challenge infection with *Citrobacter rodentium*. Whereas all of the $AHR^{-/-}$ mice (n=4), when challenged with *C. rodentium,* succumbed to death by ten days post-infection. However, ectopic expression of IL-22 in $AHR^{-/-}$ mice increased mouse survival rate to 60% upon challenge with *C. rodentium*. A higher *C. rodentium* bacterial count was present in the feces of $AHR^{-/-}$ mice as compared to $AHR^{+/+}$

mice. Furthermore, a significantly reduced *C. rodentium* bacterial count was detected in $AHR^{-/-}$ mice having ectopic expression of IL-22 [89]. These results indicate that AHR is essential for protection against a gastrointestinal *C. rodentium* infection. Furthermore, AHR modulates ILC3 in the gut to increase the production of IL-22 that protected mice from lethal *C. rodentium* infection.

Activation of AHR also negatively affects the pathogenesis of *Listeria monocytogenes*. *L. monocytogenes* is a gram-positive foodborne pathogen that causes gastroenteritis. The most susceptible population to *L. monocytogenes* infection include newborns, adults over the age of 65, and people with a weakened immune system [106]. Homozygous $AHR^{-/-}$mice on C57BL/6J background were more susceptible to *L. monocytogenes* than heterozygous $AHR^{+/-}$ mice. Upon infection with *L. monocytogenes*, $AHR^{-/-}$ mice had a higher bacterial load of this bacterium in the spleen and liver as compared to $AHR^{+/-}$. However, proinflammatory cytokine production in response to *L. monocytogenes* infection was not affected in $AHR^{-/-}$ mice as serum levels of MCP-1, IFN-gamma, and TNF-alpha were comparable between *L. monocytogenes* infected $AHR^{-/-}$ and $AHR^{+/-}$ mice. These results demonstrate that AHR activation is required for host resistance against *L. monocytogenes* infection, which cannot be mediated by cytokine production alone [107].

In another study, Kimura *et al.* [78] also examined effects of AHR activation on the *L. monocytogenes* pathogenesis in mice. C57BL/6 WT and C57BL/6 $AHR^{-/-}$ were infected with *L. monocytogenes* intra-peritoneally. $AHR^{-/-}$ mice succumbed to death at five days

post-infection, whereas no significant decrease in survival rate was observed in WT mice infected with *L. monocytogenes*. A significantly higher *L. monocytogenes* bacterial load was found in the spleen and liver of $AHR^{-/-}$ mice as compared to the WT mice. Furthermore, AHR activation promoted the survival of macrophages when infected with *L. monocytogenes*. AHR activation induced the expression of an anti-apoptotic factor in macrophages, thereby preventing macrophage cell death. AHR activation also promoted the production of reactive oxygen species (ROS) in these macrophages, which resulted in *L. monocytogenes* clearance from the cells [78]. These results shed light on possible mechanisms by which AHR enhances host resistance against bacterial pathogens.

The effect of AHR activation against *Clostridium difficile* has also been reported [108]. Two groups of C57BL/6 mice were either given regular chow or custom chow having 1000 ppm of I3C. I3C is a dietary AHR agonist which is naturally found in cruciferous vegetables [93]. 66% of the mice survived *C. difficile* infection, which were given custom chow having I3C. In contrast, only 20% of the mice survived *C. difficile* infection when given regular chow. Mice given I3C in the diet had an increased number of $FoxP3^{+}$ $CD4^{+}$ T-regulatory cells, ILC3, and neutrophils in the cecal lamina propria as compared to the mice on regular chow. Furthermore, mice fed I3C in the diet also had a significantly reduced translocation of *C. difficile* to the spleen and lungs as compared to the controls. These results indicate that activation of AHR through dietary components can be a novel therapy for the amelioration of *C. difficile* disease that is often resistant to all available antibiotics.

Does AHR plays a role in promoting host resistance against *C. jejuni?*

There is a critical knowledge gap in understanding of the direct effects of AHR activation during *C. jejuni* mediated disease. Elucidating the role of AHR activation in *C. jejuni* pathogenesis may lay the foundations for enhancing the host resistance mechanisms to ameliorate *C. jejuni* disease without the use of antibiotics. It also holds potential for decreasing the significant tissue damage that can accompany infection with this foodborne pathogen. If AHR activation plays an important role in limiting *C. jejuni* colonization it may also be important in blocking the initiation of the autoimmune consequences of this pathogen.

Concluding remarks

With the advent of antimicrobial-resistant traits in bacterial pathogens such as *C. jejuni* [59], there is a dire need to develop alternatives to antibiotics. Five decades after the identification of AHR, its important immunological roles have started to emerge. Given the established role of AHR against certain human bacterial pathogens such as *S. pneumoniae* [77]*, L. monocytogenes* [107] and *C. difficile* [108], it is plausible to determine the effect of AHR activation on other human bacterial pathogens such as *C. jejuni*. AHR can be a novel drug target to treat or ameliorate human bacterial diseases (such as campylobacteriosis) without the use of antibiotics.

The probability of AHR as a therapeutic target against *C. jejuni* prompts new research questions, such as 1) What will be the effect of AHR activation on *C. jejuni* pathogenesis? 2) How does probiotic supplement capable of AHR activation affect *C.*

jejuni disease? Answers to these questions may guide future research efforts to discover novel AHR targeted therapeutics against *C. jejuni* and disease it causes. To know the answers to the afore-mentioned questions, this study was conducted with the following aims.

Specific Aim 1

To determine the effect of AHR activation on inflammation and intestinal epithelial barrier integrity in an *in-vitro* model of colitis.

Hypothesis

Activation of AHR by probiotic or defined AHR ligands will attenuate pro-inflammatory responses and epithelial barrier damage in a cell model of colitis.

Specific Aim 2

To determine the effect of probiotic supplementation on *C. jejuni* disease in a mouse model.

Hypothesis

Inoculation of *L. murinus* having AHR activation potential will attenuate *C. jejuni* induced colitis in IL-$10^{-/-}$ mice.

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CHAPTER 2: *LACTOBACILLUS MURINUS* ACTIVATES THE ARYL HYDROCARBON RECEPTOR TO ATTENUATE TNF-ALPHA-INDUCED PRO-INFLAMMATORY RESPONSES IN A HUMAN INTESTINAL EPITHELIAL CELL MODEL.

Abstract

Background

Anti-tumor necrosis factor-alpha (anti-TNF-α) therapy is an established treatment modality for inflammatory bowel disease (IBD). Yet up to 30% of patients do not respond to anti-TNF-α therapy (primary non-responders), and almost 50% of responders lose clinical efficacy over time (secondary non-responders). A potential alternative to anti-TNF-α therapy is the use of novel probiotics capable of attenuating the damaging effects of TNF-α on the intestinal epithelium. We hypothesized that *Lactobacillus murinus* attenuates TNF-α induced pro-inflammatory responses in human intestinal epithelial cell model of colitis *in vitro* by activating a novel transcription factor, aryl hydrocarbon receptor (AHR).

Methods

We used Caco-2 cells grown on Transwell inserts to model the human intestinal epithelial barrier. To develop an *in vitro* model of colitis, Caco-2 monolayers were treated with increasing concentrations of TNF-α. Caco-2 monolayers were also treated with *L. murinus* or defined ligands (exogenous or endogenous) to determine the activation of AHR in Caco-2 cells. Next, we pre-treated the Caco-2 cells with either *L. murinus* or defined AHR ligands for 12 hours. After pre-treatment, Caco-2 monolayers were then stimulated with TNF-α for 24 hours. Finally, IL-8 protein and transepithelial electrical resistance (TEER) were quantified as endpoints to determine the pre-treatment efficacy.

Results

TNF-α stimulation decreased TEER and induced the secretion of IL-8 in a concentration-dependent manner. AHR was significantly activated in monolayers treated with *L. murinus* or defined AHR ligands. Furthermore, pre-treating the monolayers with *L. murinus* or with endogenous AHR ligand attenuated the TNF-α induced pro-inflammatory response and decreased epithelial barrier disruption.

Conclusions

These results indicate that TNF-α induced damage to the gut wall can be attenuated using novel probiotics such as *L. murinus*. These data suggest that AHR could be a novel drug target for treating IBD.

Keywords: IBD, probiotics, inflammation.

Introduction

Inflammatory bowel disease (IBD) is an umbrella term used to describe the chronic inflammatory disorders of the digestive tract [1]. The two major forms of IBD are Crohn's Disease (CD) and Ulcerative Colitis (UC) [2]. It is estimated that 1 in 209 adults in the US currently suffers from some form of IBD. There is a high incidence of IBD as 70,000 new patients are diagnosed with IBD each year in the US [3]. CD causes inflammation in the whole or any part of the gastrointestinal (GI) tract whereas, UC causes inflammation in the colon. Furthermore, CD and UC differ in their signs and symptoms; as symptoms such as pain in the lower right abdomen and thickening of the bowel wall are mainly associated

with CD whereas, symptoms such as pain in the lower left abdomen, diarrhea, weight loss, and rectal bleeding are primarily associated with UC [4]. Furthermore, certain gut bacterial pathogens such as *Campylobacter jejuni* have been associated with initiation of the pathogenesis of IBD [5, 6].

Current treatment options for IBD include the use of aminosalicylates, immunosuppressants, and monoclonal antibodies [7]. The aminosalicylates maintain remission in IBD by inhibiting the upregulation of leukocyte adhesion molecule; thereby, preventing the recruitment of leukocytes in the inflamed bowel [8]. The use of monoclonal antibodies has revolutionized IBD treatment. Infliximab was the first FDA-approved monoclonal antibody against TNF-α for the treatment of IBD [9]. Infliximab targets TNF-α which is one of the key cytokines that is upregulated in IBD [10]. At present, anti-TNFα therapy is an established treatment modality for IBD. Yet up to 30% of patients do not respond to anti-TNF-α therapy (primary non-responders), and almost 50% of responders lose clinical efficacy over time (secondary non-responders).

Moreover, numerous safety concerns, including increased risk for opportunistic infections, are associated with long-term use of anti-TNF-α agents [11]. Additionally, many of these IBD medications have side effects such as headache, nausea, vomiting and diarrhea [4]. Increased incidence of these medicinal side-effects reduces patient compliance and thus treatment efficacy. Side effects and the safety concerns of current IBD therapy have driven the need to develop innovative strategies to treat IBD.

Many factors play a role in the onset of IBD such as genetic predisposition, environmental factors and perturbations in the intestinal microflora [12]. For instance, mutations in the NOD2 gene are associated with increased susceptibility to CD [13]. Previous studies have reported an association between dysbiosis of the gut microbiome and IBD. *Manichanh* et al. [14] reported a decreased diversity of the bacterial phylum *Firmicutes* in the stool samples of patients suffering from CD. *Ott* et al. [15] analyzed the mucosa-associated colonic microflora of patients suffering from active IBD. A reduced bacterial diversity was found in the IBD patients as compared to healthy controls. All of these results suggest a role for the gut microbiota in the development of IBD.

Gut microbiota dysbiosis and disruption in mucosal immunity have been described as triggers for IBD [16]. Many scientists have suggested employing probiotics to restore the gut microbial balance and attenuate the excessive inflammatory response at the gut epithelial surface as a novel approach to treating IBD. The clinical efficacy of specific probiotic preparations has been reported in patients with mild to moderately active UC [17]. However, the mechanisms by which probiotics induce protection against some forms of IBD are not yet completely understood.

In this study we report that *Lactobacillus murinus* (a putative probiotic strain) attenuates TNF-α induced inflammatory insults in human intestinal epithelial cells by activating a novel transcription factor. We hypothesized that *L. murinus* attenuates TNF-α induced pro-inflammatory responses in the human intestinal epithelial cells by activating the AHR. AHR is a ligand-activated transcription factor that belongs to the basic-helix-

loop-helix family of transcription factors [18]. Caco-2 cells were grown on Transwell inserts to model the human intestinal epithelial barrier. The monolayers were pre-treated with either *L. murinus* or with defined AHR ligands (exogenous or endogenous) or with sham treatments. After pre-treatment, the cells were then stimulated with TNF-α. The results indicate that TNF-α induced pro-inflammatory responses and damage to the gut wall can be attenuated using *L. murinus* or an endogenous AHR ligand. The results from this study identify a novel mechanism of action for probiotics to attenuate TNF-α induced damage to the gut wall.

Materials and methods

Chemicals and Reagents

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in dimethyl sulfoxide (DMSO) was a kind gift from Dr. Norbert Kaminski, Michigan State University. 6-formylindolo[3,2-b] carbazole (FICZ) was purchased from Sigma-Aldrich (St. Louis, MO), Catalog no. SML1489. DMSO (Catalog no. D8418) was also purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human TNF-α protein (Catalog # 210-TA-005) was purchased from R&D systems (Minneapolis, MN).

Bacterial Culture

L. murinus bacterial strain was grown on De Man, Rogosa and Sharpe (MRS) agar (Neogen®, Lansing, MI). The plates were incubated at 37°C under microaerophilic conditions (5% CO_2) for 48 hours. Bacterial colonies were then suspended in sterile PBS

(Millipore-Sigma, Catalog no. 806552) and OD_{600} was measured using spectrophotometry. Co-relation between OD_{600} reading and colony-forming units (CFU)/ml was made by plating serial dilutions on MRS agar and enumerating the colonies. Next, the *L. murinus* colonies were suspended in sterile PBS, adjusted to OD_{600} of ~ 1 (5×10^6 CFU/ml) and then added to cell culture medium containing the Caco-2 cells.

Cell culture

Caco-2 cells were purchased from ATCC (Gaithersburg, MD, USA) and were used between passages 6 to 10. The Caco-2 cells were cultured with Minimum Essential Medium Eagle (Millipore-Sigma, Catalog no. M2279), supplemented with 20% Fetal Bovine Serum (Rocky Mountain Biologics, Catalog no. FBS-CBT), 1% L-glutamine (Thermofisher, Catalog no. 25030-081), and 1% MEM Non-essential amino acids solution (Thermofisher, Catalog no. 11140050). For the undifferentiated use of Caco-2 cells, the cells were cultured on 24 well tissue culture plates (Alkali Scientific, Catalog no. TPN 1024) at the density of 5×10^4 cells per well. The cells were given treatments upon reaching 90% confluence.

HT-29-MTX-E12 cells were a kind gift from Dr. Leslie Bourquin at Michigan State University. E12 cells were originally purchased from Sigma-Aldrich (St. Louis, MO) and were used between 51 – 60 passages. The HT-29-MTX-E12 cells were cultured using Dulbecco's Modified Eagle Medium (DMEM, Gibco™, Catalog # 11995073) with high glucose (4500 mg/L) and sodium pyruvate (110 mg/L). The cells were supplemented with

10% Fetal Bovine Serum (Rocky Mountain Biologics, Catalog no. FBS-CBT), 1% L-glutamine (Thermofisher, Catalog no. 25030-081), and 1% MEM Non-essential amino acids solution (Thermofisher, Catalog no. 11140050).

Cell treatments

All treatments were given for 12 hours except for TNF-α treatment which was given for 24 hours. Caco-2 monolayers were treated with increasing concentrations of TCDD (0.1 nM, 1 nM and 10 nM) and FICZ (1 nM, 10 nM and 100 nM) as a positive control to activate AHR. Vehicle control cells were treated with DMSO having a final concentration of 0.02% in cell culture medium.

Cell viability assay

Caco-2 cells were treated with increasing doses of TNF-α (0.1, 1 and 10 ng/ml), TCDD (0.1 nM, 1 nM and 10 nM) and FICZ (1 nM, 10 nM and 100 nM) for 6 hours. Lactate dehydrogenase (LDH) was measured using CytoTox 96® non-radioactive cytotoxicity assay kit (Catalog # G1780) by Promega (Madison, WI). Percent cytotoxicity was calculated according to kit protocol.

Measurement of TEER

Caco-2 cells were cultured on Transwell inserts at the density of 5×10^4 cells per Transwell. 6.5 mm Transwell inserts were purchased from Corning Life Sciences (Catalog no. 3470), having 0.4 um pore size on the polyester membrane. TEER was measured using an EVOM2 epithelial volt/ohm meter (World precision instruments, Sarasota, FL).

Once the TEER reached 500 ohms/cm^2 the monolayers were considered to have formed tight junctions. Based on preliminary trials on average it took between 16 – 21 days for the monolayers to form tight junctions in Caco-2 monolayers.

Quantification of IL-8 protein through Enzyme-Linked Immunosorbent Assay (ELISA)

Cell culture medium was collected from the basal chamber of the Transwell inserts after treating monolayers with TNF-α for 24 hours. IL-8 protein was quantified using Invitrogen IL-8 human ELISA kit (Catalog # BMS 204-3) according to the manufacturer's instructions. Optical density (OD) was measured at 450 nm on a Bio-Tek microplate reader (Winooski, VT). The concentration of the samples was calculated using the standard curve generated from the known controls.

Quantitative reverse transcription PCR

Total RNA was isolated using an Qiagen RNeasy® mini kit. The samples were processed according to the manufacturer's instructions. The RNA purity was determined by measuring absorbance ratios using a NanoDrop® ND-1000 spectrophotometer (Wilmington, DE). The samples having A260/280 absorbance ratio of ~ 2.0 and A260/230 absorbance ratio ~ 2.2 were considered pure for RNA. 300 ng of total RNA was used for cDNA synthesis using a high-capacity cDNA reverse transcription kit (Catalog # 4368814) by Applied Biosystems™ (Waltham, MA). TaqMan™ Universal PCR Master Mix (Catalog # 4304437) was used for qRT-PCR. Furthermore, TaqMan™ gene expression assays for human CYP1A1 (Hs00153120-m1, Catalog # 4453320), 18S rRNA (Hs03003631-g1,

Catalog # 4448489), tight junction protein 1 (Hs01551871-m1, Catalog # 4448892) and occludin (Hs00170162-m1, Catalog # 4453320) were used for specific gene amplifications. qRT-PCR measurements were made on QuantStudio™ 3 Real-Time PCR machine. Thermal cycling conditions consisted of initial denaturation at 95 °C for 10 minutes followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing at 60 °C for 1 minute. The fold-change in gene expression was calculated using $\Delta\Delta C_t$ method [19].

Statistical Analysis

GraphPad Prism version 9.2.0 was used to perform statistical analysis and to prepare graphs. The data is displayed as Mean ± S.E.M in all graphs. The Kolmogorov–Smirnov test was used to test for the normality of data. For the comparison between two groups, student's t-test was used. For the comparison between more than two groups one-way analysis of variance (ANOVA) was performed. Bartlett's test was used to determine the homogeneity of variance across samples before using ANOVA. The P values between different groups were summarized as ns: $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Results

Caco-2 monolayers form strong tight junctions when grown on Transwell inserts

We initially selected Caco-2 [20] and HT-29-MTX-E12 [21] cell lines to model human gut barrier *in vitro*. The monolayers for both cell-lines were grown separately on

Transwell inserts for 21 days. To access the formation of tight junctions TEER was measured between the apical and basolateral compartments of the Transwell insert. 500 ohms per cm^2 was considered a threshold for the formation of tight junctions [22]. Caco-2 cells formed strong tight junctions as their TEER was significantly above 500 ohms per cm^2 by day 21 (Fig 2.1). However, HT-29-MTX-E12 cells failed to form strong, tight junctions as their average TEER was ~ 40 ohms per cm^2 by day 21 (Fig 2.2). Therefore, we chose the Caco-2 cell line to model the human intestinal epithelial barrier *in vitro* based on their ability to form strong tight junctions when grown on Transwell inserts.

TNF- α decreased intestinal epithelial barrier integrity in a concentration-dependent manner

We then determined the concentration-dependent effect of TNF-α on the resistance readings of Caco-2 monolayers. Increasing concentrations of TNF-α caused a concentration-dependent decrease in relative TEER at 24 hours (Fig 2.3). Groups that were given 0.1 and 1 ng/ml of TNF-α had a relative change in TEER of -9.81% and -36.77%, respectively. The highest decrease in relative TEER was observed in the group given 10 ng/ml of TNF-α (-55.76%). However, there was an increase in relative TEER in the control group. These results indicate that TNF-α decreases intestinal epithelial barrier integrity concentration-dependent.

TNF- α does not affect the mRNA expression of occludin and tight junction protein 1 (TJP-1) in a concentration dependent manner

The expression of mRNA for occludin and TJP-1 was determined by qRT-PCR with 18S rRNA as an internal control. Results showed that increasing concentrations of TNF-α does not affect the mRNA expression of occludin (Fig 2.4A) and TJP-1 (Fig 2.4B).

TNF-α induced secretion of IL-8 from the intestinal epithelial cells in a concentration-dependent manner

Next, we sought to determine the concentration-dependent effect of TNF-α on the secretion of IL-8 from Caco-2 monolayers. Caco-2 monolayers were treated with 10-fold increasing concentrations of TNF-α (0.1, 1 and 10 ng/ml) for 24 hours. No IL-8 protein was detected in the negative control group (0 ng/ml of TNF-α) and cells treated with a 0.1 ng/ml concentration of TNF-α. However, there was a significant induction of IL-8 protein in groups treated with 1 and 10 ng/ml of TNF-α (Fig 2.5). These results indicate that TNF-α induces the secretion of IL-8 protein from Caco-2 cells in a concentration-dependent manner.

TCDD and FICZ activate the AHR in the Caco-2 cells in a concentration-dependent manner

After developing an *in vitro* model of colitis by stimulating Caco-2 cells with TNF-α, we next asked if AHR can be activated by its defined exogenous and endogenous ligands in the Caco-2 cell model *in vitro*. We selected TCDD as it is a well-characterized exogenous synthetic agonist of the AHR [23]. Furthermore, we selected FICZ produced

by tryptophan under ultra-violet radiation because it is the most potent endogenous ligand of AHR [24]. After treating the Caco-2 cells for 12 hours with increasing concentrations of TCDD (0.1 nM, 1 nM and 10 nM) and FICZ (1 nM, 10 nM and 100 nM), we then quantified the mRNA of the CYP1A1 gene. CYP1A1 has been shown to be a reliable biomarker for AHR activation [25]. Our results showed that TCDD and FICZ activated AHR in a concentration-dependent manner (Fig 2.6).

Lactobacillus murinus activate the AHR in MOI dependent manner

After determining that AHR can be stimulated in the Caco-2 cells by its defined agonists; we then sought to determine if *L. murinus* can activate the AHR in Caco-2 cells. *L. murinus* was suspended in PBS and the OD_{600} was measured to estimate the colony forming units per ml. The inoculum was then plated on MRS agar after limiting dilution assay to determine the absolute CFU. Caco-2 monolayers were stimulated with *L. murinus* in increasing multiplicity of infection (MOI). After 24 hours of stimulation, we then quantified the mRNA of the CYP1A1 gene. The results showed that *L. murinus* increased AHR activation in an MOI dependent manner (Fig 2.7).

AHR activation attenuated TNF-α induced gut barrier disruption and pro-inflammatory response in Caco-2 monolayers

Next, we asked if AHR activation can attenuate TNF-α induced decrease in TEER and IL-8 secretion in Caco-2 monolayers. To determine this, we first activated the AHR in the Caco-2 monolayers by pre-treating with TCDD (10 nm), FICZ (100 nm) or *L. murinus* (MOI 1:100). After 12 hours of pre-treatment the monolayers were then

stimulated with TNF-α (10 ng/ml) for a period of 24 hours. Relative TEER percentage and IL-8 protein were quantified as a measure of efficacy of the pre-treatment.

L. murinus pre-treatment significantly attenuated the TNF-α induced TEER disruption. However, pre-treating the monolayers with TCDD or FICZ was not effective in attenuating the TNF-α induced epithelial barrier damage (Fig 2.8). These results showed that *L. murinus* pre-treatment was significantly effective in attenuating the TNF-α induced damage to the intestinal epithelial barrier function *in vitro.*

There was a significant increase in IL-8 production upon the treatment of monolayers with TNF-α (10 ng/ml). However, *L. murinus* pre-treatment, significantly reduced the secretion of IL-8 upon TNF-α stimulation ($P > 0.0001$). Furthermore, activating the AHR by FICZ also significantly reduced IL-8 production upon TNF-α stimulation ($P = 0.0176$). However, pre-treating the monolayers with TCDD did not reduce the IL-8 secretion when stimulated with TNF-α (Fig 2.9). These results suggest that activation of the AHR by some but not all AHR ligands attenuate the TNF-α induced pro-inflammatory response in the human intestinal epithelial cells. Thus, these results also suggest that these protective effects of AHR activation against TNF-α induced pro-inflammatory response are ligand dependent.

Discussion

The incidence of IBD in the US is high with estimates of 1 in 209 people suffering from some form of IBD [3]. Immunosuppressants and anti-TNF-α drugs are the current

treatment modalities for IBD; however, their associated safety concerns along with increasing risks for opportunistic infections has augmented the need to develop innovative treatments for IBD. Yet, this is a difficult task because the pathogenesis of IBD is complex where genetics (mutations in NOD2), epigenetics, host immune system factors such as detrimental mucosal immune responses or imbalances between pro-inflammatory and anti-inflammatory cytokines, and alterations in gut microbiota leading to dysbiosis have all been considered as primary factors for IBD [26].

One novel approach for treating IBD that has been recently sought is identification and understanding of the role of commensal members of the gut microbiota [27]. Several studies have reported alterations in the human gut microbiota (such as lower abundance of firmicutes) and dysbiosis in patients suffering from IBD [14, 27, 28]. *Frank* et al., [28] performed rRNA sequence analysis of intestinal tissue samples obtained from patients suffering from IBD. They found a decreased abundance of bacteria belonging to phyla *Firmicutes* and *Bacteroidetes* in patients suffering from IBD as compared to healthy controls. They also showed that bacteria belonging to phyla *Actinobacteria* and *Alphaproteobacteria* were increased in IBD patients as compared to healthy controls. These results indicate that alterations in gut microbiota mediating dysbiosis play a role in the pathogenesis of IBD, but more work is needed to study bacterial taxa at a phylogenetic level where functional attributes can be identified.

Given the role of dysbiosis in the pathogenesis of IBD, therapeutics employing the commensal gut microbiota such as probiotics, are a logical approach for treating IBD.

Many studies have reported little or no beneficial effect of probiotics in treating CD [29, 30]. However, as opposed to CD, studies have shown efficacy of certain probiotics in patients suffering from UC [31]. VSL # 3 is one of the most studied probiotic preparation in treating UC. VSL # 3 consists of four strains of *Lactobacilli (L. paracasei, L. plantarum, L. acidophilus*, and *L. delbrueckii* subsp. *bulgaricus)*, three strains of *Bifidobacteria (B. longum, B. breve*, and *B. infantis)*, and one strain of *Streptococcus salivarius* subsp. *thermophilus* (*S. thermophilus).* Miele *et al*., [32] conducted a one-year-long, placebo-controlled, double-blind study to test the efficacy of VSL # 3 on induction and maintenance of remission in children with active UC. It was found that 92.8% of patients achieved remission upon treatment with VSL # 3 and IBD therapy whereas, only 36.4% of patients achieved remission upon treatment with placebo and IBD therapy. In another study, a randomized clinical trial was conducted to test the effectiveness of *Lactobacillus reuteri* ATCC 55730 in patients suffering from active distal UC [33]. Patients suffering from active distal UC received a rectal enema of *L. reuteri* ATCC 55730 or placebo for 8 weeks. Investigators found that clinical and endoscopic score for UC (Mayo score) decreased significantly in the group that was given *L. reuteri* as compared to the placebo-controlled group. The mucosal expression level of anti-inflammatory cytokine (IL-10) was increased significantly in the *L. reuteri*-treated group as compared to the placebo-treated group. Furthermore, the mRNA expression of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-8 were significantly reduced in the *L. reuteri* group compared to the placebo-controlled group [33]. These results indicate that intestinal microbiota manipulation through probiotics can be effective in treating some forms of IBD.

While probiotics are becoming an attractive therapy to treat UC, there is also a great need to understand the molecular mechanisms that govern probiotic induced beneficial effects against chronic intestinal inflammation. Previous studies have elucidated some mechanisms for probiotic-induced protection, including the production of short-chain fatty acids (SCFA) [34], increased production of mucin [35], enhancement of the intestinal epithelial barrier function [36], modulation of the immune system including production of immunoglobulins [37] and modulation of negative TLR regulators [38]. However, the mechanisms by which probiotics attenuate chronic gut inflammation are not yet fully understood.

Our study has identified a novel mechanism of action of a putative probiotic strain, *L. murinus*. We showed that *L. murinus* activates AHR in the human intestinal epithelial cells *in vitro*. To determine the activation of AHR in the Caco-2 monolayers, we quantified the CYP1A1 mRNA fold-change, since CYP1A1 is considered a biomarker for AHR activation [25]. Using 18S rRNA as a house-keeping gene [39], we used 10-fold increasing MOIs of *L. murinus* on Caco-2 monolayers to determine the activation level of AHR with increasing *L. murinus* MOIs. Our results indicate that AHR activation is directly proportional to the MOI of *L. murinus* in the Caco-2 cells (Fig 2.7).

We then treated the monolayers with defined AHR agonists in a concentration dependent manner. We chose to treat cells with TCDD or FICZ in 10-fold increasing concentrations. We specifically chose TCDD because it is the most comprehensively studied exogenous AHR agonist [23]. We also chose a second known AHR agonist, FICZ,

because it is a tryptophan derivative and an endogenous ligand having the highest affinity for binding to AHR among all other endogenous ligands [40]. Our results indicate that AHR can be activated in the Caco-2 human intestinal epithelial cell monolayer model by its defined exogenous and endogenous ligand in a concentration-dependent manner (Fig 2.6).

Next, to optimize the *in vitro* model of IBD, we treated the Caco-2 cells with increasing concentrations of TNF-α because this is one of the main cytokines that is upregulated in the gut epithelium of IBD patients [41]. Our strategy was to use TNF-α as a surrogate for *C. jejuni* infection because it is the principal cytokine that signals early inflammatory responses after infection with this bacterium [42]. Other investigators have employed TNF-α in this manner to model *C. jejuni* infection outcomes in *in vitro* models [43]. This is effective because exogenous TNF-α induces a leaky gut barrier and mimics the mucosal cytokine storm which is a prominent feature in *C. jejuni* pathogenesis [44, 45]. Campylobacteriosis is very common in the US and worldwide and previous studies have associated a role for *C. jejuni* induced enteritis in the pathogenesis of IBD [5, 6]. Therefore, stimulating Caco-2 cells with TNF-α mimics *C. jejuni* infections conditions as well as models IBD conditions *in vitro.*

Our results indicate that a decrease in barrier integrity is directly proportional to the increasing concentrations of TNF-α (Fig 2.3). These findings are consistent with findings reported by *Cui* et al.,[46] in which Caco-2 monolayer permeability was increased when treated with TNF-α. However, the increasing concentrations of TNF-α had no effect on

the mRNA expression of the occludin (Fig 2.4A) and TJP-1 (Fig 2.4B). These results suggest that TNF-α directly acts on tight junction proteins to induce barrier dysfunction and does not affect the mRNA expression of these barrier proteins.

Our results also indicate that IL-8 secretion from Caco-2 cells is directly proportional to the TNF-α concentration (Fig 2.5). *Sonnier* et al., [47] also reported similar findings where basolateral stimulation of Caco-2 cells with TNF-α resulted in apical and basolateral secretion of IL-8; however, no concentration-dependent effect of TNF-α on IL-8 secretion was determined in their studies.

Next, we determined the effect of AHR activation—either by defined AHR agonists or by *L. murinus*—on IL-8 secretion and gut barrier function *in vitro*. Our results indicate that pre-treating the monolayers with *L. murinus* attenuated the TNF-α induced decrease in barrier integrity when compared to the positive control i.e., monolayers given TNF-α only (Fig 2.8). However, pre-treating the monolayers with defined AHR agonists (TCDD and FICZ) did not protect against the TNF-α induced barrier disruption. Interestingly, we also observed a significant disruption in epithelial barrier integrity in groups given either FICZ or TCDD. One explanation for the disruption of intestinal epithelial barrier integrity with TCDD and FICZ is that AHR ligands can also activate several mitogen-activated protein kinase (MAPK) pathways [48]. MAPK signaling pathways play a dual role in regulating intestinal barrier permeability [49]. For example, it has been reported that activation of MAPK through the ERK1/2 pathway leads to intestinal barrier disruption [50]. In another study, inhibition of MAPK pathway resulted in a reduced loss of barrier function

in Caco-2 cells [51]. Furthermore, we did not observe any cytotoxic effects of TCDD and TNF-α at concentrations of 0.1 nM, 1 nM and 10 nM and 0.1, 1 and 10 ng/ml respectively (data not shown). Whereas no cytotoxic effects of FICZ were observed at 1nm concentration. However, a cytotoxicity of 4.88% and 7.47% was observed in the Caco-2 cells treated with FICZ concentrations of 10 nM and 100 nM respectively (data not shown). The cytotoxic effects of FICZ on epithelial cells at higher concentrations has also been reported by Walczak *et al*., [52].

Pre-treating the cells with *L. murinus* or FICZ also significantly reduced the secretion of IL-8 from Caco-2 monolayers upon stimulation with TNF-α (Fig 2.9). This is of significance since IL-8 is also known as a “neutrophilic chemotactic factor” as it attracts neutrophils, basophils and T-cells to the site of inflammation [53]. Neutrophils are also the primary cell type recruited to the inflamed bowel [54]. Furthermore, neutrophils contribute to the gut epithelial barrier damage by producing reactive oxygen species (ROS) and neutrophil extracellular traps (NETs) [55]. Assessing levels of neutrophil infiltration in gut mucosa is widely considered a useful means to determine disease severity in IBD [56, 57] and is employed routinely to score for UC disease severity [58, 59]. Therefore, attenuating the IL-8 secretion by *L. murinus* or endogenous AHR agonist (FICZ) may decrease the neutrophil-induced damage to the gut wall. On the other hand, activation of AHR by TCDD did not result in decreased IL-8 response. The reason for the differential FICZ and TCDD response to IL-8 secretion is not clear. However, recent evidence suggests that endogenous AHR ligands may induce differential cell type responses compared to synthetic ligands such as TCDD [60, 61]. The reason for these divergent

effects between the endogenous and exogenous AHR ligands is not completely understood; however, the differences between the half-lives of TCDD and FICZ may explain this dichotomy. TCDD is resistant to AHR-induced metabolism and has a long half-life therefore it leads to sustained AHR activation. On the other hand, FICZ is rapidly metabolized, has a short half-life and leads to a transient AHR activation [62].

In future, conducting proof-of-concept studies will further dissect the role of AHR in the pathogenesis of IBD. To the best of our knowledge, at present, $AHR^{-/-}$ Caco-2 cell-line is not commercially available. Another way to conduct proof-of-concept studies is to use AHR ligand-selective antagonists. CH223191 is a ligand-selective antagonist that inhibits the TCDD mediated nuclear translocation and DNA binding of AHR [63]. In our preliminary experiments CH223191 blocked TCDD mediated CYP1A1 upregulation in the HT-29 cells (data not shown). However, we did not use CH223191 in designing subsequent proof-of-concept studies as we did not observe any protective effect of TCDD pretreatment in attenuating TNF-α induced pro-inflammatory responses in *in vitro* model of colitis. In future, gene editing techniques such as CRISPR-Cas9 [64] can be used to develop $AHR^{-/-}$ Caco-2 cell-line to perform *in vitro* proof-of-concept studies.

In short, our results indicate a novel mechanism of action of probiotics to alleviate damaging effects of TNF-α on the gut wall. The results from this study may lead to the development of microbiome-based interventions to potentially treat IBD without the use of immunosuppressants or anti-TNF-α. Furthermore, the results from this study may lead

to the development of novel probiotics capable of treating bacterial disease which manifests similar disease pathogenesis as IBD such as human campylobacteriosis.

APPENDIX

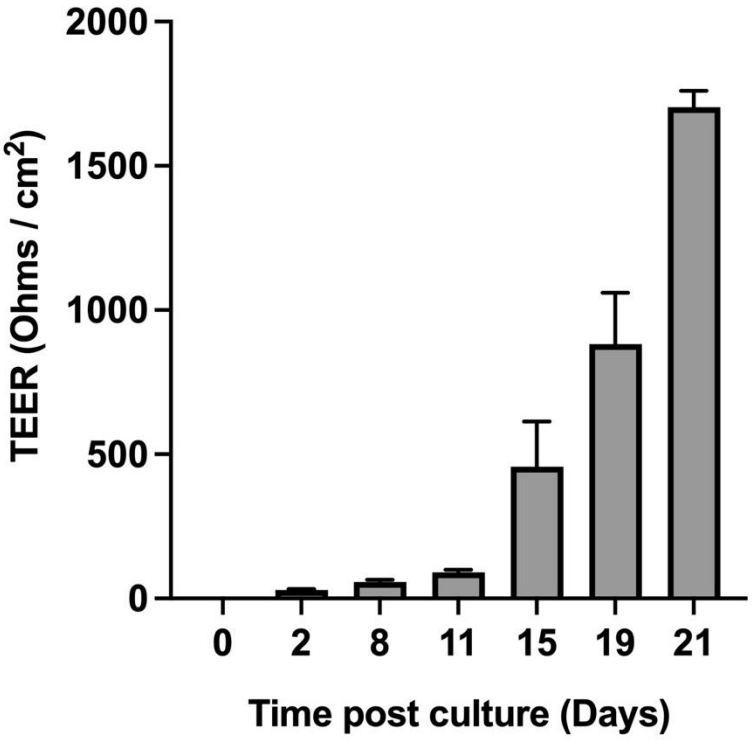

Figure 2.1) Resistance readings of Caco-2 monolayer

Transepithelial electrical resistance (TEER) of the Caco-2 monolayer was measured when grown on the Transwell™ inserts. The monolayer was grown on the Transwell™ insert for the period of 21 days. The resistance was measured every couple of days. Resistance readings are shown as mean ± SEM.

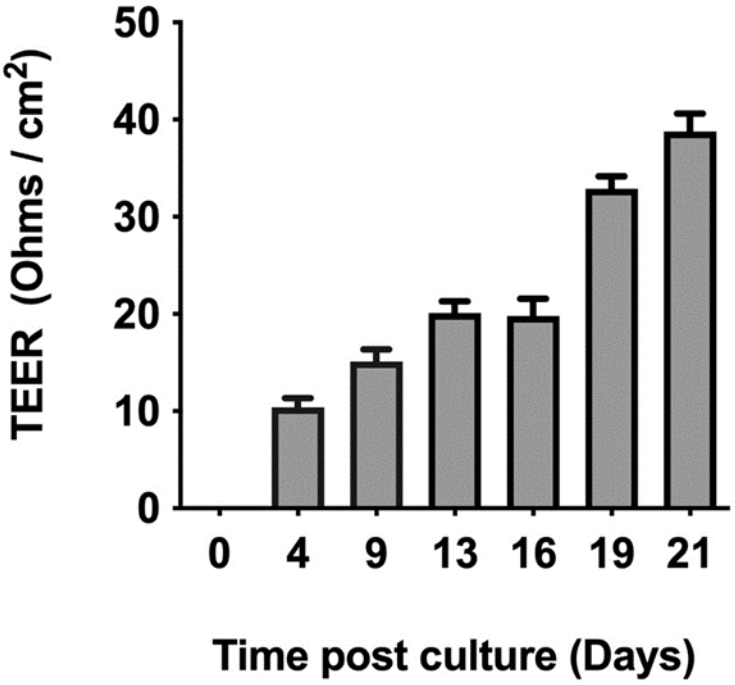

Figure 2.2) Resistance readings of HT-29-MTX-E12 monolayer

The TEER values of the E12 monolayer were measured when grown on the Transwell inserts. The monolayer was grown on the Transwell insert for a period of 21 days. The resistance was measured every couple of days. Resistance readings are shown as mean ± SEM.

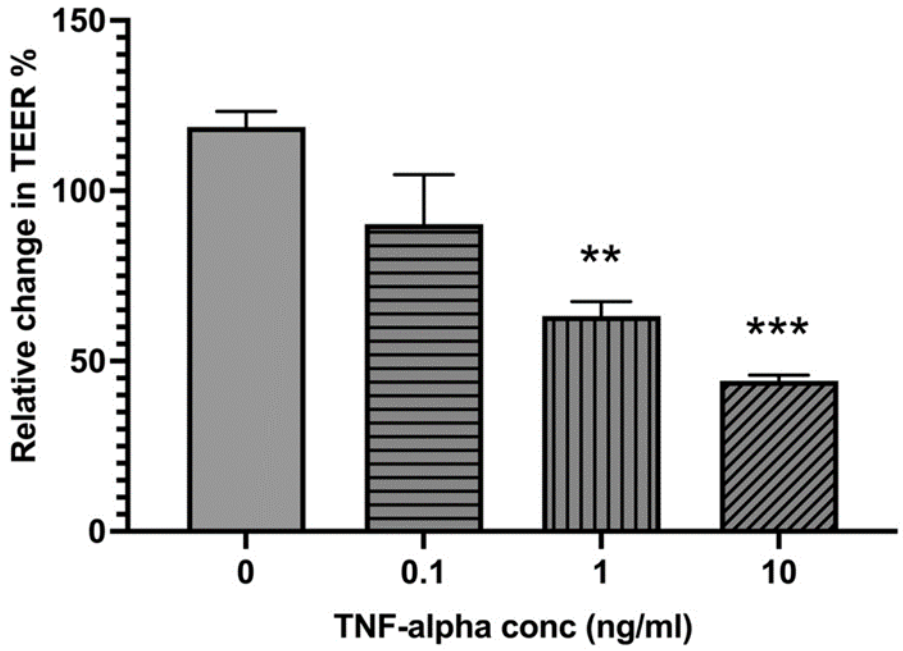

Figure 2.3) TNF-α decreases epithelial barrier integrity in a concentration-dependent manner

Caco-2 cells monolayers were treated with 10-fold increasing concentrations of TNF-α. TEER was measured at 24 hours post TNF-α stimulation. Relative TEER % was calculated as the percent change of TEER at 0 hours. The results are expressed as mean ± SEM. There were at least 3 replicates per group. The statistical analysis was performed using one-way ANOVA with post-hoc Tukey's HSD test for computing statistically significant differences relative to the control group (0 ng/ml).

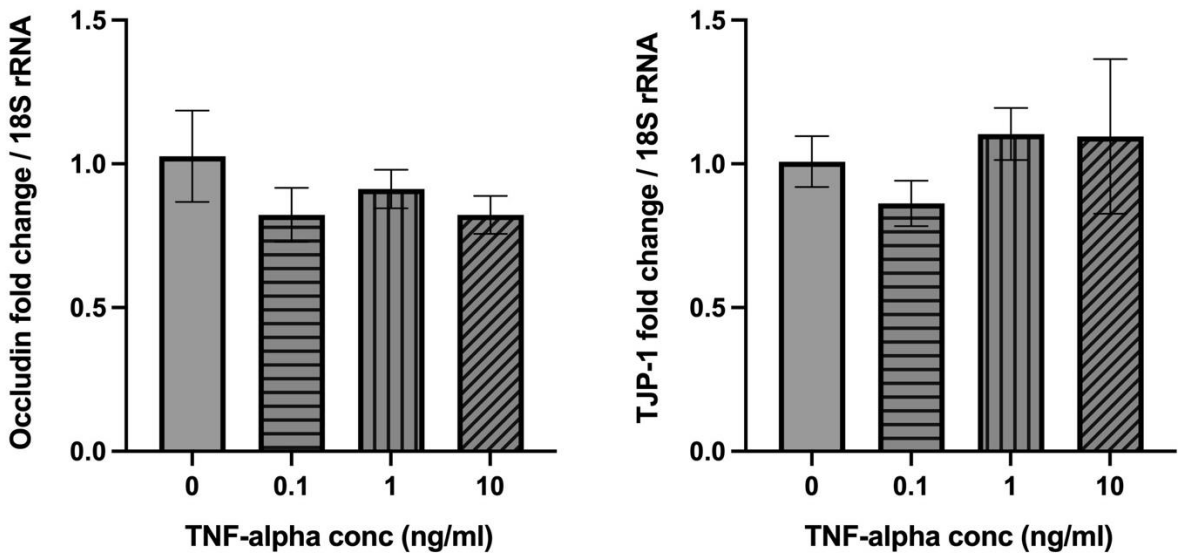

Figure 2.4) TNF-α does not affect the mRNA expression of Occludin and TJP-1

Caco-2 cells monolayers were treated with 10-fold increasing concentrations of TNF-α. The mRNA of occludin and TJP-1 was quantified by qRT-PCR using 18S rRNA as an internal control. The results are expressed as mean ± SEM. There were at least 3 replicates per group. The statistical analysis between different groups was performed using one-way ANOVA. There was no statistically significant difference between any of the groups.

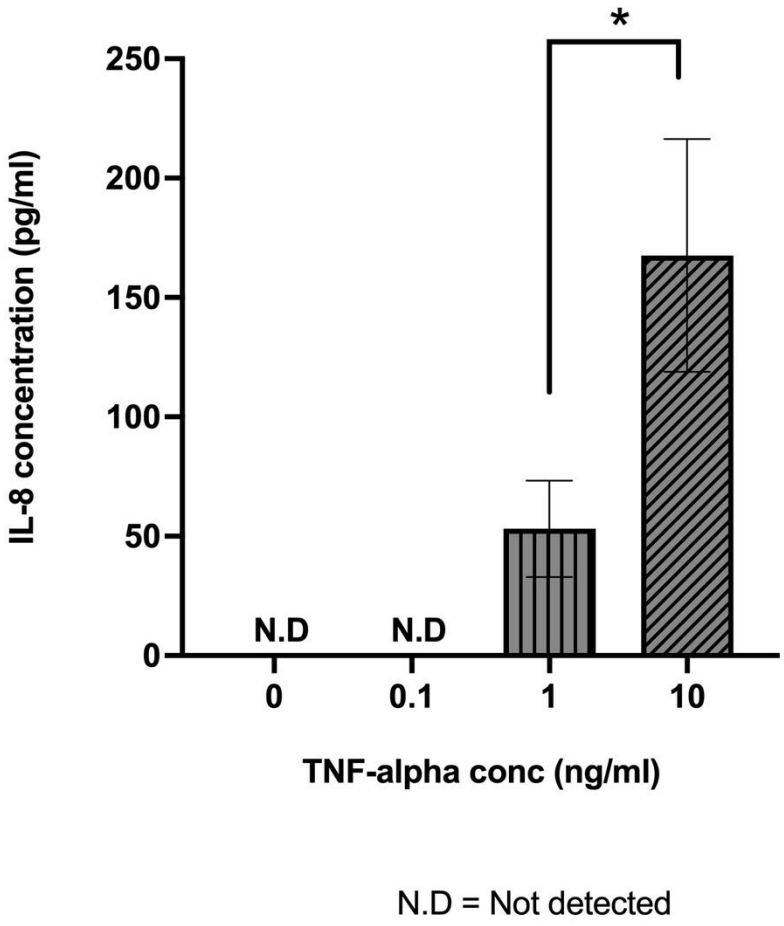

Figure 2.5) TNF-α induces IL-8 secretion from Caco-2 monolayers in a concentration-dependent manner

The Caco-2 monolayers were treated with increasing concentrations of TNF-α for 24 hours. IL-8 protein was measured from the cell culture medium collected from the basal chamber of the Transwell inserts. There were at least 3 replicates per group. The statistical analysis was performed using unpaired students t-test.

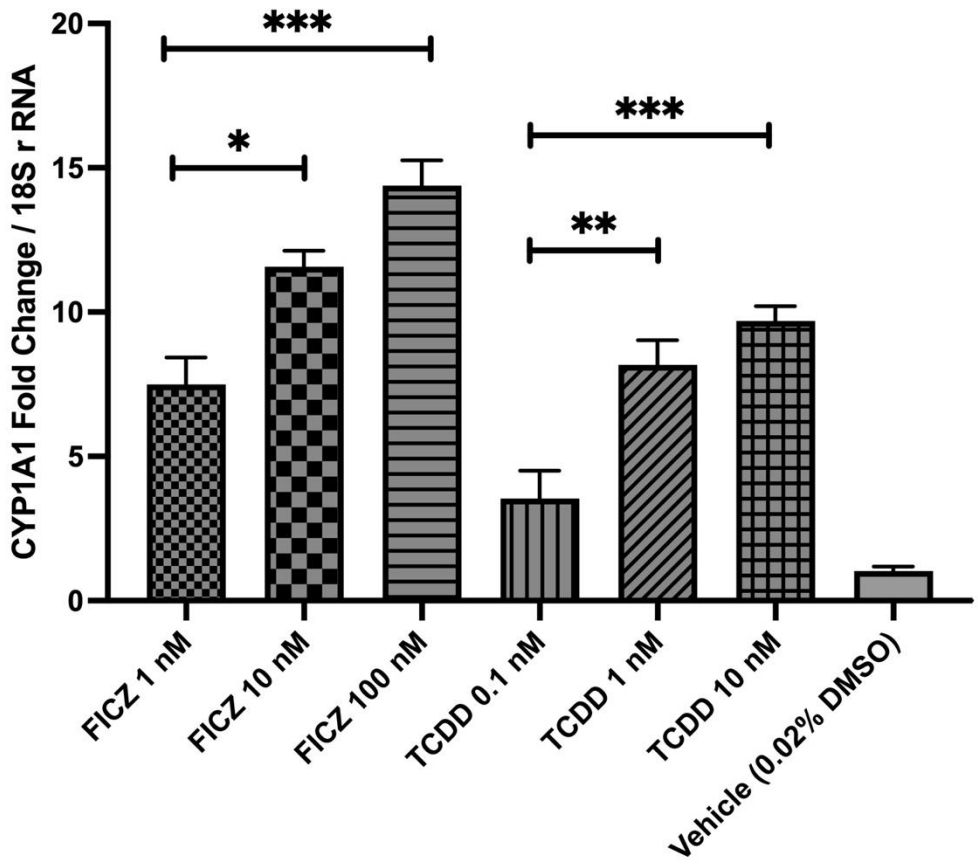

Figure 2.6) AHR ligands increase CYP1A1 transcription in a concentration-dependent manner

The expression of CYP1A1 was measured in the Caco-2 cells treated with different concentrations of the two defined AHR agonists, i.e., FICZ and TCDD for 12 hours. The expression of the CYP1A1 was determined by qRT-PCR and the results were normalized using 18S rRNA as a house keeping gene. The results are expressed as mean ± SEM. There were at least 3 replicates per group. The statistical analysis between different groups was performed using one-way ANOVA with post-hoc Tukey's HSD test for comparing multiple treatments.

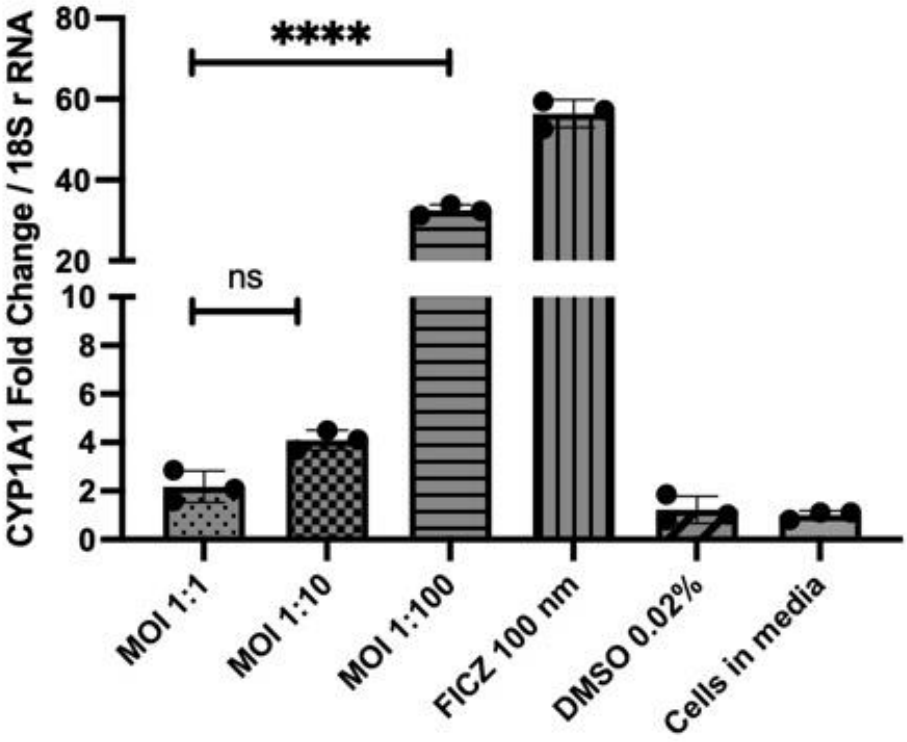

Figure 2.7) *L. murinus* upregulates CYP1A1 transcription in MOI dependent manner

Caco-2 cells were treated with *L. murinus* in 10-fold increasing MOI. CYP1A1 activity was measured by qRT-PCR with 18S rRNA used as an internal control. Cells were treated with 100 nm FICZ as positive control and 0.02% DMSO as vehicle control. All treatments were given for 24 hours. There were at least 3 replicates per group. The results are expressed as mean ± SEM. The statistical analysis between different groups was performed using one-way ANOVA with post-hoc Tukey's HSD test for comparing multiple treatments.

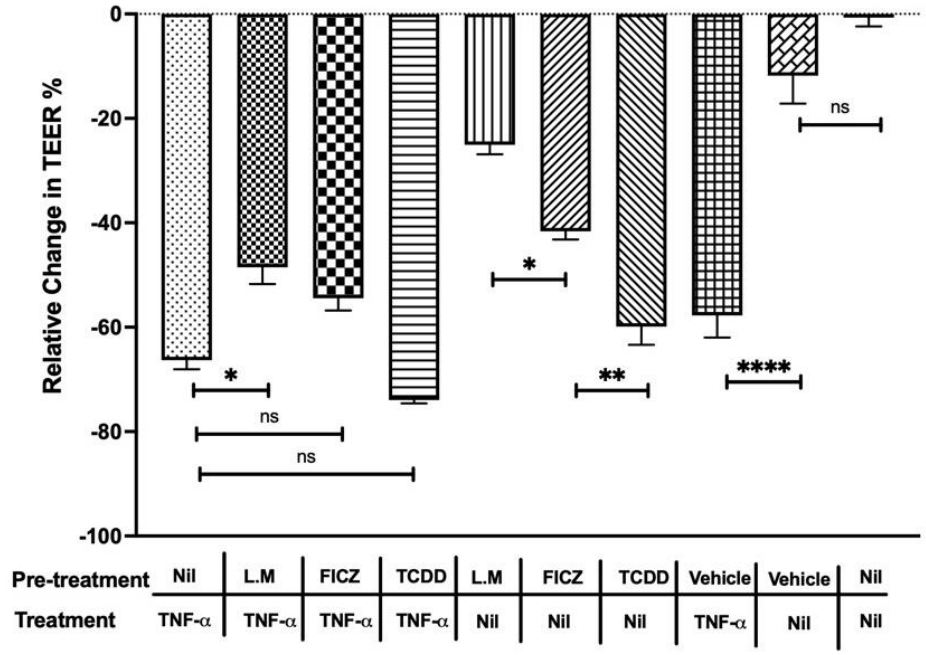

Figure 2.8) AHR activation attenuates epithelial barrier disruption

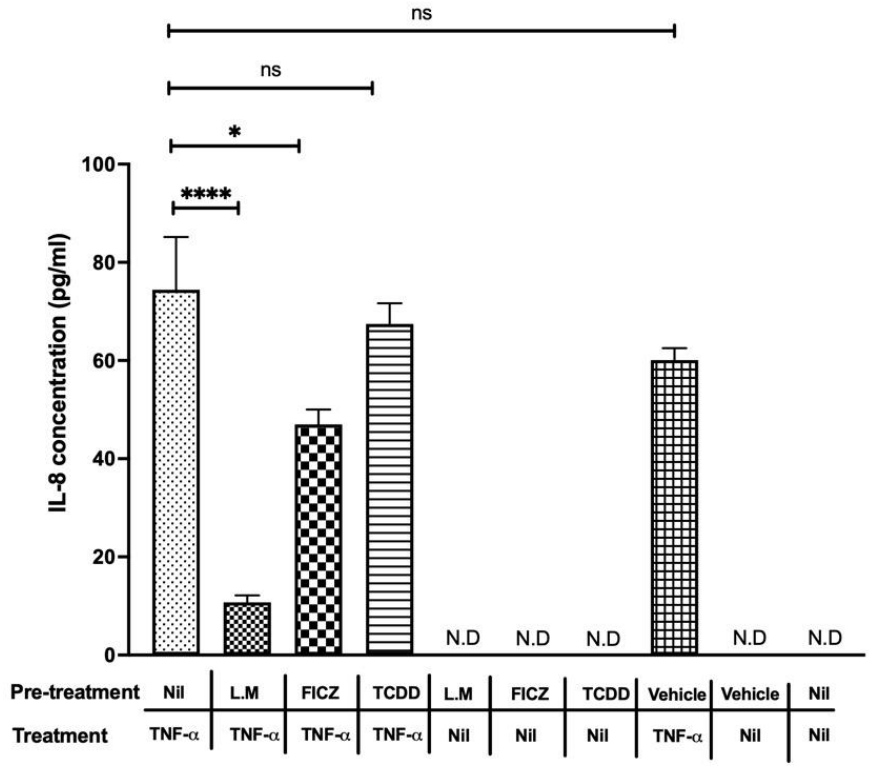

Figure 2.9) AHR activation attenuates pro-inflammatory response

AHR activation attenuates epithelial barrier disruption and pro-inflammatory response in Caco-2 monolayers

Caco-2 monolayers were given respective pre-treatments for 12 hours with either *L. murinus* (MOI 1:100) or FICZ (100 nM) or TCDD (10 nM); followed by 24 hours of treatment with TNF-α (10 ng/ml). At the end of 24 hours of treatments, TEER and IL-8 were measured to determine the effect of AHR activation on barrier dysfunction (Fig 2.8) and IL-8 secretion (Fig 2.9) from Caco-2 cells. The results are expressed as mean ± SEM. There were at least 3 replicates per group. The statistical analysis between different groups was performed using one-way ANOVA with Dunnett's multiple comparison test.

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CHAPTER 3: CO-INOCULATION OF *LACTOBACILLUS MURINUS* AND *CAMPYLOBACTER JEJUNI* ENHANCED COLONIZATION LEVELS OF BOTH TAXA AND CHANGED THE GUT MICROBIOTA LANDSCAPE BUT DID NOT PROTECT BALB/C IL-10$^{-/-}$ MICE FROM DEVELOPING COLITIS.

Abstract

Background

Interleukin-10 deficient mice are a robust model for investigating inflammatory bowel disease (IBD). When infected with the foodborne pathogen *Campylobacter jejuni*, IL-10 deficiency leads to enhanced inflammation in the gut resulting in colitis [1]. In a previous experiment, C57BL/6 IL-$10^{-/-}$ mice unexpectedly failed to develop colitis upon infection with *C. jejuni* 11168. This result contrasted with the previously reported findings where C57BL/6 IL-$10^{-/-}$ mice developed severe colitis upon inoculation with *C. jejuni* 11168 [1, 2]. Coincident with this finding, *Lactobacillus murinus* was isolated from the mice protected from *C. jejuni* induced colitis, suggesting a role for *L. murinus* in suppressing *C. jejuni* induced colitis [3]. This outcome was consistent with the ability of this *L. murinus* strain isolate to attenuate inflammatory responses in an *in vitro* intestinal cell model of colitis [4]. In this study, we hypothesized that prophylactic administration of *L. murinus* would attenuate *C. jejuni* induced colitis in an IL-10 deficient mouse model.

Methods

A total of 41 BALB/c IL-$10^{-/-}$ mice were used to test this hypothesis. 11 mice were sham inoculated (negative control), 10 mice received only *L. murinus*, 10 mice received only *C. jejuni* (positive control), and 10 mice in the test group received both *L. murinus* and *C. jejuni. L. murinus* was inoculated 32 days before *C. jejuni* infection. Thirty days post-C. *jejuni* challenge mice were sacrificed and assessed for gut pathology. During necropsy intestinal tissues and fecal samples were collected from mice to determine

histopathological changes and bacterial colonization levels in the gut through 16S sequence analysis and bacterial culture.

Results

Both the *C. jejuni* positive control and the co-inoculated (*L. murinus* and *C. jejuni*) test group mice developed severe colitis. Furthermore, both *C. jejuni* and *L. murinus* colonized more densely in the co-inoculated test group than in the *C. jejuni* only control or *L. murinus* only groups. 16S sequencing analysis of fecal DNA of all groups revealed that the Shannon-alpha diversity in the *C. jejuni* infected control and the co-inoculated (*L. murinus* and *C. jejuni*) test groups were significantly less ($P<0.001$) than in the *Lactobacillus* only and negative control groups. Presence of *C. jejuni* caused dysbiosis in the gut bacterial community with increased abundance of *Enterococcus* and *Lactobacillus* and decreased abundance of *Lachnospiraceae*.

Conclusions

These results indicate that gut microbial diversity was significantly decreased in the presence of *C. jejuni*. The microbiota landscape was also significantly altered in the presence of *C. jejuni*, particularly in co-infected mice. The increase of colony forming units of both *C. jejuni* and *L. murinus* in the co-infected group suggests an ecological interaction between these two taxa. These results showed that prophylactic administration of *L. murinus* did not protect BALB/c IL-$10^{-/-}$ mice from developing inflammatory bowel disease following *C. jejuni* infection despite its ability to attenuate inflammatory responses in an *in vitro* intestinal cell model of colitis.

Introduction

Campylobacter jejuni is a leading cause of human foodborne gastroenteritis in the US, with an incidence rate of 13.6 diagnosed cases per 100,000 individuals. Because many sporadic infections go undiagnosed, the Centers for Disease Control predict that the actual incidence is much higher, with estimates of approximately 1.5 million cases of campylobacteriosis in the US per annum [5]. *C. jejuni* resides as a commensal in the gastrointestinal tract of many agricultural animals [6]. The most frequent cause of *C. jejuni* infection in the US is the consumption of chicken contaminated during processing because *C. jejuni* resides as a commensal in chicken gut [7]. Campylobacteriosis in humans is characterized by mild to severe bloody diarrhea, abdominal pain, and severe intestinal inflammation that lasts from 7 – 10 days [6]. Various post-infectious auto-immune diseases such as Miller-Fisher syndrome, Guillain-Barre syndrome, and reactive arthritis have been associated with *Campylobacter* infections increasing its impact as an intestinal pathogen [2, 8, 9].

Macrolide antibiotics such as azithromycin and ciprofloxacin are the drugs of choice to treat *C. jejuni* infection in human populations [10]. However, over-use of antibiotics has led to the emergence of antimicrobial-resistant *C. jejuni* strains and reduced treatment efficacy with long-term shedding in patients [10, 11]. In 2015, the National Antimicrobial Resistance Monitoring System (NARMS) reported that 47% of *C. jejuni* isolates were resistant to tetracycline, whereas 25% of the isolates were resistant to ciprofloxacin [12]. More than 35,000 people die each year in the US from antibiotic-resistant infections [13]. Thus, the Centers for Disease Control (CDC) declared *C. jejuni*

a serious health threat [13]. The development of antimicrobial resistance traits in *C. jejuni* isolates has augmented the need to develop innovative strategies to prevent and treat drug-resistant *C. jejuni* infections in human and animal populations.

Probiotics can be an attractive therapy to reduce or eliminate the use of antibiotics to limit gut bacterial pathogens such as *C. jejuni* [14]. Certain members of the *Lactobacillus* spp. are commonly used as probiotics [15]. Previous studies have elucidated several mechanisms by which particular *Lactobacillus* spp. can attenuate *C. jejuni* virulence properties *in vitro* [16, 17]. Different *Lactobacillus* strains (*L. salivarius*, *L. johnsonii*, *L. reuteri*, *L. crispatus*, and *L. gasseri*) alone or in combination reduced the ability of *C. jejuni* to adhere and invade human intestinal epithelial cells *in vitro*. Furthermore, incubating these *Lactobacillus* strains (except *L. reuteri*) with *C. jejuni* reduced the expression of *C. jejuni* virulence genes, including genes responsible for motility such as *flaA, flaB*, and *flhA* and gene responsible for invasion (*CiaB*) [16]. Similarly, Wang *et al*. [17] reported that *L. casei* ZL4 reduced the adhesion and invasion of *C. jejuni* in cultured HT-29 cells, a mucus-producing human intestinal epithelial cell line. These results suggest that certain members of the *Lactobacillus* spp. possess mechanisms that can attenuate *C. jejuni* virulence.

Previously, *L. murinus* was isolated from C57BL/6 IL-10$^{-/-}$ mice that failed to develop colitis when infected with a colitogenic strain of *C. jejuni*, thus, suggesting a possible role for the *L. murinus* isolate to suppress *C. jejuni* induced colitis [3]. We also reported the ability of this isolate of *L. murinus* to attenuate TNF-alpha-induced pro-

inflammatory responses in cultured human intestinal epithelial cells by activating the aryl hydrocarbon receptor (AHR) [1]. TNF-alpha was used as a surrogate to *C. jejuni* infection to provide an *in vitro* model of colitis [1]. Therefore, in this study we hypothesized that prophylactic inoculation of *L. murinus* will protect BALB/c IL-10$^{-/-}$ mice against *C. jejuni* induced colitis. To test this hypothesis, we challenged BALB/c IL-10$^{-/-}$ mice with a colitogenic strain of *C. jejuni* known to induce TNF-alpha during infection [2-4]. We used BALB/c IL-10$^{-/-}$ mice to model *C. jejuni* disease because IL-10$^{-/-}$ mice are a robust model to study *C. jejuni* pathogenesis [3, 5]. Upon infection with *C. jejuni*, IL-10$^{-/-}$ mice develop clinical symptoms and histopathologic lesions consistent with those in humans with campylobacteriosis [3]. Yet, in this study, results indicated that prophylactic administration of *L. murinus* was not effective in attenuating *C. jejuni* induced colitis in BALB/c IL-10$^{-/-}$ mice. Interestingly, *C. jejuni* infected groups had reduced bacterial diversity of the gut microbiota when compared to the non-*C. jejuni* infected groups. Further, co-infected mice had the highest levels of *C. jejuni* and *L. murinus* and the landscape of the microbial community was significantly altered. These results demonstrate that *C. jejuni* mediates dysbiosis of the gut microbial community with reduced microbial diversity during its pathogenesis and that *L. murinus* was not capable of preventing this outcome in an IL-10 deficient environment.

Materials and methods

Animal Handling Protocols

Animal protocols were approved by Michigan State University (MSU). C.129P2(B6)-*Il10*^{tm1Cgn}/J (referred to as BALB/c IL-10$^{-/-}$) mice were originally obtained

from the Jackson Laboratories (Bar Harbor, ME). Mice were housed in a *Campylobacter*/*Helicobacter-free* facility at MSU. Mice were kept in the Flex-Air ventilated mouse rack (Alternate Design Manufacturing & Supply Inc., Siloam Spring, AR) with an ad-libitum supply of feed and water. The mice were routinely monitored for the presence of a variety of bacterial, viral, and protozoan agents by routine monitoring of the dedicated sentinel mice. Furthermore, mice developing signs of spontaneous colitis were screened for the presence of colitogenic bacteria, including *Campylobacter* spp., *Enterococcus faecalis*, *Citrobacter rodentium,* and *Helicobacter spp*., by PCR [6-9]. The detailed protocols for maintaining mice in specific pathogen-free facility at MSU has been described previously [3].

Experimental Design

A total of 41 BALB/c IL-10$^{-/-}$ mice were used in this experiment. The mice were divided into four groups. The table below describes the groups of mice for this experiment.

Table 1) Groups of mice used in this study

Group (# of mice)	Group Description	Inoculation Strain / Vehicle (Day of inoculation)
Group A (10)	Control group for *L. murinus*	*L. murinus* (1)
Group B (10)	Control group for *C. jejuni*	*C. jejuni* 11168 (32)
Group C (10)	Test group	*L. murinus* (1) and *C. jejuni* 11168 (32)
Group D (11)	Vehicle control group	Sham inoculation, Tryptic Soy Broth (1,32)

Mice in all groups were sacrificed 30 days post-*C. jejuni* infection. Fecal and intestinal tissue samples were taken from mice during necropsy.

Growing bacterial cultures and their inoculation in mice

Lactobacillus murinus

L. murinus was initially isolated from C57BL/6 mice. The detailed description regarding the isolation and characterization of *L. murinus* isolated from C57BL/6 mice has been described by J. Brudvig [1]. In brief, *L. murinus* was recovered from glycerol stocks stored at -80 °C and then streaked onto De Man, Rogosa, and Sharpe (MRS) agar plates. The MRS agar plates were incubated at 37 °C for 48 hours. After 48 hours, individual colonies were picked and Gram-stained to check for *L. murinus* morphology and culture purity. *L. murinus* were identified to species using MALDI-TOF mass spectrometry at the Veterinary Diagnostic Laboratory, Michigan State University [1].

L. murinus growth was harvested from the MRS agar plates and diluted in tryptic soy broth (TSB) to an optical density co-related with 1×10^9 CFU of *L. murinus* per 100 ul of TSB. Each mouse in groups A and C received 100 ul of TSB containing 1×10^9 CFU of *L. murinus* in the stomach through a sterile 3.5-French feeding tube (Kendall Sovereign; Tyco Healthcare Group, Mansfield, MA) attached to a 1-ml Luer-Lok syringe (Becton Dickinson and Company, Franklin Lakes, NJ). The mice in groups B and D were sham inoculated with 100 ul of TSB without *L. murinus*.

Campylobacter jejuni

C. jejuni 11168 was initially obtained from American Type Culture Collection (Manassas, VA) and stored in glycerol stock cultures at -80°C. *C. jejuni* ATCC 700819 (referred to as *C. jejuni* 11168) was streaked onto tryptone soy agar (TSA) (Oxoid, Basingstoke, United Kingdom) that was supplemented with 5% defibrinated sheep's blood (Cleveland Scientific, Bath, OH) [1]. The plates were then incubated for 48 hours at 37°C in sealed containers containing *CampyGen*® sachet (Oxoid, Basingstoke, United Kingdom). After 48 hours, individual colonies were picked and Gram-stained to check for *C. jejuni* spiral morphology and culture purity. Furthermore, wet mount preparations were made to verify *C. jejuni* darting motility under the microscope. *C. jejuni* colonies were picked from TSA agar plates using a sterile cotton swab (Puritan Medical Products, Guilford, ME) and were resuspended in TSB to an optical density that correlated with 1×10^{10} CFU of *C. jejuni* per 100 ul of TSB. Each mouse in groups B and C received 100 ul of TSB (containing 1×10^{10} CFU of *C. jejuni)* in the stomach by a sterile 3.5-Fr feeding tube (Kendall Sovereign; Tyco Healthcare Group, Mansfield, MA) attached to a 1-ml Luer-Lok syringe (Becton Dickinson and Company, Franklin Lakes, NJ). Mice in groups A and D were sham inoculated with 100 ul of TSB not containing *C. jejuni*.

Necropsy, sample collection, and gross histopathological scoring

Fecal samples from each mouse were collected prior to euthanasia in 1.5 ml Eppendorf ® tubes (Hamburg, Germany). Mice were euthanized using a gradually administered overdose of CO2 gas in a sealed chamber according to AVMA guidelines [2]. Immediately after euthanasia, blood was obtained by cardiac puncture and

immediately mixed with 0.68% sodium citrate. Plasma was collected from blood centrifugation and quickly stored at -80 °C until further analysis could be performed.

Mice were sprayed with alcohol, placed in dorsal recumbency and the abdomen opened to remove the gut in its entirety from esophagus to rectum. The gut was placed on a sterile field, and gross histopathological changes were observed, including the thickened gut wall, enlarged ileocecocolic lymph nodes, and blood in the lumen. Gross pathological changes were graded as follows: Grade 0 = no gross pathological findings were observed, Grade 1 = One of the following findings were observed, enlarged ileocecocolic (ICC) lymph node or thickened gut wall (TGW) or enlarged (ENL) colon or cecum, Grade 2 = Two of the following gross pathological findings were observed, enlarged ICC lymph node or TGW or ENL colon or cecum, Grade 3 = Three of the following findings were observed, enlarged ICC lymph node or TW or ENL colon and cecum including bloody feces or luminal contents.

The cecum and colon were divided into 3 sections; one section of each organ was stored in formalin; the second section was flash-frozen, and the third section was streaked onto TSA plates. The TSA-CVA plates contained 10 μg vancomycin per ml 20 μg cefoperazone per ml, and 2 μg amphotericin B per ml (all antibiotics were purchased from Sigma-Aldrich, St-Louis, MO). The plates were then incubated in a sealed container containing *CampyGen*® sachet for 48 hours.

Histological staining of ICC junction

Cecum, along with 1 cm of the terminal ileum and 1 cm of the proximal colon, was excised from each mouse, and its contents were removed by placing it in phosphate-buffered saline (PBS). It was then preserved for histopathology by placing it inside a histopathological cassette (Histocette II; Simport Plastics, Beloeil, Quebec, Canada) and immersing into 10% phosphate-buffered formalin. After 24 hours the formalin was then replaced with 60% ethanol. The intestinal tissues were then sent to the Investigative Histopathology Laboratory at MSU. The samples were then vacuum infiltrated with paraffin using Sakura VIP 2000® tissue processor, followed by embedding with ThermoFisher HistoCentre III embedding station. The blocks were finely sectioned at 4-5 microns using Reichert Jung 2030® rotary microtome. Sections were then dried at 56°C using a slide incubator. Once dried, the slides were then removed from the incubator and stained on a Leica Autostainer XL® using Hematoxylin and Eosin (H&E) staining method which is as follows: Two changes of Xylene – 5 minutes each, two changes of absolute ethanol – 2 minutes each, two changes of 95% ethanol – 2 minutes each, running tap water rinse for 2 minutes, endure Hematoxylin (Cancer Diagnostics – Durham, NC) for 1 ½ minute followed directly by a 10 – 15 second differentiation in 1% aqueous glacial acetic acid and place slides under running tap water for 2 minutes to enhance nuclear detail. Slides were then given the following treatments for a defined length of time; placed in 95% ethanol for 2 minutes, 1% Alcoholic Eosin-Phloxine B for 2 minutes to stain cytoplasm, placed in 95% ethanol for 2 minutes, four changes of 100% ethanol for 2 minutes each, four changes of Xylene for 2 minutes each followed by coverslip with synthetic mounting media for permanent retention and visualization with light microscopy.

Histopathological Scoring

Histopathological scoring for the H&E stained ileocecocolic junction of each mouse was performed by a single veterinary investigator who was blinded to the sample identity. The criteria for histopathological scoring have been described in detail elsewhere [1]. In brief, the ileocecocolic junction of each mouse was scored on a scale from 0 to 44. Features used for scoring the ileocecocolic junction include the presence of mucus and inflammatory exudate in the lumen. The intestinal epithelium was evaluated for its integrity, hypertrophy of goblet cells, depletion of goblet cells, presence of intraepithelial lymphocytes, crypt hyperplasia and crypt atrophy, and crypt inflammation. The lamina propria was evaluated on the presence and distribution of immune cells and the sub-mucosa was evaluated for the development of inflammation and/or fibrosis.

16S sequence analysis

Genomic DNA was isolated from the fecal samples collected from each mouse using a FastDNA SPIN Kit for stool (MP Biomedicals, Solon, OH) according to the manufacturer's protocol. Careful attention was given to preventing laboratory contamination by the operator or within the laminar flow hood. All reagents were validated free of 16S DNA before use. For all samples, standard controls (Mock Community, ATCC/BEI) were run on reagents and kits to control for random effects of the reagents and procedures used. Once DNA was extracted from the samples, Qubit assay (Thermofisher, Waltham, MA) was performed to ascertain the concentration and 16S rRNA gene PCR was performed [3] to validate the quality of the DNA samples prior to submission to the Michigan State University Research Technology Support Facility

(RTSF) for Illumina MiSeq sequencing. At RTSF, the V4/V6 regions of the 16S rRNA gene were amplified by PCR in triplicate using two sets of barcoded primers, and the PCR products purified, combined and sequenced using Illumina MiSeq and then demultiplexed. Then the raw sequences were imported into QIIME2 for quality control. The reads were denoised with Deblur, then clustered into OTU's with vsearch, both using QIIME 2. The reads were then filtered for chimeras which were removed along with borderline chimeras. Then taxonomy was classified at 97% similarity to Silva database release 128.

The quality-controlled sequences were imported into R Studio. Raw read counts were converted into relative abundances using base R studio functions, then filtered for any reads with relative abundance higher than 1% in at least one mouse. The filtered reads were subjected to principal component analysis (PCA) and visualized using package factoextra version 1.0.5. Relative abundance bar plots were created using Microsoft Excel. Package vegan version 2.5-3 was used to run similarity percentage analysis (SIMPER) and calculate the Shannon diversity index and Bray-Curtis dissimilarity index. Package ggplot2 version 3.0.0 was used for visualization.

Semi-quantitative scoring for *L. murinus* colonization

Fecal samples were suspended in PBS and 20 ul were suspended onto MRS agar containing 10 μg vancomycin per ml, 20 μg cefoperazone per ml, and 2 μg amphotericin B per ml (all antibiotics were purchased from Sigma-Aldrich, St-Louis, MO). The plates were then incubated at 37 °C for 48 hours. The plates were then ranked from 0 – 4

according to number of *L. murinus* colonies per plate as 0 (no CFU), 1 (1 – 20 CFU), 2 (20 – 200 CFU), 3 (>200 CFU) and 4 (confluent growth) [4].

Semi-quantitative scoring for *C. jejuni* colonization

At necropsy small snips of colon tissue were streaked onto TSA-CVA agar plates. The plates were then incubated in a sealed container containing *CampyGen*® sachet at 37 °C for 48 hours. The plates were then ranked from 0 – 4 according to the number of *C. jejuni* colonies per plate as 0 (no CFU), 1 (1 – 20 CFU), 2 (20 – 200 CFU), 3 (>200 CFU), and 4 (confluent growth) [3].

Statistical analysis

GraphPad Prism version 9.2.0 was used for the preparation of graphs and statistical analysis. Statistical analysis was performed using Kruskal-Wallis one-way-analysis of variance. The P values between different groups were summarized as ns: $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Results

Prophylactic inoculation of *L. murinus* did not prevent *C. jejuni* induced gross pathology in mice

Prophylactic treatment of *L. murinus* did not prevent presence of gross pathology in *C. jejuni* infected BALB/c IL-$10^{-/-}$ mice (Fig 3.1). 2/10 mice in the positive control group for *C. jejuni* (group B) had enlarged ICC lymph nodes, whereas 7/10 mice in group B had

enlarged ICC lymph nodes and thickened gut wall (TGW). In the test group (group C), 3/10 mice had enlarged ICC lymph node and 3/10 mice had enlarged ICC lymph nodes and TGW. However, 1/10 mice in group C presented all 3 gross histological findings i.e., enlarged ICC lymph node, TGW and bloody intestinal contents. Interestingly, 3/10 mice in the test group did not present gross pathological lesions, whereas only 1/10 mice in the positive control group for *C. jejuni* remained protected from developing gross pathological lesions. Furthermore, no gross pathological lesions were observed in the *L. murinus* control group (group A). However, one mouse developed mild symptoms of spontaneous colitis in the vehicle control group (group D). These results indicate that prophylactic inoculation of *L. murinus* was not effective in preventing *C. jejuni* induced gross pathological lesions in the BALB/c IL-$10^{-/-}$ mice.

Prophylactic inoculation of *L. murinus* did not reduce histopathologic lesions in the ileocecocolic junctions

L. murinus pre-enrichment in the test group mice had no significant effect on reducing the histopathologic lesions in ileocecocolic (ICC) junctions of mice in group C as compared to the mice in the *C. jejuni* positive control group B (Fig 3.2). The mean histopathology score in the test group was 22.8 ± 7.45, whereas the mean histopathology score in the positive control group was 21.9 ± 7.35. There was no significant difference ($P > 0.99$) between the histopathology scores of the test group (group C) and the positive control group for *C. jejuni* (group B). Histopathological scores below 10 are considered normal in IL-$10^{-/-}$ mice [1]. 1/10 mice in group C received a score below 10. However, no mouse in group B received a score below 10. The mean histopathology scores in the *L.*

murinus control group (group A) and the vehicle control group (group D) were 7.1 and 9.2 respectively. These results indicate that prophylactic administration of *L. murinus* was not effective in attenuating *C. jejuni* induced gut pathology in BALB/c IL-$10^{-/-}$ mice. Furthermore, these results also indicate that *L. murinus* is a commensal and does not induce gastrointestinal pathology in the BALB/c IL-$10^{-/-}$ mice.

L. murinus pre-enrichment increased *C. jejuni* colonization in the cecum

Mice in the test group (group C) had a higher *C. jejuni* count in the cecum as compared to the mice in the positive control group for *C. jejuni* (group B). 8/10 mice in the *C. jejuni* infected group received a score of 1. In comparison, 8/10 mice in the *L. murinus* treated *C. jejuni* infected group (group C) received a score of 2. Interestingly, no *C. jejuni* was detected from the cecum of 2/10 mice in the *C. jejuni* infected group. As expected, no *C. jejuni* was detected in the *L. murinus* control group or vehicle control group. These results indicate that prophylactic administration of *L. murinus* increases cecal colonization of *C. jejuni* in the BALB/c IL-$10^{-/-}$ mice.

Increased *L. murinus* colonization in the test group

Increased *L. murinus* colonization was found in the test group as compared to the *L. murinus* control group (Fig 3.5). In group A, 1/10 mice received a score of 2, 2/10 mice received a score of 3, and 4/10 mice received a score of 4. Whereas in group C, 1/10 mice received a score of 2, 4/10 mice received a score of 3, and 5/10 mice received a score of 4. Furthermore, no *L. murinus* was detected from group B and group D mice.

These results indicate that *L. murinus* colonization was enhanced in the test group as compared to the *L. murinus* control group.

C. jejuni infection changed the gut microbiota composition

16S sequence analysis revealed that the gut microbiota composition was significantly changed in the *C. jejuni* infected groups (group B and C) as compared to the non-*C. jejuni* infected groups (group A and D). The relative abundance of the genus *Enterococcus* was also higher in the *C. jejuni* infected groups as compared to non-*C. jejuni* infected groups (Fig 3.6). The relative abundance of genus *Enterococcus* was 21.68% in group B and 17.45% in group C. The relative abundance of the genus *Enterococcus* in group A was 0.022%, and in group D was 0.015%. However, the relative abundance of the *Lachnospiraceae* NK4A136 was higher in the non-*C. jejuni* infected groups as compared to the *C. jejuni* infected groups. The relative abundance of the bacterium *Lachnospiraceae* NK4A136 was 19.62% in group A, and 18.89% in group D. Whereas, the relative abundance of the bacterium *Lachnospiraceae* NK4A136 was 4.2% and 2.4% in group B and C respectively. Interestingly, members of the genus *Lactobacillus* were also identified in the 16S sequence analysis of the *C. jejuni* only group (group B). However, no bacterial growth was observed when the fecal samples of group B mice were cultured on MRS agar plates.

A principal component analysis (PCA) plot based on the genus level relative abundance profile showed that group A and group D overlapped with each other (Fig 3.8). However, groups B and C were relatively dispersed in the PCA plot. Dimension 1 of the

PCA plot (Dim 1) explained 58.8% of the variability and dimension 2 of the PCA plot (Dim 2) explained 17.4% of the variability. Furthermore, the Shannon-diversity index (Fig 3.7) revealed that *C. jejuni* infected groups (group B and C) had a significantly decreased bacterial diversity as compared to the non-*C. jejuni* infected groups (Group A and D). There was a statistically significant difference ($P < 0.05$) in the Shannon-diversity scores between group A and group C. These results suggest that *C. jejuni* infection significantly changes the gut microbiota composition in the BALB/c IL-$10^{-/-}$ mice.

Discussion

C. jejuni is the leading cause of bacterial diarrheal illness in the United States [5]. *C. jejuni* infection can lead to Guillain-Barré syndrome, an auto-immune disease in which molecular mimicry between *C. jejuni* outer surface and nerve gangliosides causes patients to produce autoantibodies against the peripheral nerves, which can lead to paralysis [6]. At present, macrolide antibiotics are the drug of choice to treat *C. jejuni* infections, yet, the high rates of antibiotic resistance in *C. jejuni* have led to reduced treatment efficacy [7]. Therefore, the CDC have categorized drug-resistant *Campylobacter* as a serious public health threat [8]. It is important to identify alternatives to antibiotics to attenuate *C. jejuni* pathogenesis in humans and animal populations. Some members of the *Lactobacilli* are commonly used as probiotics [9]. Previous studies have also reported the antimicrobial activity of *Lactobacillus* spp. against *C. jejuni* in vitro [10, 11]. In this study, we determined the effect of prophylactic administration of a putative probiotic strain (*L. murinus*) on the pathogenesis of *C. jejuni* in a mouse model.

These results indicate that *L. murinus* pre-treatment was not able to reduce the intestinal burden of *C. jejuni* in BALB/c IL-10$^{-/-}$ mice. In fact, there were higher *C. jejuni* counts in the co-infected test group C as compared to the *C. jejuni* alone control group B. Similar findings were reported by *Bereswill* et al., [12] where they tested the efficacy of another species *L. johnsonii* on *C. jejuni* virulence in a secondary abiotic mouse model (generated by broad-spectrum antibiotic treatment). *L. johnsonii* was administered orally to abiotic mice in a prophylactic or a therapeutic manner. Neither the prophylactic nor the therapeutic administration of *L. johnsonii* was able to decrease the intestinal burden of *C. jejuni* [12]. However, a previous study reported a beneficial effect of a combination of probiotics against *C. jejuni* in BALB/c mice. BALB/c mice were transplanted with the defined human gut microbiota to generate a human-microbiota-associated (HMA) mouse model. HMA mice were given a combination of probiotic strains containing *L. acidophilus*, *L. rhamnosus*, *L. casei*, *L. gasseri*, *L. reuteri, Bifidobacterium thermophilus*, *B. adolescentis,* and *B. longum* before *C. jejuni* challenge. The probiotic-fed mice were able to completely eradicate *C. jejuni* in one week after challenge [13]. However, the use of a single probiotic strain such as *L. johnsonii* was not effective in eradicating *C. jejuni* from secondary abiotic mice [12], whereas a combination of probiotics successfully eradicated *C. jejuni* in HMA mice [13]. Our results from these studies provide an example where the use of a single probiotic strain was not able to eradicate *C. jejuni* from BALB/c IL-10$^{-/-}$ mice. Although using a combination of probiotic strains may produce colonization resistance against *C. jejuni* in the BALB/c IL-10$^{-/-}$ mice, it may be more fruitful to focus on determining why some strains of *Lactobacillus* spp. enhance the growth of *C. jejuni* under *in vivo* conditions. Importantly, the differential response of probiotics in eradicating *C.*

jejuni may be due to the use of mouse models having different immunological features. BALB/c IL-$10^{-/-}$ mice are immune compromised mice and have a reduced population of intestinal Treg ($CD4^+$ $CD25^+$ $Foxp3^+$ T-cells) cells [14] and are therefore prone to develop spontaneous colitis [15]. Furthermore, IL-10 deficiency in BALB/c mice renders them more susceptible to developing spontaneous colitis as compared to C57BL/6 IL-$10^{-/-}$ mice [15]. In contrast, HMA mice are immune-competent mice having a normal population of intestinal Treg cells. Although a portion of patients that develop inflammatory bowel disease are deficient in IL-10 [16]. These patients and BALB/c IL-$10^{-/-}$ mice may not be an appropriate model for dissecting the role of probiotics in attenuating the *C. jejuni* induced inflammatory responses in the gut mucosa. This is particularly true if the mechanism of action involves an active role for IL-10 downregulation in the colon where *C. jejuni* resides.

The results from the 16S sequence analysis suggest that members of the genus *Lactobacillus* were present in all four groups. However, we were only able to detect *L. muirnus* in the fecal samples of *L. murinus* only group (group A) and the test group (group C) mice. The disparity between the 16S and bacterial culture results can be explained by the fact that 16S sequencing relies on amplifying the hypervariable regions (V1 – V9) of the bacterial 16S rRNA gene [17]. One of the limitations of the 16S sequence analysis is that the resolution is not high enough to differentiate the closely related species in a particular genus [18]. Therefore, it is likely that the *Lactobacillus* genus reads in the 16S analysis (Fig 3.6) consist of species other than *L. murinus*. However, those putative *Lactobacillus* species did not grow on the MRS agar supplemented with antibiotics such

as vancomycin and cefoperazone and thus were not successfully isolated from these fecal samples. The antibiotic resistant characteristics of the inoculated *L. murinus* strain has been described by Jean Brudvig [4]. Further work is needed to determine if they were actually *Lactobacilli* or another closely related bacterial species.

C. jejuni colonization was increased in the test group (group C) as compared to the *C. jejuni* positive control group (Fig 3.4). The reason for the increased *C. jejuni* load in the test group is not clear. However, a recent finding by Sinha *et al*., [19] help explain this phenomenon. *C. jejuni* contain a phosphate-transporter PstSCAB that utilize lactate as a substrate. Sinha *et al*., reported that the PstSCAB transporter was crucial for the growth of *C. jejuni* in the lactate containing medium [19]. Furthermore, deletion of PstSCAB transporter resulted in reduced fitness of *C. jejuni* colonization in chickens. The bacterial load of the PstSCAB mutants were significantly less in the cecum of chickens as compared to wild-type *C. jejuni* [19]. It is known that *Lactobacillus* species produce lactate as a major end product of fermentation [20]. Therefore, it is likely that the lactate produced by *L. murinus* was being utilized as a substrate by *C. jejuni* in the test group, thereby enhancing the colonization levels of *C. jejuni* in the test group.

Results from 16S sequence analysis also show that *C. jejuni* 11168 colonization of the BALB/c IL-10$^{-/-}$ mice was associated with gut microbiota dysbiosis. Shannon-diversity index scores were significantly lower in the *C. jejuni* infected groups (group B and C) as compared to non-*C. jejuni* infected groups. The gut microbiome composition was significantly changed in the *C. jejuni* infected groups as compared to the non-*C. jejuni*

infected groups. *C. jejuni* infected mice groups were having a higher relative abundance of the genus *Enterococcus*. Members of the genus *Enterococcus* can be opportunistic pathogens [21], particularly *E. faecalis* and *E. faecium* are important nosocomial infections [22]. The role of *Enterococcus* in the pathogenesis of *C. jejuni* warrants further investigation.

C. jejuni associated gut microbiota dysbiosis has also been reported by *Ione* et al [23]. Interestingly, certain members of the gut microbiota were severely depleted in the *C. jejuni* infected groups as compared to the non-*C. jejuni* infected groups. The relative abundance of the genus *Lachnospiraceae NK4A136 group* was 19.62% and 18.89% in groups A and D respectively, whereas groups B and C were only having a relative abundance of 4.2% and 2.4% respectively. *L. NK4A136 group* is associated with health and anti-inflammatory properties [24]. Members of the genus *Lachnospiraceae* are important butyrate producers residing in the colonic gut mucosa [25]. Butyrate is one of the most abundant short-chain fatty acids (SCFA's) found in the gut, along with acetate and propionate [26]. The functional role of SCFA's in protection against intestinal inflammation is well-known [27]. However, the mechanisms by which SCFA's attenuate inflammatory responses at the gut mucosa has remained elusive.

A novel role of butyrate as a ligand of AHR has been described recently [28]. AHR is a ligand-dependent transcription factor, and its role in maintaining intestinal homeostasis has recently begun to be unraveled [29]. AHR appears as a promising target to develop novel therapeutics to attenuate pro-inflammatory responses in the gut [30]. In

the previous study, we reported that isolated *L. murinus* strain activated AHR in the intestinal epithelial cells to protect them from TNF-alpha-induced inflammatory insults [31]. In this study, we do not know if *L. murinus* was able to activate AHR in BALB/c IL-$10^{-/-}$ mice. However, the ability of *Lactobacillus* strains, including *L. murinus* CNCM 1-509 to activate AHR has been reported by *Lamas* et al [32]. Inoculating mice with *L. murinus* CNCM I-5020, *L. reuteri* CNCM I-5022, and *L. taiwanensis* CNCM I-5019 or treatment with AHR agonist attenuated chemically induced colitis in *Card9*-deficient mice [32]. These results suggest that *L. murinus* can activate AHR and attenuate some forms of colitis in mice.

Taken together, our results indicate that prophylactic inoculation of *L. murinus* alone was not effective in providing colonization resistance against *C. jejuni* in the BALB/c IL-$10^{-/-}$ mice. Furthermore, the results demonstrate that *C. jejuni* infection induces gut microbiota dysbiosis and inflammation in the BALB/c IL-$10^{-/-}$ mice and show the dominant bacterial taxa associated with that outcome. These results are also important because they show that *C. jejuni* colonization of the gut of an immunocompromised mouse model produces dysbiosis and reduce butyrate producing commensals in the gut. Furthermore, these results shed light on the role of probiotics in exacerbating *C. jejuni* disease under IL-10 deficient conditions and demonstrate the need to discern the underlying *L. murinus* gene expression and ecological conditions that fostered this outcome. Future research to study the effect of a combination of probiotics on the pathogenesis of *C. jejuni* is warranted.

APPENDIX

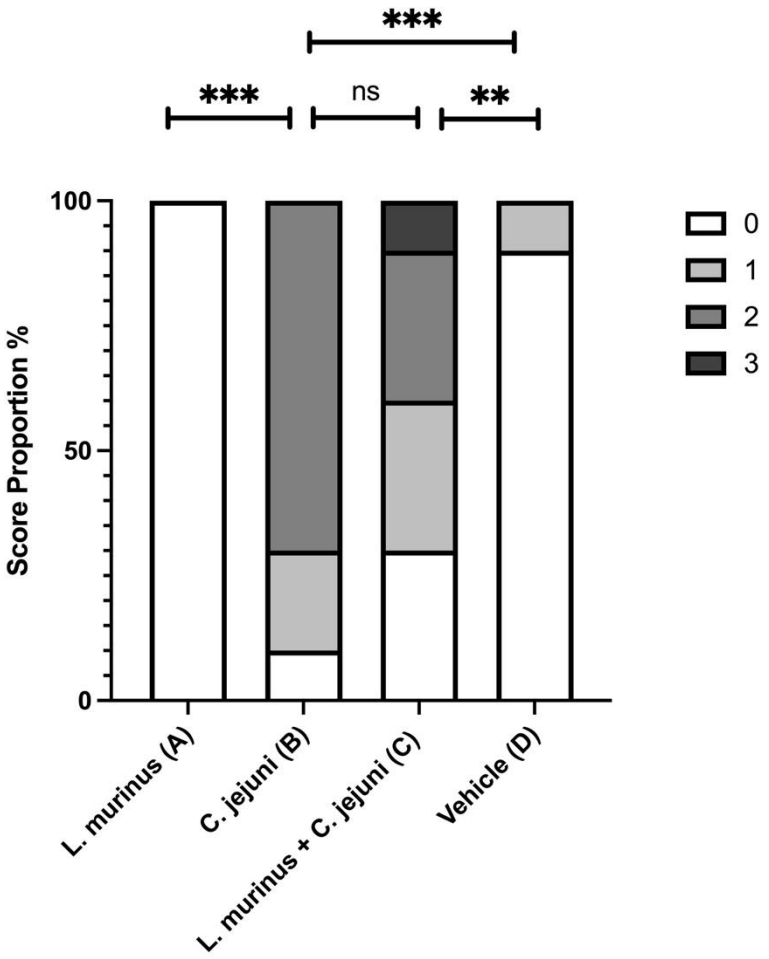


Figure 3.1) Gross pathology scoring

Mice were scored for gross pathological lesions at necropsy on a scale of 0 – 3. Statistical analysis was performed using Kruskal-Wallis one-way analysis of variance (P = 0.00026). Pairwise comparisons were made by Mann-Whitney U-test with Bonferroni correction. There was no statistically significant difference between the *C. jejuni* positive control group (group B) and the test group (group C). There was a statistically significant difference (P < 0.001) between the *L. murinus* control group (group A) and the *C. jejuni* control group (group B). Furthermore, there was a statistically significant difference (P < 0.01) between the test group (group C) and the vehicle control group (group D).

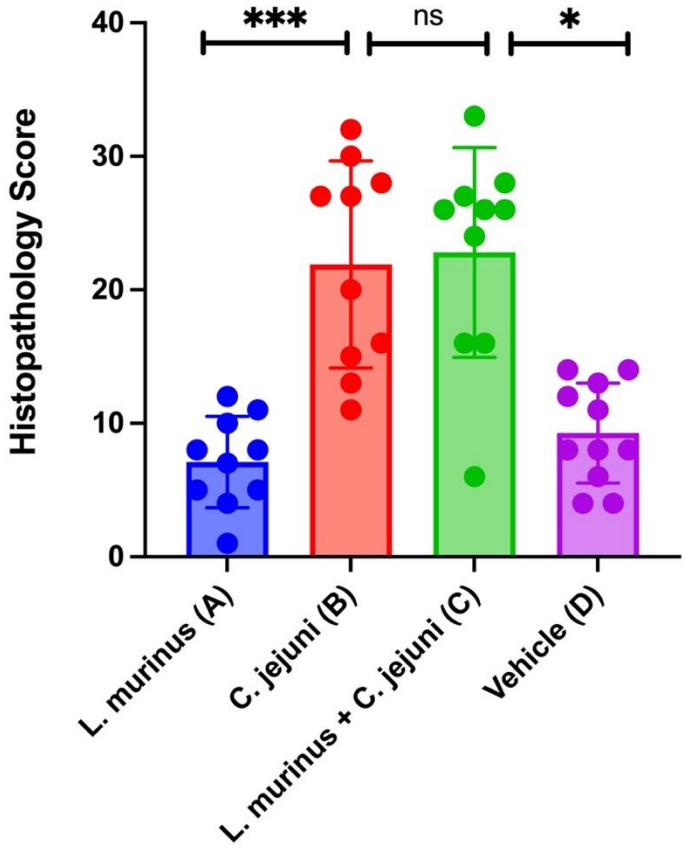


Figure 3.2) Histopathological scoring of the ileocecocolic junctions

Mice were scored for histopathological lesions on a scale from 0 to 44. Statistical analysis was performed using Kruskal-Wallis one-way analysis of variance ($P < 0.0001$). The pairwise comparisons between different groups were performed using Dunn's multiple comparison test. There was no statistically significant difference between the group B and group C mice. However, there was a statistically significant difference ($P < 0.001$) between the *L. murinus* control group and the *C. jejuni* control group. Furthermore, there was a statistically significant difference ($P < 0.05$) between the test group (group C) and the vehicle control group (group D).

Figure 3.3 A (L. murinus only group)

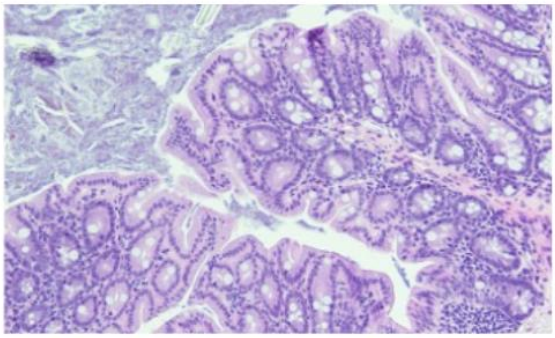


Figure 3.3 B (C. jejuni only group)

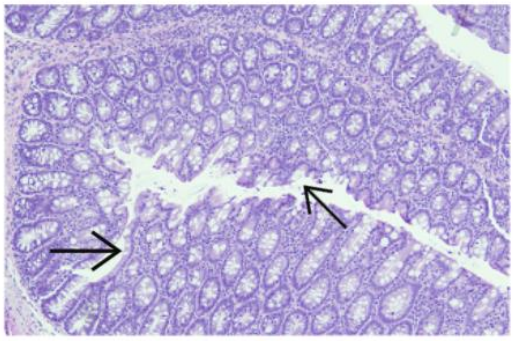


Figure 3.3 C (L. murinus + C. jejuni group)

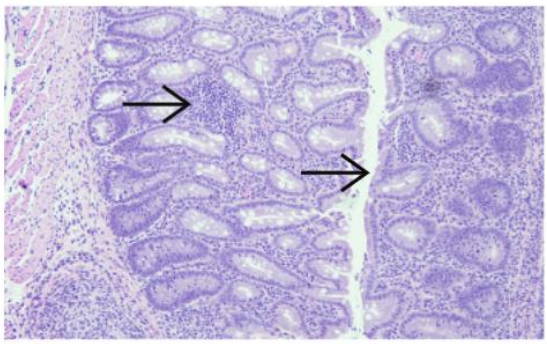


Figure 3.3 D (Vehicle control group)

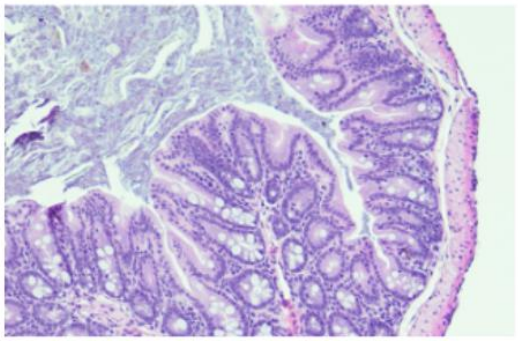


Figure 3.3) Hematoxylin and Eosin-stained (H&E) sections of the ileocecocolic junctions

Images of the H&E-stained sections of the ileocecocolic junctions. Figure 3.3A, represents the H&E-stained ICC junction of a mouse in group A. No significant histopathological lesions were seen in group A mice. In figure 3.3B, erosion of the intestinal epithelium and hyperplasia can be seen in a group B mouse. Severe histopathological lesions were observed in group B mice. In figure 3.3C, erosion of the intestinal epithelium and infiltration of monocytes can be seen in a group C mouse. Similarly, severe histopathological lesions were observed in group C mice. In figure 3.3D, no significant histopathological lesions were seen in vehicle control group mouse.

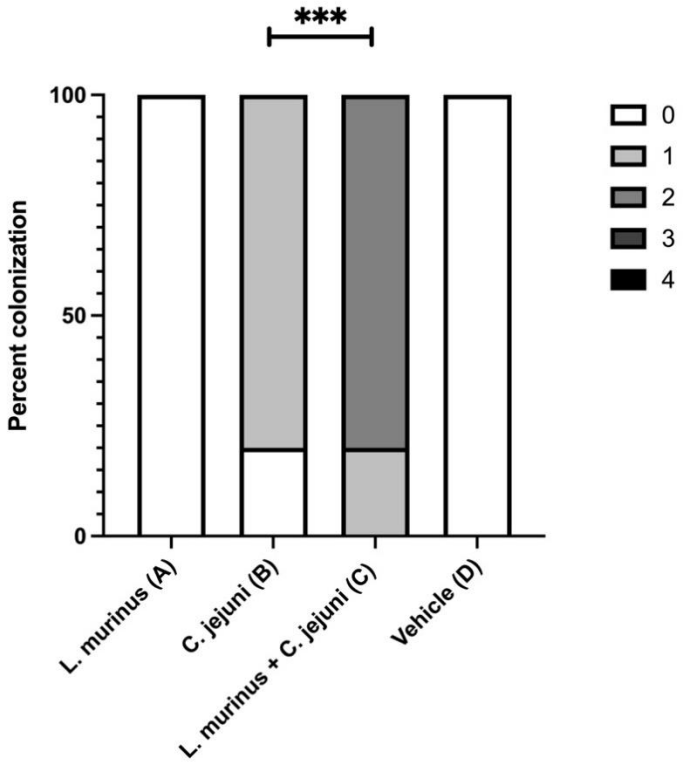


Figure 3.4) *C. jejuni* colonization scores

Cecal tissue was collected from each mouse during necropsy and was cultured for *C. jejuni* under microaerophilic conditions. The plates were ranked from 0 – 4 according to number of *C. jejuni* colonies per plate as 0 (no CFU), 1 (1 – 20 CFU), 2 (20 – 200 CFU), 3 (>200 CFU) and 4 (confluent growth). Statistical analysis between groups was performed using Kruskal-Wallis one-way analysis of variance ($P < 0.0001$). Pairwise comparisons were made by Mann-Whitney U-test with Bonferroni correction. There was a statistically significant difference ($P < 0.001$) between the *C. jejuni* control group (group B) and the test group (group C).

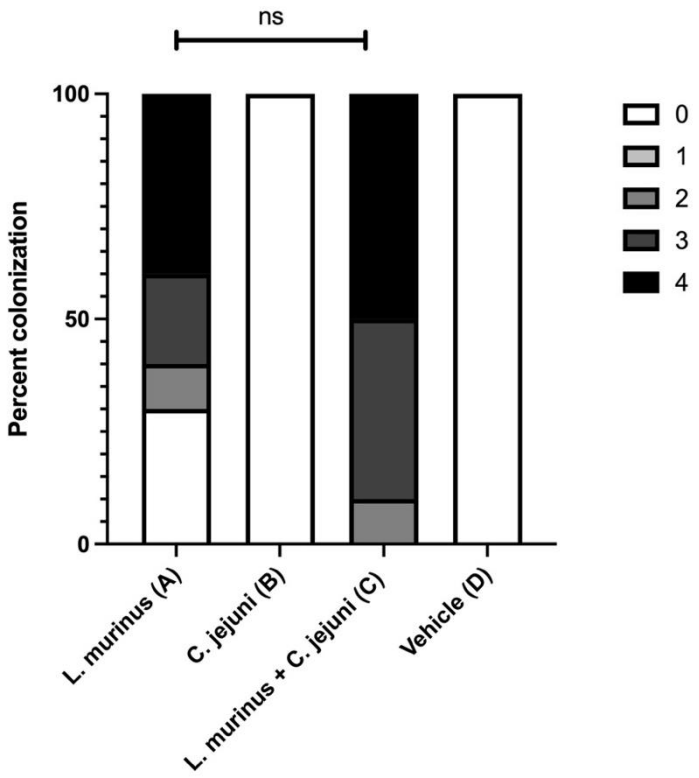


Figure 3.5) *L. murinus* colonization scores

Fecal samples were taken from each mouse during necropsy and were cultured for *L. murinus*. The plates were then ranked from 0 – 4 according to the number of *L. murinus* colonies per plate as 0 (no CFU), 1 (1 – 20 CFU), 2 (20 – 200 CFU), 3 (>200 CFU), and 4 (confluent growth). Statistical analysis between groups was performed using Kruskal-Wallis one-way analysis of variance ($P < 0.0001$). Pairwise comparisons were made by Mann-Whitney U-test with Bonferroni correction. There was no statistically significant difference between the *L. murinus* control group (group A) and the test group (group C).

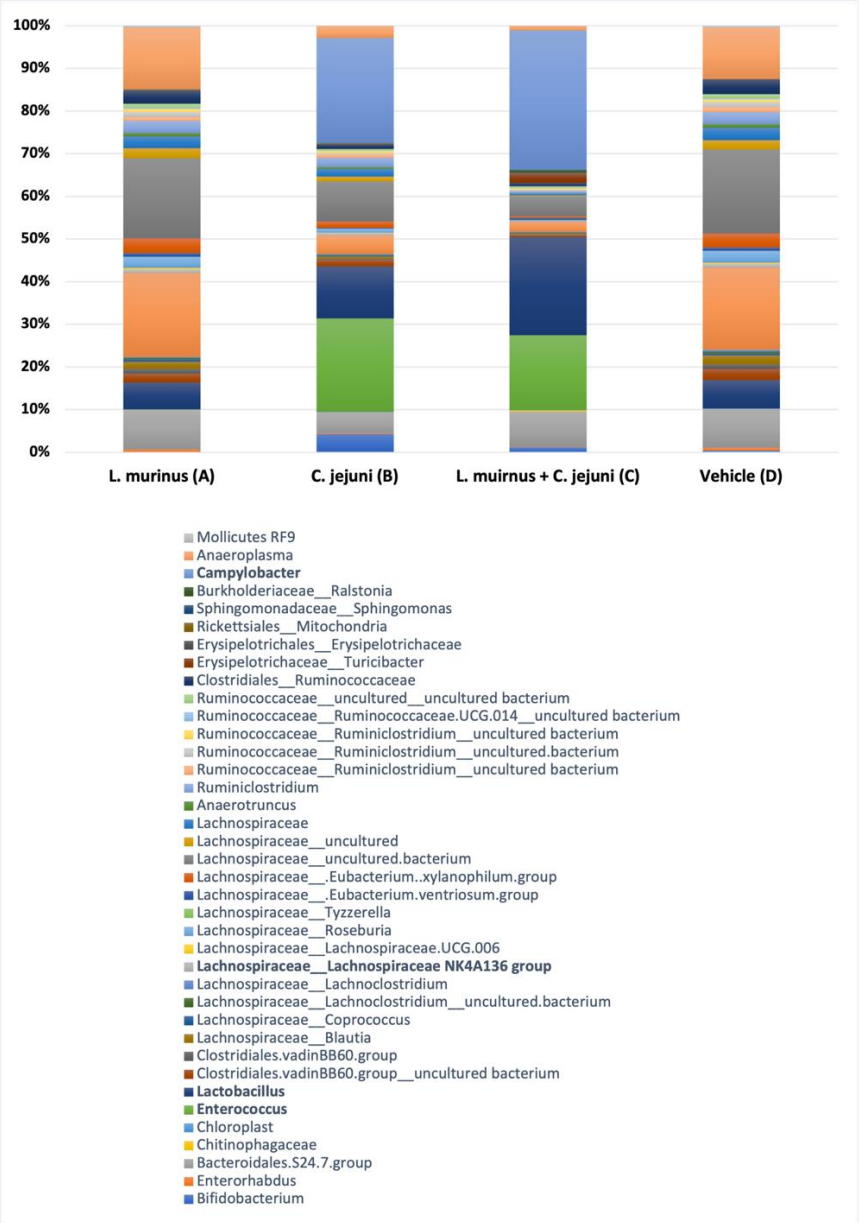


Figure 3.6) 16S rRNA gene sequence analysis of fecal DNA

16S rRNA gene sequence analysis was performed on fecal samples. The bar plot describes the percent relative abundance of OTU's on the y-axis. The different groups are plotted against the x-axis. The landscape of the gut microbiome composition was significantly changed in the *C. jejuni* infected groups (B and C).

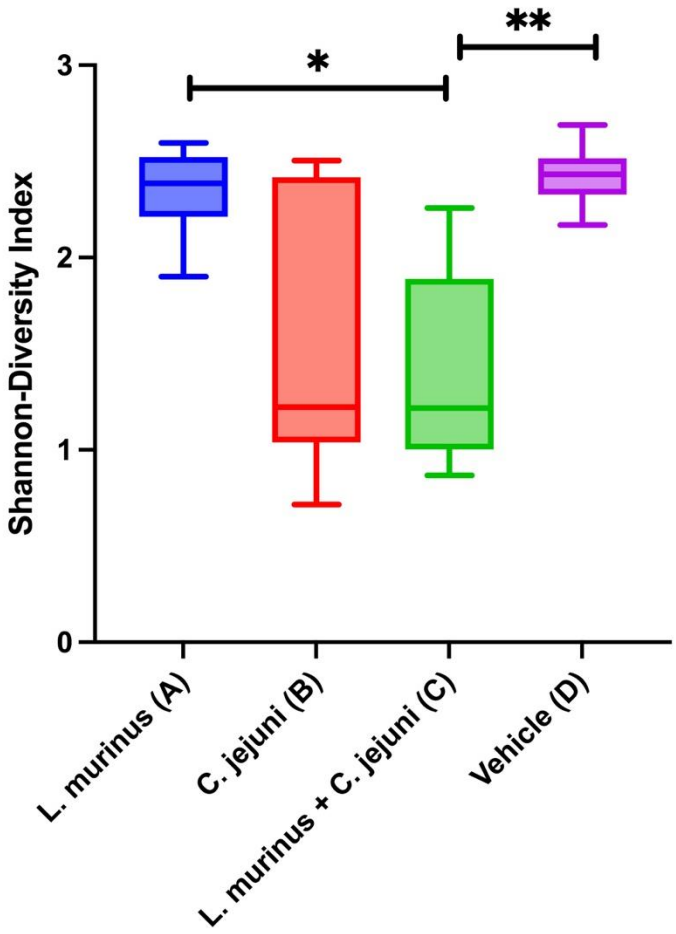
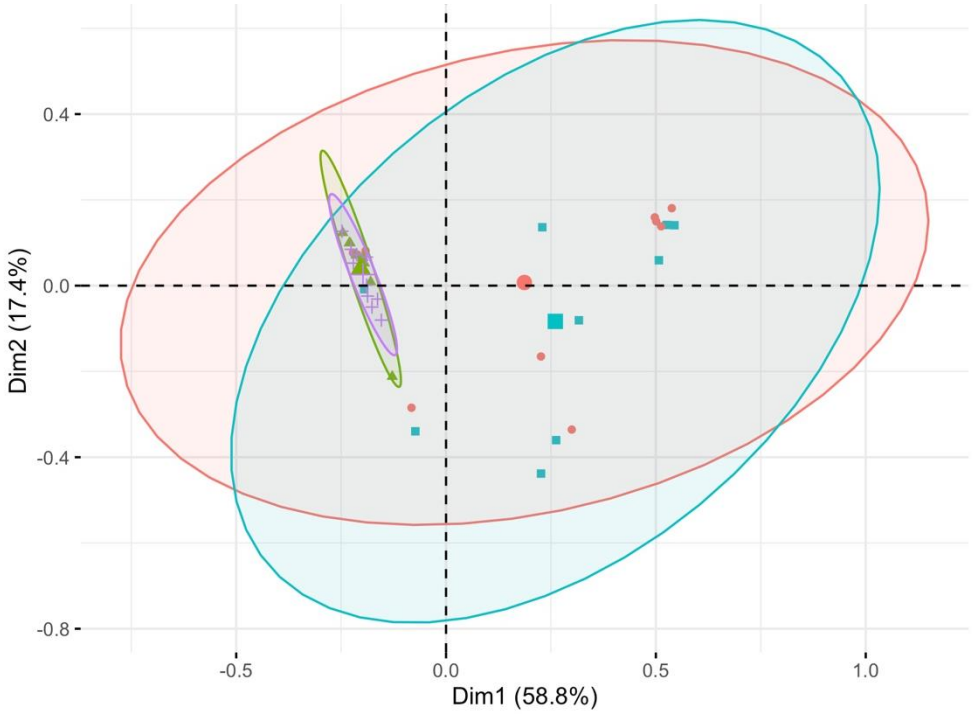


Figure 3.7) Shannon-Diversity Index

The Shannon-diversity index was calculated for all the experimental groups. Statistical analysis was performed using Kruskal-Wallis one-way-analysis of variance ($P = 0.0004$). The pairwise comparisons between different groups were performed using Dunn's multiple comparison test. There was a statistically significant difference ($P < 0.05$) between the *L. murinus* control group and the test group. Furthermore, there was a statistically significant difference ($P < 0.01$) between the vehicle control group and the test group.



Groups

L. murinus only (A)
C. jejuni only (B)
L. murinus + *C. jejuni* (C)
Vehicle (D)

Figure 3.8) Principal Component Analysis

Principal Component Analysis (PCA) of the 16S rRNA gene sequencing of the fecal samples. Dimension 1 explains 58.8% of variation whereas dimension 2 explains 17.4% of variation between the samples.

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CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS.

IBD is an umbrella term encompassing ulcerative colitis and Crohn's disease [1]. Chronic, relapsing inflammation of the gut is a typical manifestation of IBD. In addition, the mechanisms of disease manifestation in human campylobacteriosis, such as damage to the gut epithelium [2] and dysbiosis of the gut microflora [3] are similar to the pathogenesis of IBD. Furthermore, *C. jejuni* infection may contribute to the pathogenesis of IBD [4]. Therefore, the pathogenesis of *C. jejuni* infection and disease manifestation of IBD greatly overlap one another.

In the 2nd chapter, we described a novel mechanism for the beneficial effect of probiotics against inflammatory responses operating at the gut epithelial surface. We reported that probiotics such as *L. murinus* activate AHR to maintain intestinal homeostasis against inflammatory insults [5]. We also described the role of AHR activation (by defined AHR ligands or *L. murinus*) in the attenuation of pro-inflammatory responses in the *in-vitro* model of colitis [5]. This finding is significant as it identifies a novel therapeutic target to attenuate inflammation in IBD.

However, we still don't know what specific secreted product by *L. murinus* was able to activate the AHR. In the future, identifying the secreted product(s) from *L. murinus* that act(s) as AHR ligand(s) would be significant. To identify the secreted products of *L. murinus,* we can use fractionation-based assays such as liquid-chromatography mass-spectrometry (LCMS) [6]. LCMS technique is best suited for bacterial secreted proteins since identifying membrane proteins by LCMS is challenging [6]. Since we will be interested in identifying the secreted products of *L. murinus*, the LCMS technique can be

a powerful approach. After identifying secreted proteins through LCMS, we can employ *in-vitro* assays to validate if a secreted protein(s) activates AHR. Once we identify the secreted protein(s) that acts as an AHR ligand, we can generate *L. murinus* mutants lacking the AHR ligand protein to conduct proof-of-concept assays. Previously, we have reported that *L. muirnus* activates AHR in an MOI (multiplicity of infection) dependent manner, and *L. murinus* pre-treatment of the human intestinal epithelial cells attenuated the TNF-alpha induced inflammatory responses [5]. Therefore, generating *L. murinus* mutants lacking the AHR binding protein(s) and evaluating their pre-treatment efficacy in the attenuation of colitis can be valuable in providing a proof-of-concept for the functional role of these secreted protein(s). Furthermore, identifying specific secreted products from *L. murinus* having AHR activation potential may result in the development of synthetic dietary compounds with beneficial health effects.

The results in the 2nd chapter indicate an association of AHR activation with the attenuation of pro-inflammatory responses in the epithelial cell model of colitis [5]. However, a proof-of-concept for the role of AHR in the attenuation of colitis is lacking. Future experiments can be designed to provide a proof-of-concept for the role of AHR in gut homeostasis. An AHR deficient intestinal epithelial cell-line can be used to conduct *in-vitro* proof-of-concept studies. To the best of our knowledge AHR$^{-/-}$ Caco-2 cell line is not commercially available. However, gene-editing techniques such as CRISPR-Cas9 can be used to knock out (KO) AHR gene in the Caco-2 cells. The development of AHR$^{-/-}$ Caco-2 cell line will be a valuable resource for elucidating the role of AHR in maintaining gut homeostasis.

In the 3rd chapter, we tested the ability of *L. murinus* to protect against *C. jejuni* induced colitis in BALB/c IL-$10^{-/-}$ mice. The results indicate that prophylactic inoculation of *L. murinus* did not protect BALB/c IL-$10^{-/-}$ mice from developing *C. jejuni* induced colitis. Furthermore, the gut microbiome composition was significantly changed in the *C. jejuni* infected groups compared to the non-*C. jejuni* infected groups. These findings are of significance as they indicate that probiotics, although "generally recognized as safe" (GRAS) [7] may complement the growth and pathogenesis of pathogenic bacteria such as *C. jejuni* in IL-$10^{-/-}$ mice.

We used BALB/c IL-$10^{-/-}$ mice to determine the prophylactic effect of *L. murinus* on the *C. jejuni* pathogenesis in a mouse model. We chose IL-$10^{-/-}$ mice based upon their ability to mimic clinical features of human *Campylobacteriosis* (such as enteritis) upon infection with a colitogenic strain of *C. jejuni* [8]. Since wild-type (WT) mice serves as *C. jejuni* colonization models but do not develop enteritis upon infection with *C. jejuni* [9]. Although IL-10 deficient mice are a robust model for *C. jejuni* induced colitis [8] they also have certain limitations. One limitation of the IL-$10^{-/-}$ mouse model is that they have a reduced population of $CD4^+$ $CD25^+$ $Foxp3^+$ T-reg cells in the gut compared to WT mice [10]. This limitation of the IL-$10^{-/-}$ deficient mice hinders their use to study the beneficial effect of the class of probiotics which specifically function by targeting $CD4^+$ $CD25^+$ $Foxp3^+$ T-reg cells in the gut [11]. Most probiotic strains including those of genus *Lactobacillus* target T-reg cells in the gut to provide beneficial health effects [11, 12]. A previous study has reported that oral inoculation of *L. rhamnosus* significantly increased the percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ T-reg cells in the intestinal tissues of the wild-type

(WT) BALB/c mice [13]. These results indicate that IL-10$^{-/-}$ mice are not a suitable model to study the beneficial effect of probiotics which function by stimulating CD4^{+} CD25^{+} Foxp3^{+} T-reg cells in the gut.

To address this problem, future studies can be designed to determine the direct effect of AHR activation on *C. jejuni* colonization in the WT mice. WT C57BL/6 mice are a model for *C. jejuni* colonization, however, they do not develop enteritis upon infection with a colitogenic strain of *C. jejuni* [14]. AHR can be activated in WT C57BL/6 mice by oral gavage of TCDD for multiple days [15]. After multiple gavages of TCDD for consecutive days, mice will be challenged with *C. jejuni*. Mice will be sacrificed 5-weeks post-C. *jejuni* challenge and intestinal samples will be collected to access *C. jejuni* colonization in the gut. Furthermore, intestinal tissue sample will also be subjected to flow cytometry to determine the effect of TCDD on CD4^{+} CD25^{+} Foxp3^{+} T-reg cells in the lamina propria. Fecal samples will also be collected from mice during the necropsy to assess the level of IgA in mice in response to the TCDD treatment. The results from this experiment will determine if AHR activation by TCDD reduces *C. jejuni* colonization in a mouse model.

Due to rising antimicrobial resistance in *C. jejuni,* it is imperative to identify novel therapeutic targets to ameliorate *C. jejuni* pathogenesis. Recently, AHR appeared as a promising target to enhance host resistance against lethal bacterial pathogens such as *Streptococcus pneumoniae* [16, 17], *Listeria monocytogenes* [18] and *Clostridium difficile* [19]. However, there is a knowledge gap in elucidating the role of the AHR in enhancing

host resistance against *C. jejuni* infection. The results of this thesis provide an initial understanding of the role of AHR in *C. jejuni* pathogenesis. However, future studies are warranted to further dissect the role of this receptor in the *C. jejuni* disease manifestation.

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