AKKERMANSIA MUCINIPHILA & INTESTINAL INFLAMMATION

EFFECTS OF THE NEXT GENERATION PROBIOTIC, AKKERMANSIA MUCINIPHILA, ON INTESTINAL INFLAMMATION AND BARRIER FUNCTION

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Descriptive note

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Lay abstract

Akkermansia muciniphila is a bacterium that accounts for 1-5% of the human fecal microbiota and has been shown to stimulate intestinal mucus production and strengthen the gut barrier. Though several studies have linked inflammatory bowel disease (IBD) with decreased levels of *A. muciniphila,* the precise role of this microbe in gut inflammation is unknown. In this research, we investigate the role of *A. muciniphila* in gut barrier function and inflammation. Across several experimental models, we find that supplementation with live, and in some cases, pasteurized *A. muciniphila,* can help curb established inflammation and promote a more anti-inflammatory gut environment. We also identify that these changes are independent of this bacteria's ability to influence mucin 2, the main building block of intestinal mucus. This study has the potential to both enhance our understanding of microbial influence in intestinal inflammation and may also lead to the development of future treatments for IBD.

Abstract

Inflammatory bowel disease (IBD), characterised by chronic intestinal inflammation, is hypothesised to arise from the interplay between susceptibility genes, the immune system, environmental factors, and gut microbiota.

Akkermansia muciniphila is a symbiotic bacterium that accounts for 1-5% of the human fecal microbiota. This microbe has been hailed as a next-generation probiotic, principally with regards to its plethora of beneficial host interactions, including the ability to influence mucin secretion and strengthen the intestinal barrier. Though a clear-cut role and mechanism by which *A. muciniphila* influences inflammatory conditions is unknown, evidence indicates this microbe is depleted in IBD, suggesting it may have protective effects that are lost in these conditions.

Here, we investigate the role and mechanism of *A. muciniphila* in intestinal inflammation and its influence on intestinal barrier function by utilizing barrier-disrupting models of colitis, including dextran sulphate sodium (DSS) and *Trichuris muris*. Though only minor benefits were derived from this microbe in germ-free mice, in specific pathogen-free (SPF) mice, administration of pasteurized *A. muciniphila* in a DSS recovery model ameliorated inflammation severity and promoted recovery compared to controls. When gavaged prior to DSS administration, both live and pasteurized *A. muciniphila* failed to diminish inflammatory markers indicating minimal preventative effects. *T. muris*-infected SPF mice treated with live *A. muciniphila* showed increased levels of Th2 and anti-inflammatory cytokines, decreased worm burden, and enhanced levels of the mucin, *Muc5ac,* compared with those receiving control broth or pasteurized

bacteria. Further, both live and pasteurized *A. muciniphila* ameliorated the severity of inflammation in mucin 2 deficient (*Muc2*$^{-/-}$) mouse model of spontaneous colitis, indicating that these protective effects are Muc2-independent.

These observations provide us not only with an enhanced understanding of the role *A. muciniphila* plays in the pathogenesis of intestinal inflammatory conditions but also may fuel novel avenues of treatment for those with IBD.

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undoubtedly fostered my love of learning and who was always proud of me no matter my accomplishments.

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List of Abbreviations and Symbols

5-HT	5-hydroxytryptamine or serotonin
AGU	Axenic gnotobiotic unit
AKK	*Akkermansia muciniphila*
AMP	Antimicrobial peptide
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
BHI	Brain-heart infusion
BSA	Bovine serum albumin
℃	Degrees Celsius
CD	Crohn's disease
cDNA	Complimentary deoxyribonucleic acid
CFU	Colony forming units
DADA2	Divisive Amplicon Denoising Algorithm 2
DAI	Disease activity index
DC	Dendritic cell
DSS	Dextran sulfate sodium
ELISA	Enzyme-linked immunosorbent assay
ESP	Excretory/secretory product
EV	Extracellular vesicles
FDR	False discovery rate
Foxp3+	Forkhead box protein P3
g	Gram
GALT	Gut-associated lymphoid tissue
GF	Germ-free
GI	Gastrointestinal
GPCR	G-protein coupled receptor
GWAS	Genome-wide association studies
H&E	Hematoxylin & eosin
hBD	Human β-defensin
Hes-1	Hairy and enhancer of split-1
HFD	High-fat diet
HTAB	Hexadecyltrimethylammonium bromide
Hz	Hertz
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IFN	Interferon

Ig	Immunoglobulin
IL	Interleukin
JAM	Junction adhesion molecule
Kg	Kilogram
KO	Knock-out
L	Litre
LP	Lamina propria
LPS	Lipopolysaccharide
MAMP	Molecular-associated molecular pattern
Math1	Murine atonal homologue 1
mBD	Mouse β-defensin
MDP	Muramyl dipeptide
mM	Millimolar
ng	Nanogram
NLR	NOD-like receptors
nm	Nanometre
nM	Nanomole
NOD	Nucleotide-binding oligomerization domain-containing protein
PAMP	Pathogen-associated molecular pattern
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
PCoA	Principal coordinates analysis
PERMANOVA	Permutational multivariate analysis of variance
pg	Picogram
PIC	Protein inhibitor cocktail
PRR	Pattern recognition receptors
qPCR	Quantitative polymerase chain reaction
RegIII	Regenerating islet-derived III
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcriptase
SCFA	Short chain fatty acid
SEM	Standard error of the mean
sIgA	Secretory Immunoglobulin A
SPDEF	SAM pointed domain-containing ETS factor
SPF	Specific pathogen-free
Th	T helper cell
TJP	Tight junction protein

TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tph	Tryptophan hydroxylase
T_{regs}	Regulatory T cells
UC	Ulcerative colitis
WT	Wild-type
wt/vol	Weight to volume
ZO	Zonula occludens
α	Alpha
β	Beta
γ	Gamma
µg	Microgram
µl	Microlitre
µM	Micromole

Declaration of Academic Achievement

I, Jensine A. Grondin, am the sole author of this thesis. The work presented within was designed by Jensine A. Grondin and Dr. Waliul I. Khan. The research herein was conducted by Jensine A. Grondin with technical contributions and support from Huaqing Wang, Sabah Haq, Suhrid Banskota, and Yun Han Kwon. This thesis was completed with feedback from Dr. Waliul I. Khan, Dr. Michael Surette, and Dr. Leslie MacNeil. While undertaking the work involved in this thesis, I was a contributing author on the manuscript, "Autophagy: roles in intestinal mucosal homeostasis and inflammation" (Haq et al. *J Biomed Sci*, 2019), co-author on the manuscript entitled "Parenteral BCG vaccine induces respiratory mucosal-resident memory macrophages and trained innate immunity via the gut-lung axis" (Jeyananthan et al., submitted), co-author on the manuscript entitled "Disruption of autophagy by increased mucosal serotonin alters gut microbiota and enhances susceptibility to experimental colitis and Crohn's disease" (Haq et al., submitted), as well as a first author on the manuscript, "Mucins in intestinal mucosal defense and inflammation: learning from clinical and experimental studies" (Grondin et al., *Front Immunol.,* 2020), and co-first author on "Tryptophan-derived serotonin-kynurenine balance in immune activation and intestinal inflammation" (Haq et al., *FASEB, 2021*).

CHAPTER 1: INTRODUCTION

1.1 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD), characterized by chronic intestinal inflammation (1), is currently hypothesized to arise from the interplay between the gut microbiota, environmental factors and the immune system in a genetically susceptible individual (2,3). IBD can be broken down into two symptomatically similar but physiologically different diseases, Crohn's disease (CD) and ulcerative colitis (UC) (4). CD can manifest at any site in the gastrointestinal (GI) tract and is characterized by sporadic transmural inflammation (5). UC, on the other hand, is characterized by superficial mucosal ulcerations and varying degrees of continuous inflammation largely confined to the colon (5). Though it is thought that these two major forms of IBD stem from differing pathophysiological events, the root causes of both conditions remain elusive (4).

Increased prevalence and incidence of IBD around the world, particularly as countries become more industrialized, has emphasized the role that environmental influences play in these conditions (3,6,7). Factors including the increasingly processed "Western" diet, amplified antibiotic exposure, as well as overall improvements in sanitation may, at least in part, account for these increases. Encompassing these factors, the aptly named "hygiene hypothesis" proposes that the overly sanitized environments and diminished microbial exposure associated with modern life may promote an uneducated immune system; an immune system more prone to dysfunction and unable to appropriately deal with routine challenges which may result in the manifestation of allergic and autoimmune conditions (3,8).

Both the innate and adaptive branches of the immune system appear to play significant roles in IBD pathogenesis (3). Alterations in innate inflammatory cells, including neutrophils, mast cells, and macrophages, as well as adaptive T cell and B cell populations, occur in IBD. These changes are often accompanied by a striking reduction in suppressor T cells, indicating that a balance between the pro-inflammatory and anti-inflammatory systems seems to hold a distinct place in the pathogenesis of IBD (3,4). In conjunction with these altered cell populations, upregulated proinflammatory cytokines including tumour necrosis factor-α (TNFα), interleukin-1β (IL-1β), and IL-6 are shared between UC and CD (9).

Despite the umbrella classification of IBD, CD and UC differ in their characteristic immune responses. CD is classically associated with a T-helper type 1 (Th1) immune response, whereas UC displays a more Th2-like response (10). It should be noted that these "categorizations" greatly simplify the complex and dynamic immunological processes underlying these conditions. A prime example of this oversimplification is the role Th17 cells and their associated cytokines play in IBD. Recent work by Nemeth et al., demonstrated that Th17 cell infiltration occupies the inflamed regions in both CD and UC patients (10); however, CD but not UC patients display significant increases in Th17-associated cytokine expression, including IL-17, IL-23 and IL-32. These findings suggest a greater influence of Th17-associated cytokines in CD compared with UC (10) and emphasize that the complex immune interactions underpinning these conditions are still being uncovered. An overview of the similarities

and differences between the presentation, location, and distinct inflammatory responses of CD and UC are represented in Table 1.

Though the initiation and pathogenesis of these debilitating diseases are currently unknown, evidence suggests that the resident microbial community of the gut may play a role in triggering the inappropriate inflammatory response characteristic of IBD (3,11,12). Data regarding the role of infectious agents in IBD is relatively weak; however, evidence supports the idea that an aberrant reaction and/or failure in the regulation of the immune response to commensal microorganisms may underlie these conditions (3,13).

Despite decades of research, the cause and cure of IBD remain elusive and although several efficacious treatment options exist, long term effects, safety, and expense remain of concern for patients. In recent years, significant progress has been made in understanding the pathogenesis of IBD. These advances have led to improved strategies to control inflammation and diminish symptomatology through the use of immunosuppressive drugs and biologics (e.g. antibody targeting TNF-α) (14). However, treatment with these drugs, particularly immunosuppressive agents, is associated with several side effects, including toxicity and acute infusion reactions. With continued use, the development of antibodies to anti-TNF-α treatments also threaten long term efficacy. In addition, surgical intervention to remove diseased tissue remains a precarious option as reoccurrence risk is high. Therefore, the shortcomings associated with current treatment options prompt the need for the development of better therapeutic strategies in IBD (7,15)**.** By working with the inherent biology surrounding IBD, non-immunosuppressive

therapies involving microbial manipulation of the gut may present a more advantageous approach to altering the pathophysiology and symptomatology of IBD (16).

Crohn's Disease	***Ulcerative Colitis***
▪ Transmural inflammation/ deeply penetrating inflammation ▪ Can affect any site in the GI tract but most commonly the terminal ileum ▪ Characterized by skip lesions/ "patchy" inflammation ▪ Macrophage aggregation common (non-caseating granulomata) ▪ Associated with Th1 immune responses (IFNγ, IL-12,TNFα) and Th17-associated cytokines (IL-23, IL-17 etc.)	▪ Superficial mucosal inflammation or ulcerations ▪ Continuous inflammation within the colon, extending proximally from the rectum ▪ Associated with Th2 immune responses (IL-4, IL-13, IL-5)

Table 1. Comparison of the presentation, location, and distinct inflammatory responses of Crohn's disease and ulcerative colitis. Information obtained from Xavier & Poldolsky (2007) and Podolsky (1991) (4,5).

1.1.1 Barrier function in IBD

Within the GI tract, the gut barrier is the first line of defence in protecting the host from exogeneous threats. This barrier consists of the continuous epithelial lining of the gut, the overlying mucus layer of secreted gel-forming mucins which harbours antimicrobial peptides (AMPs), and the anchoring glycocalyx consisting of transmembrane mucins (17,18). Several studies have implicated impaired barrier function

in IBD; however, whether impairment of, or damage to, this layer is a primary causal factor or simply a consequence of exacerbated inflammation is still under investigation (19–21).

A single layer of epithelial cells makes up the lining of the gut and consist of several specialized cell types. Multipotent stem cells at the base of intestinal crypts differentiate into distinct cell types, including absorptive enterocytes, as well as secretory enteroendocrine cells, Paneth cells and goblet cells (22,23). Between cells, protein complexes interact to anchor cells together, maintaining the epithelial barrier and appropriate permeability within the gut. Differentiation of cells towards the absorptive pathway is reliant on the transcription factor, hairy and enhancer of split-1 (Hes-1), whereas murine atonal homologue 1 (Math1) controls the secretory lineages (24). The transcription factor, SAM pointed domain containing ETS factor (SPDEF) is largely responsible for facilitating the terminal differentiation of secretory cells into goblet cells (25). Goblet cells are the main producers of mucins, high molecular weight glycoproteins that make up the structural components of the mucus layer (26). Up to 21 different mucin genes have been identified in humans, with the majority of their homologues recognized in mice and rats (27). Among these mucin genes, MUC2 (Muc2 in mice) codes for the major gel-forming mucin in the gut, a critical factor in determining goblet cell morphology (28,29).

Alterations in mucins and the mucus layer are associated with IBD (18). In CD and UC, both goblet cells and the structural mucin, MUC2, are depleted (30–33). Interestingly, in both conditions, *de novo* expression of MUC5AC, which is normally

found in the stomach but absent from the colon, occurs (31,34). Though mucus layer thickness seems to be consistently depleted in UC, reports in CD vary; some findings suggest increased mucus thickness is a common trait, while others report that there is no change in thickness compared to healthy controls (35,36). Aberrant expression of AMPs, including human β-defensins (hBD), which permeate the mucus layer and act as broad-spectrum microbial retardants, are also found in IBD patients (4,37,38).

As mentioned above, genetic susceptibility is thought to be a contributing factor to IBD development. Indeed, evidence from genome-wide association studies (GWAS) have identified several IBD susceptibility genes implicating altered barrier function in disease pathogenesis (39–42). Intriguingly, anti-TNF therapies, in conjunction with dampening the uncontrolled immune response and mitigating symptoms, have also been shown to restore appropriate intestinal permeability in IBD patients (43). Intestinal permeability is largely maintained by a number of protein complexes, including tight junctions, adherens junctions and desmosomes, by forming bridges and anchors between epithelial cells (22). Of these, tight junctions are the most influential in IBD pathogenesis (23,44).

Tight junctions, which control paracellular permeability, are composed of a number of proteins, including zonula occludens (ZO), occludin, junction adhesion molecules (JAM) and claudins (45). These proteins form a complex with both extra- and intracellular components anchoring cells along the intestinal epithelium (Figure 1). Under pathophysiological stress, rearrangements and altered expression of these dynamic proteins greatly affect the infiltration of unwanted luminal contents, notably microbes and microbial antigens, into the intestinal mucosa (46,47). Indeed, several aspects of modified

barrier function in IBD, including altered tight junction protein (TJP) expression, is evident (48) and increased intestinal permeability has been reported (4,19–21). In intestinal biopsies, IBD patients exhibited altered expression of several claudins (20,49), as well as downregulation of occludin (20,21) and ZO-1 (21). In addition, in areas of active inflammation, adherens junctions, particularly the expression of the protein components, E- and α-cadherin, were severely downregulated (21). Whether cause or consequence, impaired barrier function is of great concern in IBD.

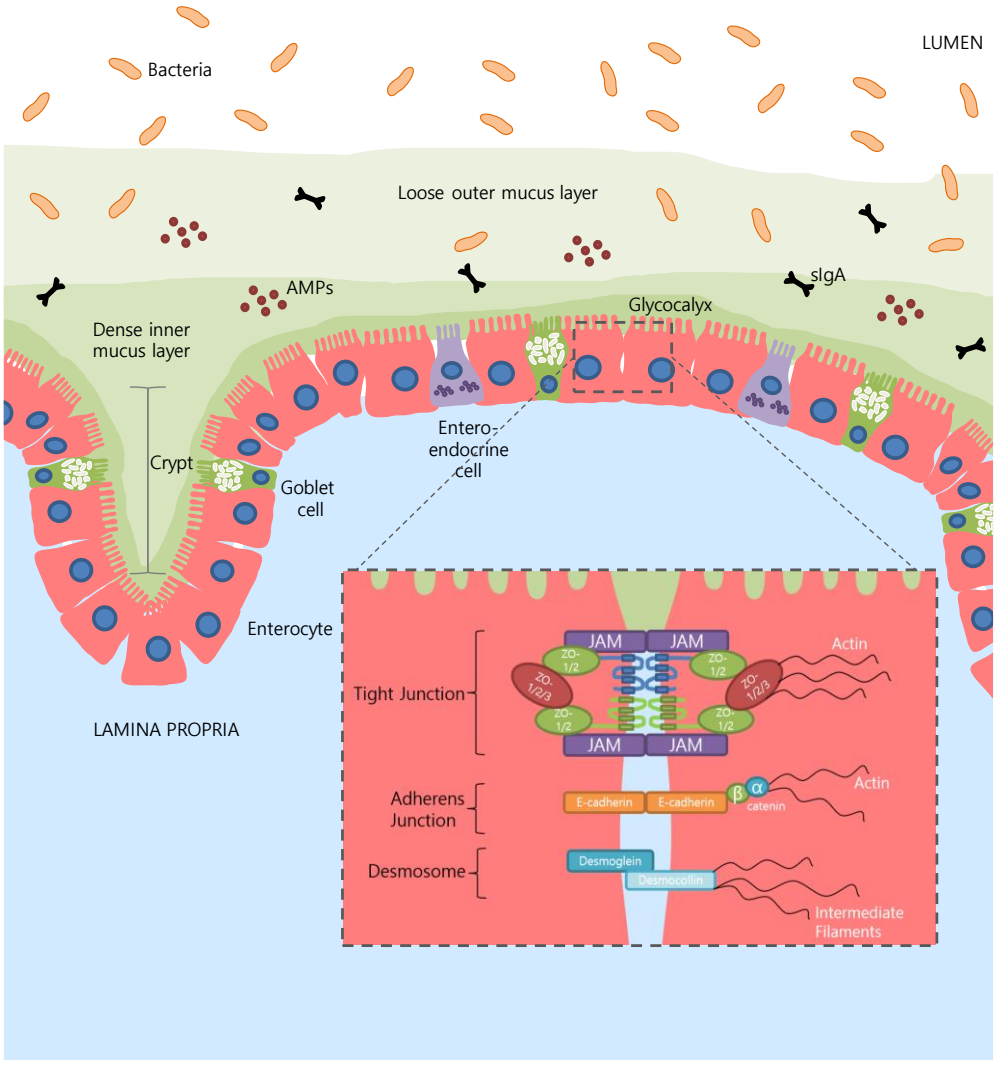

Figure 1.1. *Intestinal Barrier and Cell-Cell Junctions*

Maintenance of intestinal barrier function is a critical component in the pathogenesis of IBD. Displayed are the components of this barrier within the colon. Here, the mucus layer consists of a loose upper layer, which bacteria can penetrate, and a lower dense impenetrable layer. Below lies the glycocalyx composed of transmembrane mucin, which help anchor the above mucus layers to the underlying epithelium. Within these layers of mucus, antimicrobial peptides including defensins, and secretory immunoglobulin A (sIgA) prevent toxins, antigens and microbes from infiltrating the mucosa. Complexes including tight junctions, adherens junctions and desmosomes anchor cells to each other and maintain barrier function and appropriate intestinal permeability under homeostatic conditions.

1.2 Gut Microbiota

The trillions of commensal microorganisms that inhabit the GI tract play a critical role in GI physiology, aid digestion, protect the host from pathogen invasion and contribute to immune system development (50,51).

Along the GI tract, local environmental gradients, including those pertaining to oxygen levels, nutrient availability, pH, and immune meditators, shape microbial composition (50). Notably, increasing numbers of bacteria are found distally, with the distal colon and distal ileum harbouring the highest concentrations (52–54). The gut microbiota is a dynamic system, and it can be shaped throughout life by a number of factors, including illness and diet (50). With that being said, however, the gut microbiota tends to stabilize in adulthood and is largely dominated by the phyla Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria (50,55) and Verrucomicrobia (56).

The gut microbiota is implicated in various GI disorders, including IBD. Clinical and animal studies have suggested that gut bacteria trigger and perpetuate chronic colitis (57).

1.3 Gut Microbiota and Intestinal Inflammation

1.3.1 IBD and Gut Microbiota

The connection of the microbiota with the host immune system and IBD has been advanced by work using animal models. Interestingly, colitis does not develop in germ-free (GF) mice, suggesting that inflammation is reliant on the presence of the enteric microbiota (58–60) and is not simply an inherent feature of the gut (5,61). It is thus not

surprising that the ulcerations and lesions common in IBD often cluster in parts of the GI tract that have the greatest concentrations of microbes (3).

The influence of the intestinal microbiota within the context of IBD is further pronounced by the amelioration of colonic inflammation in IBD patients with the use of certain antibiotics (3). In contrast, antibiotic use early in life is associated with an increased risk of CD in children (62). Though seemingly contradictory, these findings suggest that the context of antibiotic usage can greatly influence outcome; in children, antibiotic use may disrupt an already dynamic microbiota and influence which species can eventually colonize in adulthood, whereas in adult IBD patients, disruption via antibiotics to a stable, yet disease-promoting, microbiota maybe beneficial.

Gut microbes can also manipulate the host immune system, promoting defence against invasive pathogens and boosting the innate immune response (63). For instance, microbes such as *Bacteroides fragilis*, can encourage the differentiation of regulatory T cells, key effectors of the adaptive immune response, and, thus, influence the production of anti-inflammatory IL-10, helping to alleviate colonic inflammation (62,64–66). Additionally, the gut microbiota has been shown to strengthen gut barrier function (63) by maintaining the integrity of the mucosa and physically impeding potentially pathogenic bacteria (4).

The genes associated with IBD susceptibility may also elucidate the connection between the immune system, IBD pathogenesis and the intestinal microbiota. Genetically, CD has been linked with the NOD2/CARD15 gene (40–42,67). This gene's associated protein participates in bacterial sensing by recognizing the bacterial peptidoglycan

component muramyl dipeptide (MDP) (68–70) and is located chiefly in innate immune cells, intestinal epithelial cells (IECs) and Paneth cells (5). Similarly, mutations in toll-like receptors (TLRs) are also associated with CD (5,62). These cell surface molecules are found largely in dendritic cells, monocytes and epithelial cells and play a key role in maintaining gut homeostasis, sensing commensal and pathogenic microbes and initiating host defensive mechanisms (5,62). Increased colitis susceptibility and inhibited barrier function has been illustrated both in mice with genetically altered, and chemically manipulated, TLR2 (71–76).

Furthermore, TLRs and NOD-like receptors (NLRs) stimulate T helper cells, activate inflammasomes and trigger the release of pro-inflammatory cytokines (5,62). These receptors also trigger the secretion of AMPs and epithelial repair factors as well as promote the activation of regulatory T cells (T_{reg}) and subsequent IL-10 secretion (5,62). These NLRs and TLRs, collectively known as pattern recognition receptors (PRRs), play a crucial role in distinguishing between innocuous commensals and problematic pathogens (77,78). Therefore, increased reactivity, or lack thereof, may break tolerance and/or elicit immunological cascades in situations where such extreme responses are not needed (67,77,78). Thus, it is not surprising that alterations in these recognition processes, which tie the intestinal microbiota to the host immune response, are suspect in the unwarranted inflammatory response found in IBD (5,68).

Additionally, the depletion of commensal microbes in IBD such as *Faecalibacterium prausnitzii*, *Akkermansia muciniphila*, and *Bacteroides uniforms* may play a role in controlling niche availability and affect bacterial cross-feeding (1,79–81).

Multiple studies suggest that microbial diversity is diminished in IBD and that microbial shifts occur, predominantly characterized by increased levels of Proteobacteria and Actinobacteria and decreased abundance of Firmicutes and Bacteroidetes (16,81–83).

Changes to microbial composition inevitably alter metabolites available to the host, and these changes likely influence IBD pathophysiology (62). In an analysis of the microbiome in IBD, Morgan et al. detected only a 2% change in microbial composition compared to a 12% alteration in the metabolic pathways between healthy controls and IBD patients (84). These results suggest functional differences in microbiota rather than broad phylogenetic changes may be of some consequence in IBD. Of particular importance within the intestinal context is the microbial production of short-chain fatty acids (SCFA) such as butyrate (79,84). In the lumen, butyrate is not only the favoured source of energy for colonic cells but also reinforces the intestinal barrier, increases the secretion of AMPs and mucins, and modulates the expression of TJPs (79).

Unfortunately, results regarding current probiotic therapy as a supplemental treatment for IBD patients have been largely inconsistent (79,85). However, the use of touted "next-generation probiotic" (79,86–88) including butyrate producer *F. prausnitzii* (89), and the mucin degrader *A. muciniphila* (88,90,91) show potential. For instance, Sokol et al. demonstrated that *F. prausnitzii* has the potential to shift the disrupted intestinal microbiota in murine colitis models, increase the secretion of anti-inflammatory IL-10 and decrease colitis severity (89). Intriguingly, several studies have suggested that many of these microbes can stabilize and strengthen the gut barrier (92).

1.3.2 Enteric Infection-Induced Inflammation and Gut Microbiota

In order to promote their own survival, enteric pathogens, such as *Trichuris* and *Salmonella*, have evolved to manipulate and exploit the interaction between the host and the resident gut microbiota (93). These pathogens can significantly alter the host intestinal environment, resulting in modulation of the gut microbiome and alterations in the host immune response (94). For instance, infection with the enteric pathogen *Salmonella enterica* serovar Typhimurium, a common cause of gastroenteritis, has been shown to restructure the resident gut microbiota in murine models by a number of mechanisms (95,96). One such mechanism includes stimulating host expression and release of the antibacterial C-type lectin, RegIIIβ, and subsequently causing a significant decrease in the representation of the Bacteroidia family within the gut microbiota (97,98). The modulation that these organisms can impose on the host microbiota becomes particularly influential during states of inflammation (93).

Trichuris muris, an infectious whipworm that can inhabit the cecum and colon of mice, can also manipulate the intestinal microbiota of its host. Several studies have demonstrated that changes in microbial composition in chronic *T. muris* infection were dominated by a significant reduction in overall diversity as well as a reduction in Bacteroidetes and increased Firmicutes (94,99). *T. muris* infection also affects the epithelial cells of the gut, and hyperplasia of goblet cells has been observed during infection (26,100,101). With this being said, mucins which are largely secreted by goblet cells within the gut, have been shown to play an important role in the innate defence to enteric infection by inhibiting parasite motility and feeding capacity (26,100–102). These

findings are particularly interesting with regards to the ability of certain gut microbes such as *A. muciniphila* to alter mucin production (91,103). Our laboratory has demonstrated, in a number of studies, that the ability of mice to expel *T. muris* relies heavily on increased Muc2 production, particularly in the initial stages of infection (102,104,105). We have also demonstrated that treatment of live *Lactobacillus rhamnosus* (JB-1) in *T. muris* infection significantly enhances worm expulsion in resistant C57BL/6 mice, *Muc2* $^{-/-}$ and susceptible AKR mice. This treatment was associated with increases in IL-10 levels, goblet cell number, and epithelial cell proliferation (105).

1.4 Akkermansia muciniphila

A. muciniphila, of the phylum Verrucomicrobia, is a symbiotic bacterium found in the digestive tract of a number of species (106,107). This wide array of *Akkermansia*-colonized species implies a long co-evolutionary history and hints at a beneficial relationship between microbe and host (106).

A. muciniphila is an oval-shaped, non-spore forming, Gram-negative bacterium (107,108). It is an obligate chemoorganotroph which largely degrades intestinal mucin as its main source of energy, carbon, and nitrogen, and accounts for 1-5% of the human fecal microbiota (91,107–109). It grows optimally at 37°C and can survive a pH varying from 5.5 to 8 which reflects the conditions it encounters as an intestinal resident (107,108). *In vitro*, *A. muciniphila* forms a filamentous capsule which may play a role in its ability to colonize the gut (108).

Interestingly, though it was initially thought to act as a strict anaerobe, *A. muciniphila* has been shown to tolerate nanomolar concentrations of oxygen (110),

possibly even utilizing this oxygen to enhance growth (111). In fact, Reunanen et al. demonstrated that *A. muciniphila* cells have an 80% survival rate when exposed to atmospheric oxygen for one hour and, thus, classify *A. muciniphila* as an aerotolerant anaerobe (112). A more recent study has corroborated and expanded these findings suggesting that *A. muciniphila's* viability under stress is more robust than once thought; Machado et al. found that it exhibited high oxygen tolerance for at least 72 hours with cultivable cell numbers remaining largely steady at both 4°C and 22°C (88).

Reflecting its status as a mucin-degrading specialist, metaproteome analyses indicated that a significant portion of *A. muciniphila's* secreted proteins (11%) were involved in mucolytic activity (106). These analyses also identified the unique outer membrane protein, namely Amuc_1100 (91), which has been shown to have beneficial effects on diet-induced obesity, intestinal barrier function, insulin resistance, and intestinal inflammation (113). As well, *A. muciniphila* has been found to be negatively correlated with several disease states, including type 1 and type 2 diabetes, depression and anxiety, psoriasis, appendicitis, obesity, metabolic syndrome, and IBD (91,114–117).

1.5 Akkermansia muciniphila - Gut Interactions

1.5.1 Mucus Degradation and Production

The mucus layer of the intestinal tract acts as a physical and chemical barrier for underlying epithelial cells (Figure 2). MUC2 (Muc2 in mice), the major glycoprotein in the intestine, forms the backbone of this protective barrier (118). Any damage to or loss of this layer exposes the epithelium to potentially pathogenic bacteria and is a substantial factor in the pathogenicity of IBD (119).

A. muciniphila degrades mucin glycoproteins using secreted mucolytic enzymes which convert the major sugar components, N-acetyl galactosamine and N-acetyl glucosamine into, primarily, the SCFAs, propionate and acetate (107,108). Possessing specialized machinery, this microbe is able to fully access the amino acids and monosaccharides present in these complex glycan chains (91). Interestingly, the mucolytic nature of this bacteria provides a competitive ecological advantage, particularly during periods of fasting and malnutrition. In these states, while exogenous dietary resources are unavailable for breakdown, the constant presence of mucin supplies ample nutrients for this microbe (91,108,114). However, despite its mucolytic nature, *A. muciniphila* has been found to stimulate mucin production, increase goblet cell number, promote mucus thickness, and enhance gut barrier integrity (91,103).

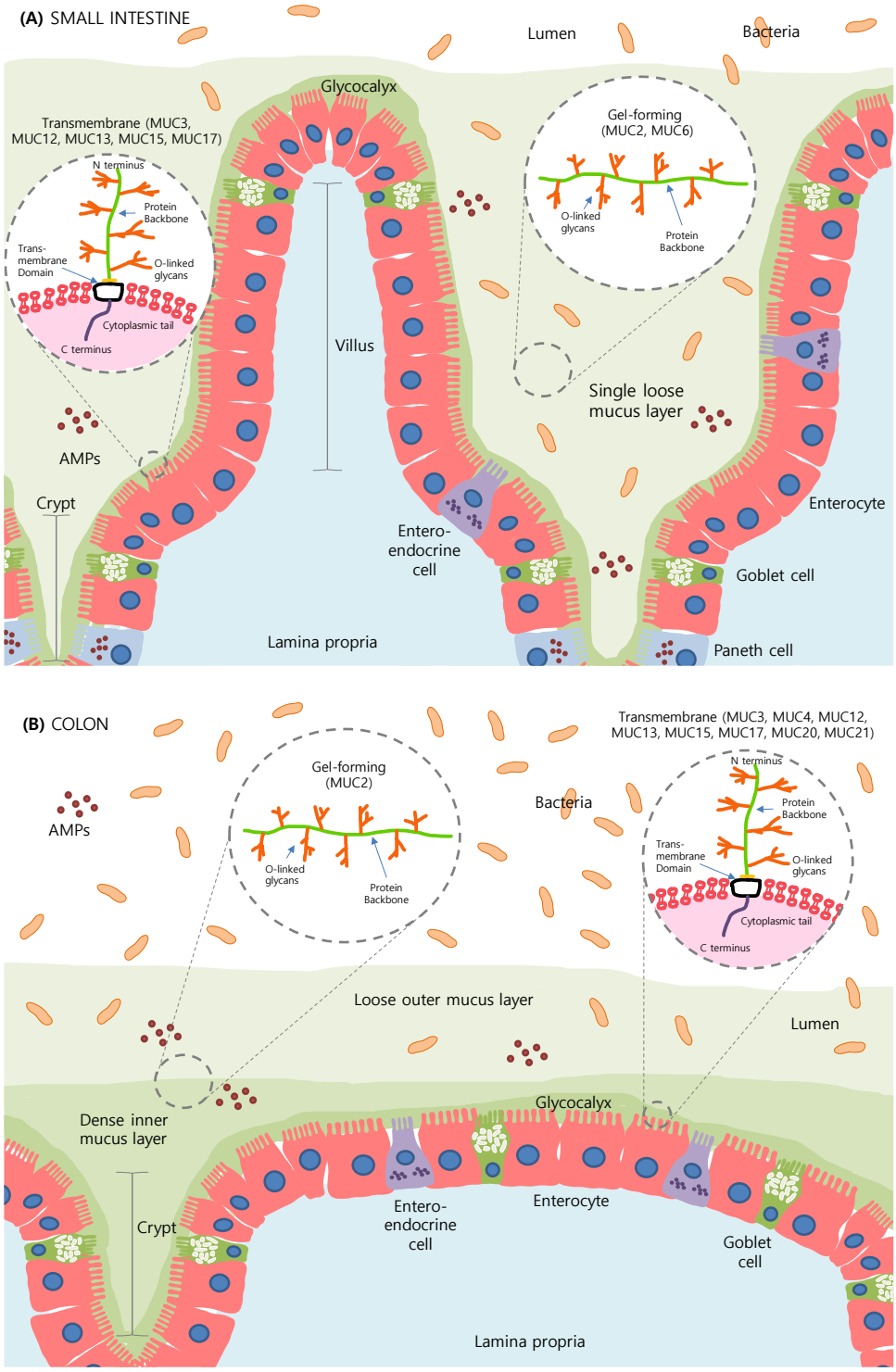

***Figure 1.2**. The mucus layer of the small intestine and colon.*

(A) In the small intestine, only one layer of loosely attached mucus is present and is penetrable by resident microbes. (B) Primarily produced by goblet cells, colonic mucus is comprised of two layers: an outer layer permeable to bacteria and a tightly adhered inner layer impermeable to bacteria. Here, secreted gel-forming mucins, largely MUC2, are the main components of this mucus layer and provide its viscoelastic properties. Transmembrane mucins, including MUC3A/B, MUC12, MUC13, MUC15 and MUC17, form a carbohydrate-rich layer called glycocalyx lying between the secreted mucins and the underlying epithelial cells in both the small intestine and colon. Simplified structures of transmembrane mucins and gel-forming mucins can be seen in the magnified sections. Transmembrane mucins are generally comprised of two subunits; the heavily glycosylated and larger extracellular subunit, and the shorter subunit consists of a small extracellular domain, a transmembrane domain and a cytosolic compartment. The extracellular protein backbone contains tandem repeat units of varying lengths consisting of the amino acids proline, serine and threonine, which create binding sites for O-linked oligosaccharides. This protein backbone and O-linked glycan structure are also present in secretory/gel-forming mucins.

1.5.2 Microbial Interactions: Cross-feeding and Competitive Exclusion

Via its metabolic products, *A. muciniphila* is able to interact with, and provide substrate for, other resident microbes and, thus, aids in the maintenance of a healthy microbiota (120). As mentioned previously, the breakdown of intestinal mucins by *A. muciniphila* produces a variety of SCFAs, including propionate and acetate as well as oligosaccharides (106). These products, particularly oligosaccharides and acetate, provide nutrients for other resident bacteria promoting their growth and metabolic activity and, thus, hamper the ability of pathogenic bacteria to colonize the mucus layer of the gut via competitive exclusion (106).

A study by Chia et al. provided insight into *A. muciniphila's* ability to interact with other commensal microbes (121). By mono- and co-culturing *A. muciniphila* with the butyrate-producer *Anaerostipes caccae*, the authors observed the occurrence of several bidirectional interactions, including unique trophic and transcriptional influences. For instance, when both of these microbes were present, *A. muciniphila* exhibited an upregulation in several mucin degradation genes and a downregulation in ribosomal genes indicating slower growth but also increased substrate availability for neighbouring microbes. Concurrently, *A. caccae* utilized these available substrates, largely mucin sugars and acetate, to produce the anti-inflammatory SCFA butyrate via the acetyl Co-A pathway (121). These experiments, as the authors suggest, provide further evidence that *A. muciniphila* plays a role as a keystone species of the gut by supporting the health of the microbial community.

1.5.3 Immune Interactions

The exact role that *A. muciniphila* plays in affecting the host immune response is still being examined. However, it is known that its metabolite products not only affect other luminal microbes but also influence host immune function. Propionate and acetate produced by mucin breakdown by *Akkermansia*-like bacteria can interact with the host via Gpr43 and Gpr41 receptors which have been linked to several chronic inflammatory disorders, including colitis (122,123). These receptors are largely localized to enterocytes and enteroendocrine cells within the colon (124–126). The interaction between microbial-derived SCFA and these epithelial-associated receptors can prompt changes in host expression machinery, metabolic signalling, and influence host immune activation (119).

In addition, Amuc_1100, a protein involved in the formation of pili-like structures on the *A. muciniphila's* outer membrane, is directly involved in immune regulation and strengthening the epithelial barrier (91,112,127). Within the context of metabolic syndrome and obesity, this outer membrane protein has been shown to reduce systemic inflammation by binding to the epithelial barrier, induce a number of cytokines including IL-1β, IL-6, TNFα and IL-10, and increase transepithelial resistance (128).

Intriguingly, Ottman et al. found that human-derived peripheral blood mononuclear cells (PBMCs) stimulated with *A. muciniphila* produced significantly more IL-10 than those stimulated with either *F. prausnitzii* or *Lactobacillus plantarum*, two known beneficial gut microbes (127). In comparison to these two microbes, the relatively low ratio of the proinflammatory TNFα to anti-inflammatory IL-10 associated with *A. muciniphila* is indicative of a low inflammatory potential (127).

In addition, *A. muciniphila* induces multiple immune cascades involved in chemotaxis and the complement system as well as enhances the proliferation of anti-inflammatory regulatory T cells (91,107). Administration of *A. muciniphila* also stimulates the production of AMPs such as RegIIIγ in the murine colon (91). Interestingly, mice colonized with *A. muciniphila* showed altered gene expression, particularly those genes involved in the immune response and cell fate determination, membrane metabolism, antigen-presenting pathways, and cell death and proliferation (119).

1.5.4 Enhancing Epithelial Barrier Integrity

A. muciniphila is particularly effective in increasing mucus thickness and enhancing gut barrier integrity (91). Reunanen et al., using Caco-2 and HT-29 colonic cell lines, demonstrated *A. muciniphila's* ability to fortify the gut barrier by binding directly to epithelial cells (112). This microbe has also been shown to increase the number of goblet cells in high-fat diet (HFD) mice (103) and stimulate mucus production (120). By acting on TLR2 and restoring appropriate tight junction expression, both live or pasteurized *A. muciniphila* and its outer membrane protein, Amuc_1100, have been found to enhance gut barrier function (129). Evidence suggests that *A. muciniphila* may also decrease intestinal permeability as indicated by decreased levels of circulating lipopolysaccharide (LPS), a component of Gram-negative bacteria (130–132). *A. muciniphila*-derived extracellular vesicles (EVs) have also exhibited effects on gut permeability and barrier function. In HFD-induced diabetic mice, oral administration of these EVs has been shown to improve epithelial barrier function by reversing the enhanced permeability found

within HFD-fed mice and increasing the expression of several tight junction proteins, including claudin-5, occludin and ZO-1 (133). These findings were also reflected in Caco-2 cells showing increased levels of occludin, decreased transepithelial resistance and, ultimately, improved gut barrier function in an AMPK-dependent manner. *A. muciniphila* itself has also been shown to increase the expression of occludin, claudin-4 and ZO-2 and ZO-3 in Caco-2 cells (134). Further, pasteurization of *A. muciniphila*, which maintains the integrity of Amuc_1100, was shown to increase goblet cell number and significantly decrease levels of circulating LPS, as well as increase claudin 3 and occludin in regions of the small intestine in HFD-induced metabolic syndrome (128).

A. muciniphila's known host interactions are summarized in Figure 1.3.

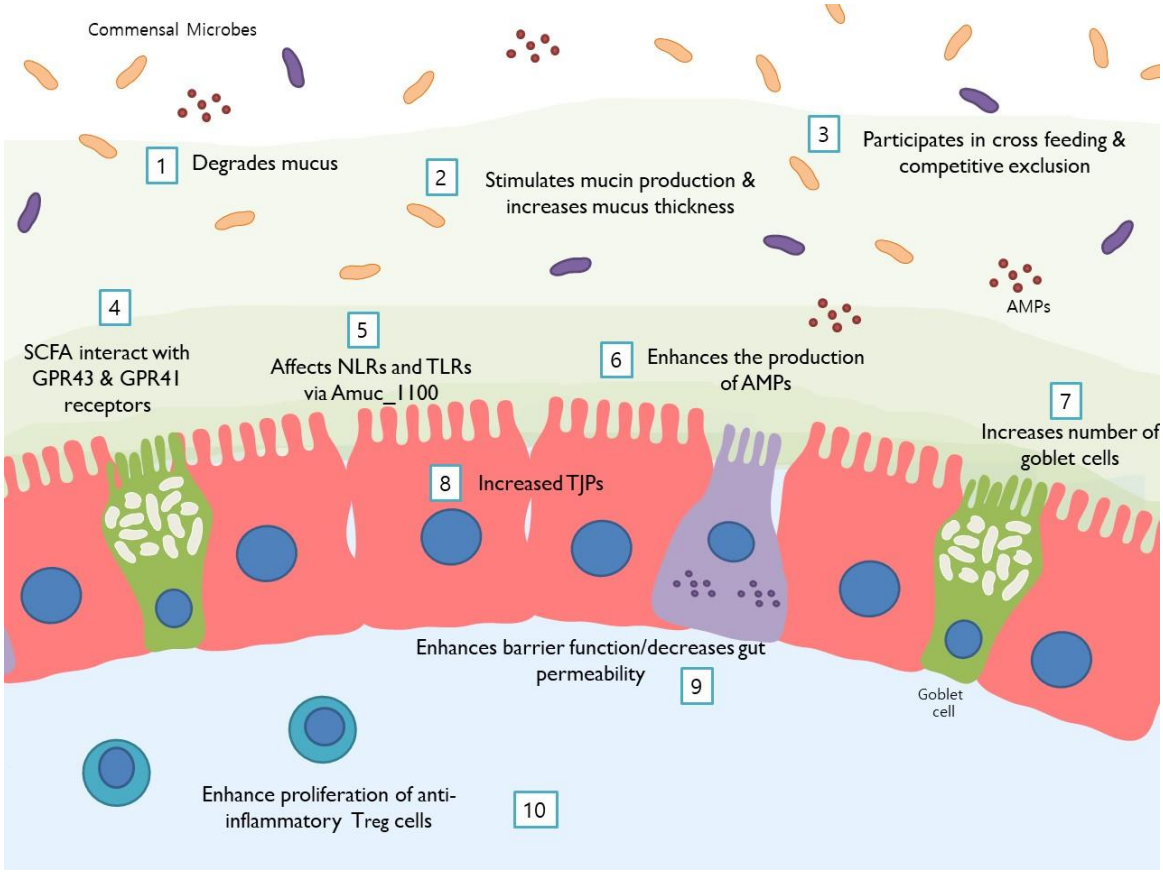

***Figure 1.3**. A. muciniphila host interactions.*

A. muciniphila (represented in purple) can interact with the host in myriad ways. This microbe has been shown to (1.) degrade mucus, (2.) stimulate mucus production and secretion and boost mucus thickness. (3.) In a process termed cross-feeding, metabolic products generated by *A. muciniphila*, including SCFA and oligosaccharides, act as substrates for other neighbouring microbes stimulating their own growth and survival and, in this process, also competitively excluding potentially harmful or invasive species. (4.) These SCFA have also been shown to interact directly with host cells via GPR43 and GPR41. In addition, *A. muciniphila* has been found to (5.) affect bacterial sensing NLRs and TLRs via its outer membrane protein, Amuc_1100, to (6.) enhance the production of AMPs, particularly RegIIIγ, and to increase goblet cell number (7.) and TJPs (8.). In addition, this mucin degrader also enhances intestinal barrier function (9.) and enhances the proliferation of anti-inflammatory T_{reg} cells (10.).

1.6 Akkermansia muciniphila and Intestinal Inflammation

Research elucidating the role of *A. muciniphila* in inflammation has largely been focused within the context of obesity and metabolic syndrome (103,129,131,132,135,136). Though a clear-cut role and mechanism by which *A. muciniphila* may influence chronic inflammatory conditions have not been determined, there is evidence indicating that this microbe is depleted in patients with IBD (135). Indeed, patients with active UC had a lower abundance of *A. muciniphila* compared to both healthy controls and those with quiescent disease (137). Intriguingly, this change in abundance was positively correlated with the proportion of sulphated mucins found in colonic biopsies as well as negatively correlated with higher inflammatory scores (137). Zhang et al. also corroborated these findings; substantially lower levels of *Akkermansia* colonization were found in both CD and UC patients compared to healthy controls; however, diminishment was most associated with UC. The authors also found that patients receiving a washed microbial transplant had significantly increased levels of *Akkermansia* colonization which also correlated with increased therapeutic effectiveness in inducing remission (138).

Similar findings to the above have been confirmed in chemically-induced colitis animal models. Mice administered dextran sulfate sodium (DSS) were found to have significantly decreased levels of *A. muciniphila*, suggesting that changes in mucus layer thickness and the inflammation induced by DSS had a detrimental effect on this microbe's abundance (113). Recent findings suggest that *A. muciniphila* can ameliorate several markers of disease severity, including improved histopathological scores, as well

as reduced weight loss, colonic shortening and levels of pro-inflammatory cytokines in both acute and chronic models of DSS colitis (139,140). Conversely, it should be noted that some studies have reported increased abundance of *A. muciniphila* in colitis, possibly explained by outgrowth in response to altered mucus thickness (141,142). In addition, EVs produced by this microbe have been shown to have anti-inflammatory effects in DSS-induced colitis (142). The above evidence suggests that *Akkermansia muciniphila* could have protective or anti-inflammatory effects that may be lost in IBD (80).

A. muciniphila's ability to influence goblet cell number and mucus secretion may provide advantageous effects in the context of colitis (5,103). Further, *A. muciniphila's* ability to strengthen the intestinal epithelial barrier, as shown in *in vitro* studies (112), suggests that it may have a beneficial influence not only on the mucus layer but also on the epithelia itself.

Furthermore, *A. muciniphila's* immunomodulatory effects, including the ability to influence the production of AMPs (132), effect NLRs and TLRs via the Amuc_1100 (143), upregulate genes involved in the immune response (119) and increase the number of anti-inflammatory regulatory T cells (103), suggest that *Akkermansia* may have a function in ameliorating colitis and play a protective role in IBD. However, the exact role and mechanism are still unclear.

CHAPTER 2: HYPOTHESIS AND AIMS

Based on the literature presented, this thesis examines the role of the bacterium *A. muciniphila* in intestinal inflammation and its potentially protective impact within this state. Since *A. muciniphila* is known to strengthen barrier function in the context of metabolic syndrome (5,91,103,112,113,129–132) and a lower abundance of *A. muciniphila* is observed in IBD patients (135,137,138), we hypothesize that *Akkermansia muciniphila* induces protective effects in intestinal inflammation and can ameliorate the severity of colitis by strengthening intestinal barrier integrity. Here, by utilizing three different experimental models of colitis (chemical, spontaneous, and enteric infection) we explore the role of *A. muciniphila* in intestinal barrier function and in the pathogenesis of colitis.

Aim 1: *To investigate the protective role of A. muciniphila in intestinal inflammation in barrier disrupting chemically-induced and spontaneous models of colitis.*

Rationale: Though the initiation and pathogenesis of these debilitating diseases are currently unknown, evidence suggests that the gut microbiota may play a role in triggering the inappropriate inflammatory response characteristic of IBD (3,11,12). Multiple studies suggest that microbial diversity is diminished in IBD and that microbial shifts occur predominantly characterized by increased levels of Proteobacteria and Actinobacteria and decreased abundance of Firmicutes and Bacteroidetes (16,81) .

Recently, our laboratory investigated the impact that serotonin (5-HT) has on the severity of colitis and on the intestinal microbiota (144). In this study, microbial differences between *Tph1*$^{+/-}$ and *Tph1*$^{-/-}$ mice which have significantly reduced levels of intestinal 5-HT, were found to play a key role in host susceptibility to colitis. The transfer

of *Tph1*$^{-/-}$ cecal content into GF and *Tph1*$^{+/-}$ mice increased gut barrier integrity, upregulated *Muc2* and decreased inflammation severity after administration of DSS. Notably, these mice also had an increased abundance of the bacterial species, *Akkermansia*.

A. muciniphila, a commensal mucin degrader, accounts for 1-5% of the human fecal microbiota (91,107–109). *A. muciniphila* has been found to be negatively correlated with several disease states, including type 1 and type 2 diabetes, appendicitis, obesity, metabolic syndrome and IBD (91,114,115). Though a clear-cut role and mechanism by which *A. muciniphila* may influence chronic inflammatory conditions have not been determined, there is evidence indicating that this microbe is depleted in patients with IBD and in chemical models of colitis (113,135) suggesting that *A. muciniphila* could have protective or anti-inflammatory effects, which are lost in IBD (80). Additionally, by acting on TLR2 and restoring tight junction proteins, both live and pasteurized *A. muciniphila* and its outer membrane protein, Amuc_1100, have been found to enhance gut barrier function and decrease intestinal permeability (112,129–132). *A. muciniphila's* influential host interactions, including its ability to influence the production of AMPs, increase goblet cell number and thickness of the mucus layer, enhance barrier function and the expression of several TJPs (145,146) and increase the number of anti-inflammatory regulatory T cells (103), suggests that it may ameliorate colitis and play a protective role in IBD. Utilizing barrier disrupting models of colitis, including chemically-induced DSS colitis (which has been shown to disrupt the integrity of the mucosal barrier, reduce the expression of tight junction proteins, and alter gut

permeability (60,147–149)) and the spontaneous colitis developed in *Muc2*$^{-/-}$ mice provides a unique opportunity to study these effects *in vivo*. Thus, supplementation of *A. muciniphila* in barrier disrupting models of colitis may elicit beneficial effects by re-establishing or strengthening intestinal barrier integrity.

Aim 2: *To investigate the protective role of A. muciniphila in intestinal inflammation and host defence in a model of enteric parasitic infection.*

Rationale: In order to promote their survival and proliferation, enteric pathogens such as intestinal nematodes, have evolved the ability to manipulate host biology and resident microbes. The host-parasite-microbe interface, thus, provides an interesting model to observe the altered function of the gut/mucosal barrier physiology and microbial influence during periods of local intestinal inflammation (93,150). *T. muris,* a non-invasive intestinal nematode that does not penetrate beyond the mucosal layer, provides an infection-induced model of colitis. The ability of mice to expel *T. muris* infection is highly mucin dependent (102,105). Our research has previously shown that treatment of live *L. rhamnosus* (JB-1) in *T. muris* infection significantly enhances worm expulsion and increases IL-10 levels, goblet cell number, and epithelial cell proliferation (105). *A. muciniphila* has the potential to elicit similar, and perhaps greater effects, due to its cacophony of beneficial host interactions seen within the context of metabolic syndrome (103,129,131,132,135,136). Perhaps the most influential of these effects is that *A. muciniphila* possesses specialised machinery to fully break down MUC2 glycan chains, the main structural components in the protective mucus layer of the intestine (91,118).

However, despite this mucolytic nature, *A. muciniphila* has been found to stimulate mucin production, increase goblet cell number, promote mucus thickness, and enhance gut barrier integrity (5,91,103). The capacity of this gut microbe to boost anti-inflammatory T_{reg} cells numbers (103) and, subsequent IL-10 levels (127), may also have indirect beneficial impacts on the mucus layer (151). *A. muciniphila's* ability to elicit these effects are likely highly advantageous in the clearance of enteric infection (102,105,151,152) and may work to ameliorate local inflammation by greatly influencing intestinal barrier integrity.

CHAPTER 3: METHODS

Animals

Mice were housed at McMaster University Central Animal Facility (CAF) and were kept in sterilized, filter-topped cages. All experiments were performed in accordance with the guidelines set forth by the Canadian Council on Animal Care (CCAC) and the McMaster University Care Committee, and all experiments were approved by the Animal Research Ethics Board (AREB) at McMaster University. Mice had access to autoclaved food and water (when not substituted by DSS) and were subjected to a 12-hour light-dark cycle. Germ-free C57BL/6 mice (10-12 weeks old) were housed in the Farncombe Family Axenic-Gnotobiotic Facility at McMaster University until the time of gavage. All specific pathogen-free (SPF) mice had a minimum 7-day acclimatization period upon arrival. C57BL/6 mice (6-8 weeks old) were purchased from Taconic Biosciences (Germantown, NY) and Charles River Laboratories (Wilmington, MA). *Muc2*$^{-/-}$ mice were created by gene mutation and were acquired from Dr. Kris Chadee (University of Calgary) and were housed in SPF conditions as above. These mice spontaneously develop colitis characterized by mucosal thickening and ulcerations as early as five weeks of age due to loss of epithelial barrier function (153).

Bacterial Culture

Akkermansia muciniphila GC7 was obtained from Dr. Michael Surette's lab at McMaster University. Bacteria were grown at 37°C for approximately seven days under anaerobic conditions (5% CO_2, 5% H_2 90% N_2) in basal-mucin based media and then inoculated into brain heart infusion (BHI) broth. Bacteria/CFU count was measured using a Vitek colourimeter (bioMérieux, Hazelwood, MO).

PTFE septum screw cap glass vials containing bacteria were prepared and maintained under anaerobic conditions. To maintain cells under anaerobic conditions for as long as possible prior to gavage, bacteria were removed from vials using a syringe. Pasteurized bacteria were prepared by heating bacterial samples for 70°C for 30 minutes (128,154–157). GF mice were gavaged with 200µl of bacterial treatment or control for three days, whereas SPF mice were gavaged with the same volume over the course of 15 days. In SPF *T. muris* experiments, resistant C57BL/6 mice were given *A. muciniphila* for a total of 15 days, beginning one day before infection. BHI broth was used as a control.

Chemically-Induced Model of Intestinal Inflammation

Chemically-induced models of gut inflammation are the best described and the most commonly used experimental models of IBD(158). Among these, the DSS-mouse model is widely utilized to induce acute or chronic colitis based on altered concentrations, duration or frequency of administration (159). Though the mechanism of action is not currently known (149), DSS has been shown to disrupt the integrity of the mucosal barrier, reduce the expression of tight junction proteins including ZO-1 and occludin, alter gut permeability, induce barrier defects as well as elicit direct cytotoxic effects to the colonic mucosa, and cause granulocyte infiltration largely confined to the colon (60,147–149,159–162). In DSS, this insult to the mucosal layer is a major inflection point in the transition from the initiation to the perpetuation of the inflammatory response (163) by allowing the infiltration of luminal contents (159). DSS-induced colitis is also associated with mucus depletion and goblet cell loss (149,163). Interestingly, DSS seems to promote a model that is a mix of CD and UC initiating a classic Th1 immune response (IL-12,

IFNγ, IL-1 and TNFα) associated with CD but with inflammation limited largely to the colon similar to UC (148,149).

As per our previously established methods (164), DSS (molecular mass 40 kDa; CAS NO. 9011-18-1, BOC Sciences, Shirley, NY, USA) was prepared in autoclaved drinking water to a final concentration between 1.5 - 5% (w/v) depending on the experimental context. Mice had *ad libitum* access to DSS for five days which replaced normal drinking water. Control mice had *ad libitum* access to normal drinking water over the full time-course. DSS consumption per cage was recorded throughout the experiment. IBD often presents with symptoms that include diarrhea, rectal bleeding, abdominal pain, and weight loss; animals treated with DSS mirror this symptomatology and display crypt erosions, epithelial damage, shortening of the large intestine and inflammatory cell infiltration (60,147,148,159,165,166). DSS has been described by Solomon et al. as having "similarities to human IBD in etiology, pathology, pathogenesis, and therapeutic response" (149).

During the administration of DSS, a disease activity index (DAI) score, the combined score of weight loss, stool consistency, and fecal and rectal bleeding, was used to assess the progression and severity of colitis. Mice were monitored and scored daily for the duration of DSS. Scores were defined as follows: weight loss: 0 (no loss), 1 (1-5%), 2 (5-10%), 3 (10-20%), and 4 (>20%); stool consistency: 0 (normal), 2 (loose stool), and 4 (diarrhea); and bleeding: 0 (no blood), 1 (Hemoccult positive), 2 (Hemoccult positive and visual pellet bleeding), and 4 (gross bleeding, blood around anus). Changes in daily body

weight were recorded and given a score based on the percent change compared to day 0/baseline starting weight of each mouse.

Enteric Parasite Infection-induced Model of Intestinal Inflammation

Over a long co-evolutionary history, enteric pathogens such as intestinal nematodes, have developed the ability to manipulate host biology and resident microbes in order to promote their own survival and proliferation. The host-parasite-microbe interface, thus, provides an interesting model to observe the altered function of the gut/ mucosal barrier physiology and microbial influence during periods of local intestinal inflammation (93,150).

Trichuris trichiura is a parasitic roundworm and the causative agent of trichuriasis in humans. Largely infecting children in warmer climates, this roundworm accounts for an estimated 1 billion parasitic infections worldwide (167). *T. muris, T. trichiura's* murine equivalent, is a non-invasive intestinal nematode that does not penetrate beyond the mucosal layer and provides an infection-induced model of colitis (168). This parasitic model is ideal for studying local responses in the gut without the confounding influence of a systemic response and for understanding gut inflammation and host defence. In mice, within two to three weeks of infection with *T. muris,* the intestinal mucosa becomes thickened, crypt hyperplasia develops, and inflammatory cells infiltration is present (169). Interestingly, different inbred strains of mice show differential immunological responsiveness to *T. muris* (169,170). The resistant strains (BALB/c, C57BL/6) completely clear infection by day 35 post-infection (p.i.) with activation of Th2 type response (IL-4, IL-13) (102,169,171). In contrast, susceptible strains (AKR, SCID)

harbour a chronic infection with activation of Th1 type response (94,99,170). Type of response is critical in determining resistance or susceptibility (150,172). The primary response in both resistant and susceptible strains to *T. muris* excretory/secretory products (ESPs) is predominantly IgG.

T. muris worms harvested from immunodeficient/susceptible mice were extracted and incubated in RPMI 1640 media with 500U/ml penicillin and 500µg/ml streptomycin for 4 hours and then overnight at 37℃. Eggs from this culture were harvested and resuspended in sterile H_2O. In preparation for administration, stored eggs were washed and resuspended using autoclaved DNAase free water and centrifuged at 1500rpm for 6 minutes. Under a microscope, live, mature eggs were counted per 50µl, and subsequently, the initial egg/water solution was adjusted to 300 eggs per 100µl in preparation for oral gavage. Mice were then infected with one dose of approximately 300 *T. muris* eggs by oral gavage (173,174). Post-infection, GF mice were housed in a ventilated rack.

The majority of worms in resistant mice are expelled between 14-28 days, with the most rapid reduction between 16 and 18 days p.i. (175). The timepoint of sacrifice was set at 14 p.i. to assess immune alterations and cecal worm burden.

Upon sacrifice, cecal samples were collected and stored at -20℃. The cecum and proximal colon are the predominant infection site of *T. muris* (150). Thus, cecal worm burden was assessed by scraping tissue and cecal content in dilutions of sterile H_2O; worms were counted under a dissecting microscope.

Tissue Preparation

Upon sacrifice, colonic tissue was collected. Cecal content and fecal matter were also collected for 16S rRNA analysis and other downstream applications. Colonic tissue was divided into five sections of approximately 1cm each. These sections were flash-frozen in liquid nitrogen and then stored at -80°C. These five sections were sequestered for assays based on location within the colon. From proximal to distal, the segments were utilized as follows: qPCR, 5-HT (if needed), cytokine assays, histology (approximately 2mm section, formalin-fixed), myeloperoxidase assay (if needed) and one section of extraneous tissue for any additional analysis.

At the time of sacrifice, colitis severity was assessed by macroscopic scoring based on colonic and rectal bleeding, diarrhea/stool consistency, and rectal prolapse, all represented by scores ranging from 0 to 3, with 3 representing the most severe case of each condition. Each mouse was given an individualized macroscopic score based on the summation of the aforementioned criteria.

Histology

Colonic samples were formalin-fixed, washed in 50% ethanol solution and then placed in 70% ethanol solution until embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) stain to assess histological damage and loss of structure, and periodic acid-Schiff (PAS) for goblet cell quantification. PAS-stained goblet cells were quantified per 10 villus-crypt units.

H&E-stained tissue sections were analyzed by a blind observer for alterations in histology, particularly goblet cell depletion, inflammatory cell infiltration, loss of crypt

architecture, muscle thickening, and the presence of crypt abscesses in mucosal tissue (147,164).

Enzyme-Linked Immunosorbent Assays (ELISA)

Pro- and anti-inflammatory cytokines, including IL-1β, IL-6, TNFα, (for DSS experiments) and IL-4 and IL-13 (for *T. muris* experiments), were assessed within colonic tissue using commercially available ELISA kits (Quantikine ELISA; R&D Systems, Minneapolis, MN, USA). The anti-inflammatory cytokine, IL-10, was measured for all experiments using a commercially available high sensitivity ELISA kit (Cat. # EKN49384; Biomatik, Kitchener, Canada). As previously described, colonic tissue was sectioned into five segments of approximately 1cm. For all experiments, section 4 was homogenized in 1ml lysis buffer supplemented with protease inhibitor cocktail (PIC) (Cat. # P8340; Sigma-Aldrich, Oakville, Canada). This solution was prepared with a ratio of 50µl PIC to 10ml lysis buffer. After homogenizing for 5 minutes at 30Hz, samples were subsequently centrifuged for 5 minutes at 3300g. Supernatants were collected and stored at -80°C until use. Protein quantification of each sample was performed according to the manufacturer's protocol using DC Protein Assay Kit (Cat. # 5000111; Bio-Rad Laboratories) and compared to a standard curve of known concentration using bovine serum albumin (Cat. # ALB001.250 CAS # 9048-46-8, BioShop, Burlington, ON, Canada). ELISAs were run according to the manufacturer's protocol. All cytokine measures were expressed in ng/µg protein. Cytokine levels (IL-1β, Cat. # MLB00C; IL-6, Cat. # M6000B; TNFα, Cat#MTA00B, IL-13, Cat. # M1300CB; IL-4, Cat. # M4000B)

were determined according to the manufacturer's instructions (Quantikine Murine; R&D Systems, Minneapolis, MN, USA).

Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR)

To investigate the effects that *A. muciniphila* has on barrier function in the context of colitis, reverse transcriptase qPCR was used to explore the relative gene expression of several factors, including several TJPs, AMPs, and mucins.

RNA extraction was performed using TRIzol® reagent (Ref. # 15596018; ThermoFisher Scientific/Ambion by Life Technologies, Carlsbad, CA, USA). Briefly, cleaned and weighed tissue sections were homogenized for 4 minutes at 30Hz in 500µl of TRIzol® reagent regardless of weight. After incubating at room temperature for 5 minutes, 100µl of chloroform was added to each sample and mixed by hand until cloudy and incubated again for 3 minutes at room temperature. Samples were then centrifuged at 12000rcf for 15 minutes at 4°C. After separation, the aqueous layer of each sample was collected, 250µl of ice-cold isopropanol was added to each and samples were incubated at room temperature for 10 minutes and subsequently centrifuged at 12000rcf for 10 minutes at 4°C. The remaining pellet was resuspended in 500µl of 75% ice-cold ethanol, centrifuged at 7500rcf for 5 minutes at 4°C, the supernatant was discarded, and pellets were left to air dry for ~10 minutes. Pellets were then resuspended in 20µl Ultra PureTM Distilled water DNAase and RNAse free (Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 15 minutes at 55°C.

Total RNA yield and quality were determined by the UV-Vis spectrophotometer, NanoDrop™ One (Thermo Scientific). Sample purity post-TRIzol extraction was

assessed by comparison of the ratio of absorbance at wavelengths of 260 to 280nm and 260 to 230nm. These ratios typically lie at approximately 2.0 for pure RNA. Deviation away from this value can signify contamination either by proteins, urea or phenols and negate the use of the sample for further application. RNA was measured in µg/mg. Samples were equalized to 1µg before subjecting to complementary DNA (cDNA) synthesis.

BIORAD iScript cDNA Synthesis Kit (Cat. # 1708891; Bio-Rad Laboratories, Mississauga, ON, Canada) was used to convert equalized RNA samples to cDNA. cDNA was synthesized according to the manufacturer's instructions.

To quantify the genes of interest, real-time quantitative polymerase chain reaction (RT qPCR) was used. The forward and reverse primer sequences, which were used at a concentration of 10 µM, can be found in Table 2. Each reaction mixture contained 1 µl of cDNA, 10 µL SsoAdvanced ™ Universal SYBR® Green Supermix (Cat. # 1725271; Bio-Rad Laboratories), 1 µl respective forward and 1 µl respective reverse primer and 7 µl of Ultra PureTM Distilled water DNAase and RNAse free (Thermo Fisher Scientific). RT- qPCR was executed using the CFX96 real-time PCR system (Bio-Rad). The resultant data were analyzed using the $2^{-\Delta\Delta CT}$ method. Results are expressed as relative abundance compared to the housekeeping gene coding for 18S ribosomal RNA (rRNA). At the end of each run, melting curve analysis was performed to verify the quality and specificity of the employed primers.

Primer	Forward Sequence (5'- 3')	Reverse Sequence (5'- 3')
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
mβdef1	GGTGTTGGCATTCTCACAAG	ACAAGCCATCGCTCGTCCTTTATG
RegIIIγ	CCGTGCCTATGGCTCCTATTG	GCACAGACACAAGATGTCCTG
Muc5ac	GTGATGCACCCATGATCTATTTTG	ACTCGGAGCTATAACAGGTCATGTC
Muc2	CTGACCAAGAGCGAACACAA	CATGACTGGAAGCAACTGGA
Ocln	ATGTCCGGCCGATGCTCTCTC	CTTTGGCTGCTCTTGGGTCTGTAT
TJP1 (ZO-1)	ACCCGAAACTGCTGCTGTGGATAG	AAATGGCGGGCAGAACTTGTGTA

Table 2. Mouse Primer Sequences for qPCR *in vivo*

Microbial Profiling and Analysis

To analyse the composition of the microbiota and changes in diversity with *A. muciniphila* supplementation, 16S rRNA sequencing of fecal samples was performed via amplification of the V3 regions. Amplification products were sequenced via Illumina Mi Seq Illumina (Farncombe Institute) as previously described (144), processed via the DADA2 pipeline and compared against the SILVA taxonomy database. Microbial data was analyzed using the online tool MicrobiomeAnalyst developed by the Xia Lab at McGill University based on the Java Server Faces technology using the Primefaces component library and found at https://www.microbiomeanalyst.ca/. This online tool utilizes the MicrobiomeAnalyst R package for backend statical analysis and graphical/visual outputs, which can be obtained at https://github.com/xia-

lab/MicrobiomeAnalystR (176). Data was rarefied to the minimum library size to account for any uneven sequencing depth, under-sampling etc. in the data.

The impact *A. muciniphila* supplementation exhibited on the bacterial community was visualized by Bray-Curtis beta diversity, and statically significant differences were examined using permutational multivariate analysis of variance (PERMANOVA) and dissimilarities were visualized using principal coordinates analysis (PCoA) plots generated in MicrobiomeAnalyst. To account for both evenness and richness, alpha diversity was analysed utilizing the Shannon Index.

Student's *t*-test and one-way analysis of variance (ANOVA) were used, where appropriate, for univariate statistical comparison. *P* values were adjusted using false discovery rate (FDR). A corrected *P* values of <0.05 was considered statistically significant.

Statistical Analysis

Statistical analysis was completed using GraphPad Prism version 9.0.0 for Windows (GraphPad Software, San Diego, California, USA). All results were expressed as mean ± standard error of the mean (SEM). Where appropriate, comparison between two groups was performed using unpaired Student's *t*-test. In contrast, ANOVA with Tukey's HSD post-hoc multiple comparison test was used when comparing more than two treatment groups. *P* values of <0.05 were reported as statically significant.

CHAPTER 4: EXPLORING THE EFFECTS OF *AKKERMANSIA MUCINIPHILA*-MODULATED MICROBIOTA IN CHEMICALLY-INDUCED AND ENTERIC PARASITIC MODELS OF INTESTINAL INFLAMMATION IN GERM-FREE MICE

4.1 Introduction

The resident gut microbiota and the pathogenesis of intestinal inflammation are inextricably linked. Altered disease severity with use of antibiotics in patients with IBD (3,62), lack of colitis development in GF mice (58–60), the ability of certain microbes to modify host immune responses (62–66), altered microbial diversity and composition associated with IBD, and several IBD susceptible genes that code for bacterial sensors (5,40–42,62,67) all point to an intimate relationship between the colonizers of the gut and intestinal inflammation. Indeed, several clinical and animal studies have suggested that gut bacteria trigger and perpetuate chronic colitis (57).

Our laboratory recently investigated the impact that serotonin (5-HT) has on the severity of colitis and on the intestinal microbiota (144). In this particular study, microbial differences between littermate *Tph1*$^{+/-}$ and, *Tph1*$^{-/-}$ mice, which have significantly reduced levels of intestinal 5-HT, were found to play a key role in host susceptibility to colitis. The transfer of *Tph1*$^{-/-}$ cecal content into both GF and *Tph1*$^{+/-}$ mice increased gut barrier integrity, upregulated *Muc2* and decreased inflammation severity after administration of DSS. Notably, these mice had an increased abundance of the bacterial genus, *Akkermansia*. Largely within the context of metabolic syndrome and obesity, *A. muciniphila* (AKK) has been touted as a "next-generation probiotic" due to its influential host interactions, including its ability to influence the production of AMPs (132), increase goblet cell number and thickness of the mucus layer, enhance both barrier function and the expression of several TJPs (145,146), and increase the number of anti-inflammatory regulatory T cells (103). In conjunction with our findings in GF and *Tph1*$^{+/-}$

mice, *A. muciniphila*'s aforementioned ability to impact host physiology suggests that it may ameliorate colitis and play a protective role in IBD. However, the exact role and mechanism are still unclear.

In this chapter, we investigate the impact of *A. muciniphila*-modulated microbiota in both chemically-induced and enteric parasitic models of intestinal inflammation in GF mice. The relative "blank slate" of GF mice provides a unique opportunity to manipulate the administered microbial contents and establish a fairly uniform initial microbiota across multiple mice. By utilizing both DSS and *T. muris* models, we investigate the impact of this microbe on both intestinal inflammation and barrier function.

4.2 Results

Concurrent and subsequent *A. muciniphila* supplementation in GF mice provides only minor beneficial effects in an acute DSS model of colitis

To examine the impact of *A. muciniphila*-supplemented microbiota in a chemically-induced model of colitis, two alternative strategies were used; concurrent administration of *A. muciniphila* along with SPF C57BL/6 cecal contents or administration of SPF C57BL/6 cecal contents followed by a subsequent administration of *A. muciniphila* alone.

In preliminary work, GF C57BL/6 mice were gavaged for three days with wild-type cecal contents or wild-type cecal contents supplemented with *A. muciniphila* (courtesy of the Surette lab), followed by a 7-day period of colonization. 2.5% DSS was then administered for five days via autoclaved drinking water. A slight difference in disease activity index (DAI) score was found between the *A. muciniphila* group and the wild-type microbiota group on day 5 of DSS treatment. This difference was also observed

macroscopically upon sacrifice. Pro-inflammatory cytokines assessed did not prove significant; however, the data suggests lower levels of these cytokines in the group that was administered *A. muciniphila* (Figure 4.1).

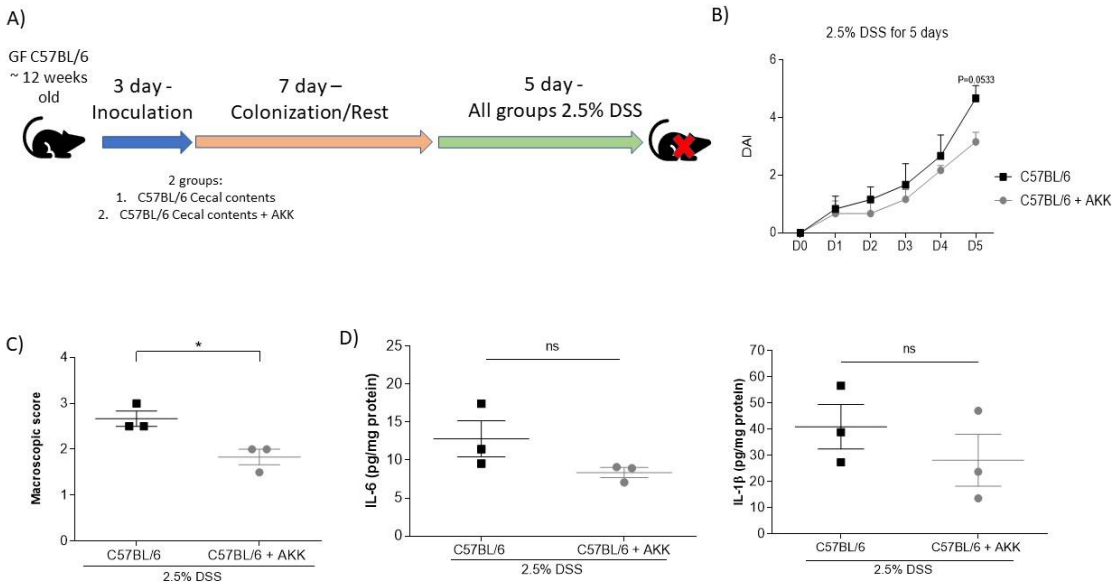

Figure 4.1. *Effect of A. muciniphila on DSS-induced colitis in GF mice.*

GF C57BL/6 mice were given wild-type cecal contents or wild-type cecal contents supplemented with *A. muciniphila* for three days, followed by a 7-day period of colonization. 2.5% DSS was then administered for five days. (A) represents a brief experimental outline. Inflammation was scored by (B) DAI, (C) macroscopic scoring, and the pro-inflammatory cytokines (D) IL-6 and IL-1β. Each value represents the mean ± SEM (n = 3-4 mice per group). *$P < 0.05$.

A follow-up experiment was performed to examine the effects of separating and delaying the *A. muciniphila* gavage from the gavage of wild-type cecal contents. Here, GF C57BL/6 mice received wild-type cecal contents followed by a 7-day colonization period. Starting three days prior to the administration of 2.5% DSS, mice were inoculated daily with *A. muciniphila* by oral gavage. This delayed gavage (C57BL/6 + dAKK) was

significant in improving DAI score (day 3) and macroscopic score compared with controls (Figure 4.2 B & C). These findings were also reflected histologically (Figure 4.4). The pro-inflammatory cytokines, IL-6 and TNFα, had a slight, though not significant, decrease in those mice gavaged with *A. muciniphila* (Figure 4.2D). No difference in the levels of IL-1β were detected between the groups (Figure 4.2D). Interestingly, IL-10 was elevated in this delayed *A. muciniphila* group (Figure 2E), suggesting that this microbe has an influence on the inflammatory balance of the gut. Indeed, when comparing the pro-inflammatory cytokine, TNFα, to the anti-inflammatory, IL-10 (127), *A. muciniphila*-gavaged group displayed a significant decreased in this ratio compared to controls (Figure 4.2E), suggesting it may not be the influence on individual cytokines but the overall shift to a more anti-inflammatory microenvironment that lies behind this microbes effect in colitis.

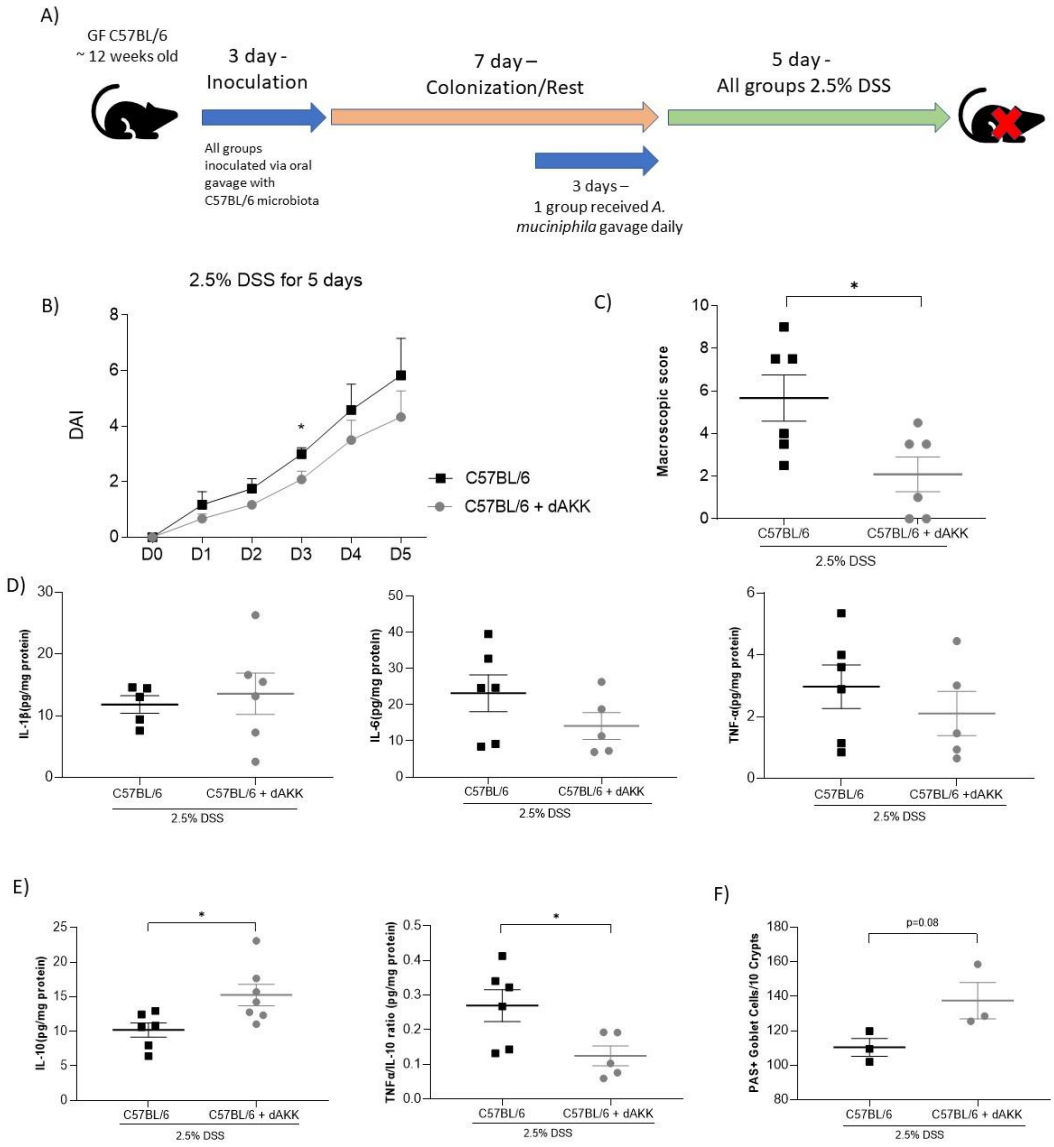

Figure 4.2. *Markers of inflammation in A. muciniphila gavaged three days prior to induction of DSS-induced colitis in GF mice.*

GF C57BL/6 mice received wild-type cecal content for three days, followed by a 7-day colonization period. Starting three days before administration of 2.5% DSS, mice were inoculated with *A. muciniphila* daily. (A) shows the overall experimental layout. Disease progress and severity of inflammation were assessed by (B) DAI, (C) macroscopic scoring, (D) the pro-inflammatory cytokines IL-1β, IL-6 and TNFα, and (E) the anti-inflammatory cytokine IL-10, and the pro- to anti- inflammatory ratio as represented by TNFα/IL-10. (F) shows the number of PAS+ stained goblet cells per 10 crypts, representative pictures of

which are shown in Figure 4.4. Each value represents the mean ± SEM (n = 5-7 mice per group). *$P < 0.05$.

Gene expression levels for several markers of barrier function, including the TJPs, occludin, and the AMPs, mouse β-defensin-1 and *RegIIIγ,* did not prove significantly different between groups (Figure 4.3). The tight junction protein, zonula occludens-1, proved slightly upregulated in the *A. muciniphila* treated group compared with controls though statistical significance was not reached (Figure 4.3B).

There was no difference in the expression levels of the major structural mucin of the gut, *Muc2*, between *A. muciniphila*-gavaged mice and control mice (Figure 4.3E). Despite this lack of difference, distinct changes in the number (Figure 4.2F) and robustness of goblet cells were apparent between the groups (Figure 4.4); PAS staining revealed that the *A. muciniphila*-treated group displayed goblet cells with a more "filled" appearance as well as slightly increased numbers of these mucin-producing cells compared to those mice which received unsupplemented microbial contents.

Within this GF model, 16S rRNA sequencing showed successful establishment of the microbiota post-DSS administration. However, at this timepoint, only alpha diversity (P= 0.049) proved different between those mice gavaged with C57BL/6 content and *A. muciniphila* versus those gavaged with C57BL/6 cecal content alone. Classical univariate analysis indicated no significant difference in *Akkermansia* between the groups (Figure 4.5).

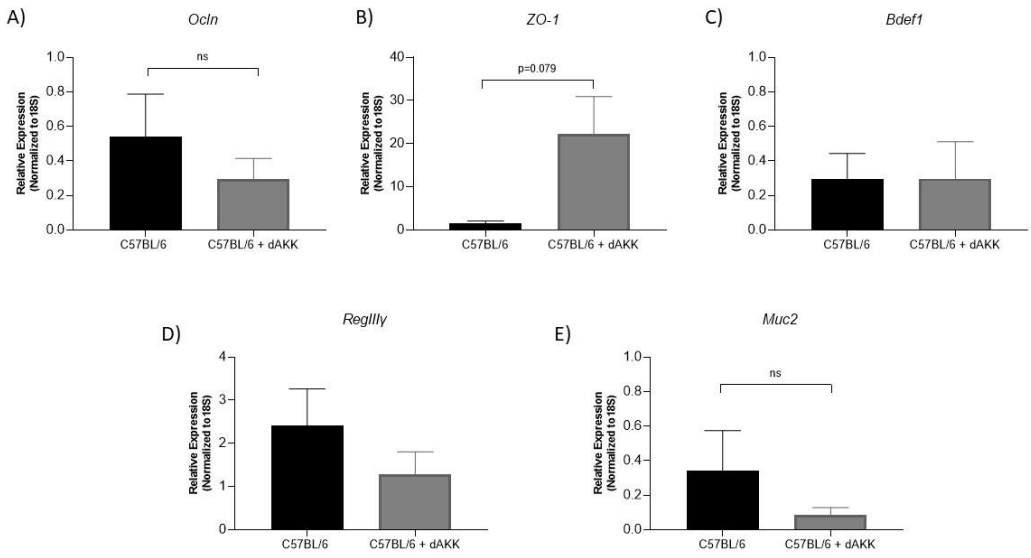

Figure 4.3. *Markers of barrier function in GF mice gavaged with A. muciniphila three days prior to induction of DSS-induced colitis.*

GF C57BL/6 mice received wild-type cecal content for three days, followed by a 7-day colonization period. Starting three days before administration of 2.5% DSS, mice were inoculated with *A. muciniphila* daily. Genes involved in barrier function including the tight junction proteins, (A) occludin and (B) zonula occludens-1, the antimicrobial peptides, (C) mouse β-defensin 1 and (D) *RegIIIγ*, and the major structural mucin of the gut, (E) *Muc2*, were assessed by qPCR. Each value represents the mean ± SEM (n = 5-7 mice per group). **P <0.05.*

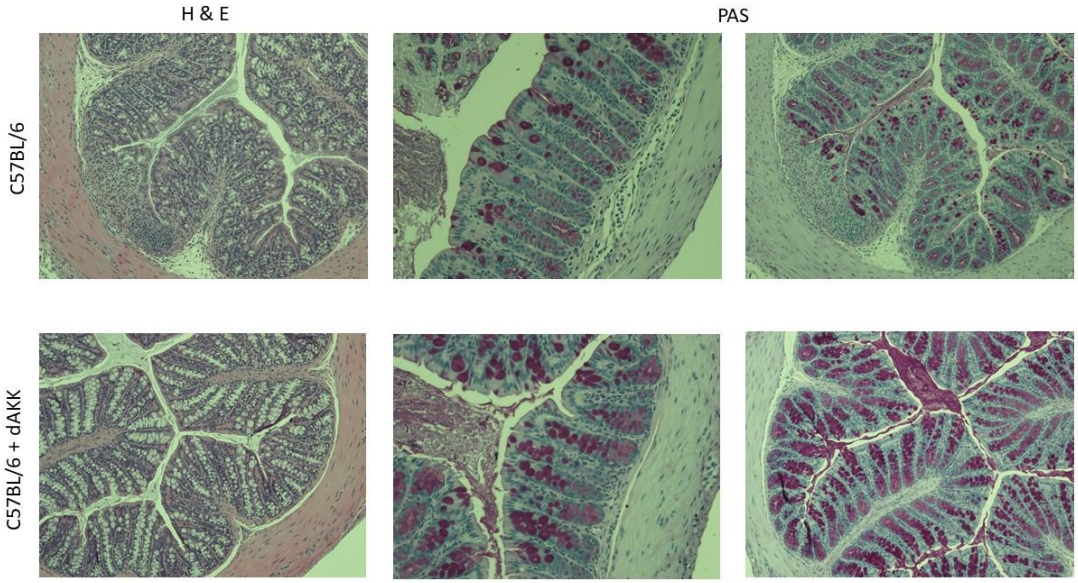

Figure 4.4. *Histological alterations in DSS-induced colitis in GF mice supplemented with A. muciniphila.*

GF C57BL/6 mice received wild-type cecal content for three days, followed by a 7-day colonization period. Starting three days before administration of 2.5% DSS, mice were inoculated with *A. muciniphila* daily. Representative H&E-stained micrographs and PAS-stained micrographs of colonic tissue in mice gavaged with either C57BL/6 or C57BL/6 + dAKK cecal contents are shown.

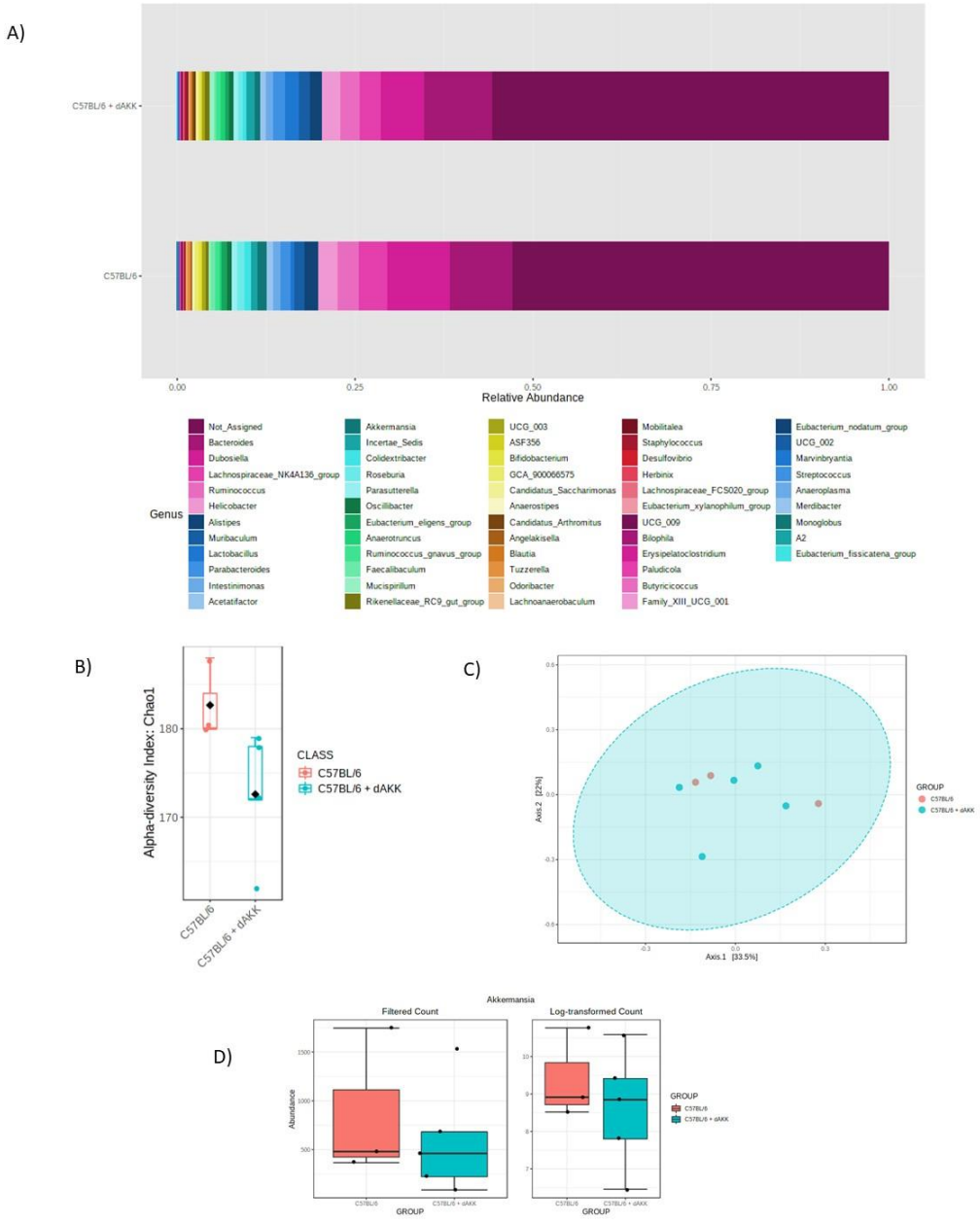

Figure 4.5. *16S rRNA analysis of A. muciniphila gavage in DSS-induced colitis in GF mice.*

GF C57BL/6 mice received wild-type cecal content for three days, followed by a 7-day colonization period. Starting three days before administration of 2.5% DSS, mice were inoculated with *A. muciniphila* daily. (A) Relative abundance, represented at the genus level, in mice gavaged with wild-type (C57BL/6) cecal contents or wild-type cecal contents supplemented with *A. muciniphila* (C57BL/6 + dAKK) on Day 5 of DSS

administration. (B) Alpha diversity, (C) beta diversity, and (D) classical univariate analysis of *Akkermansia* are also shown.

Supplementation with *A. muciniphila* alters infection severity and markers of intestinal barrier function in a *T. muris* infection-based model of colitis in GF mice

The host-parasite-microbe interface provides an interesting model to observe the altered function of the gut/mucosal barrier physiology and microbial influence during periods of local intestinal inflammation (93,150). Thus, in addition to the DSS chemical model of colitis, the intestinal nematode, *T. muris* was utilized as an infection-induced model of colitis.

To investigate whether *A. muciniphila* provides protective effects against *T. muris* infection-induced changes in intestinal epithelium and gut inflammation, *A. muciniphila* was gavaged along with wild-type cecal contents into GF C57BL/6 mice for three days. Following a 3-week colonization period, mice were infected with ~300 *T. muris* eggs via oral gavage. The ability of mice to clear *T. muris* infection is greatly influenced by increased production of Muc2. Thus, *A. muciniphila's* known ability to influence mucin turnover, upregulate goblet cells, and enhance mucin production may prove beneficial in the expulsion and clearing of this infection (102,104). Mice were sacrificed 14 days post-*T. muris* infection (Figure 4.6). Upon sacrifice, the severity of infection and inflammation were assessed. Cecal worm burden was used as a marker of infection severity; at 14 days post-*T. muris* infection, mice receiving *A. muciniphila* trended toward decreased worm burden compared to those receiving only wild-type cecal contents (Figure 4.6B). The number of PAS+ goblet cells (Figure 4.6C & D) and the expression of *Muc2* (Figure

4.7E) and *Muc5ac* (Figure 4.7F) also trended toward increased levels in mice supplemented with *A. muciniphila* versus those gavaged with wild-type cecal contents alone. Interestingly, though normally elevated in resistant mice, the Th2 cytokine, IL-4, was decreased in mice supplemented with *A. muciniphila* (Figure 4.6E). This decrease may be the result of the timepoint of collection or simply the altered colonic microenvironment in GF mice. Despite lower levels of IL-4, the anti-inflammatory cytokine, IL-10 (Figure 4.6G), was elevated in mice receiving *A. muciniphila* versus those receiving wild-type contents alone. This finding prompted an investigation into measures of the pro-inflammatory cytokine, interferon-γ (IFNγ), as an overall tamping down of the immune response in conjunction with elevated IL-10 may be responsible for the increased worm expulsion and decrease in Th2 cytokines associated with *A. muciniphila*-gavaged mice. However, no statistically significant differences between the groups were detected in levels of IFNγ or in the pro- to anti-inflammatory ratio (IFNγ/IL-10) (Figure 4.6H & I).

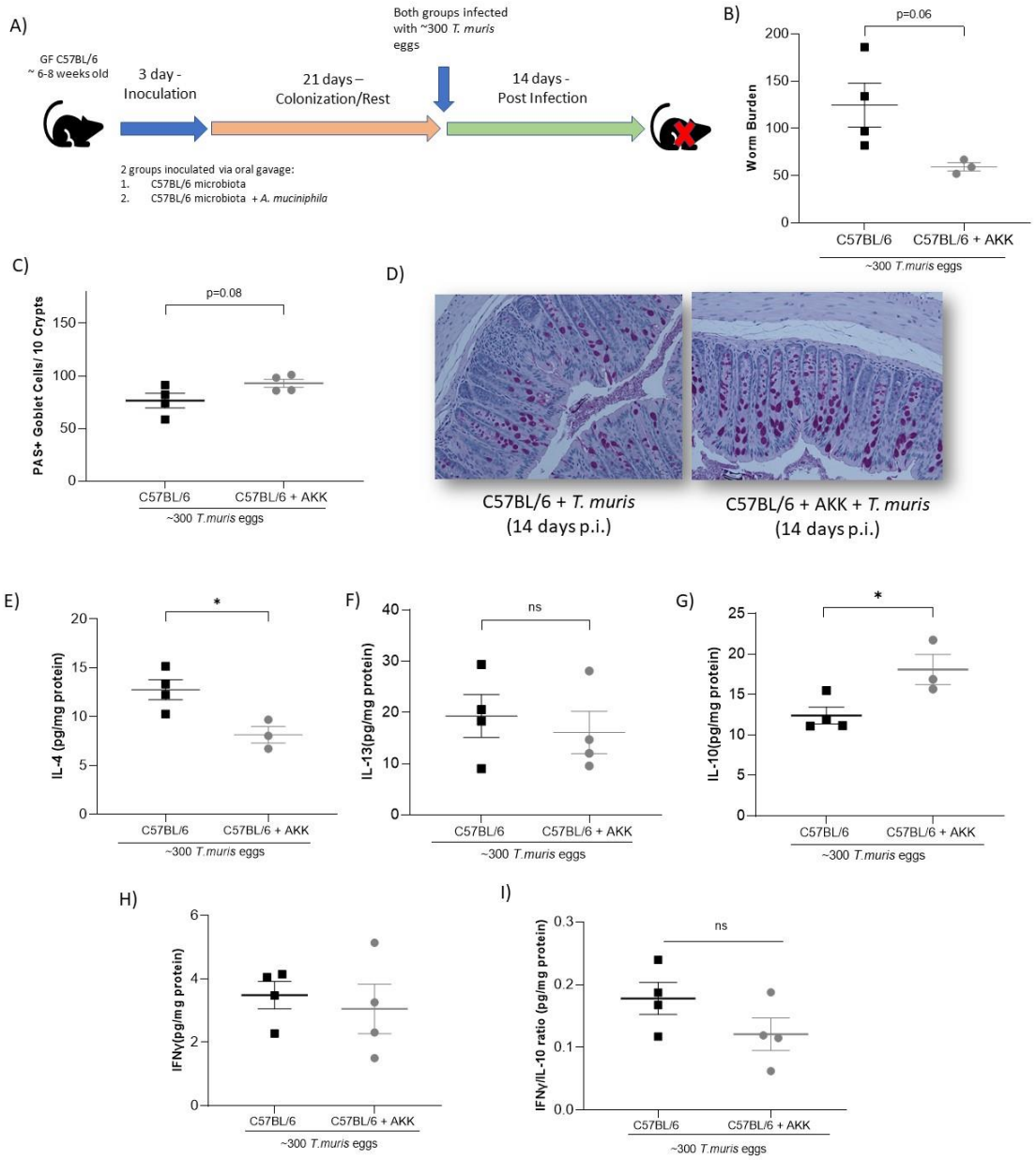

Figure 4.6. *Effect of A. muciniphila against T. muris infection-induced changes in intestinal epithelium and gut inflammation in GF mice.*

GF C57BL/6 mice were given wild-type cecal contents or wild-type cecal contents supplemented with *A. muciniphila* for three days, followed by a 21-day period of colonization. ~300 *T. muris* eggs were then administered. Mice were sacrificed 14 days post-infection. (A) represents a brief experimental outline. Infection severity and inflammation were analyzed by (B) worm burden, (C) the number of PAS+ stained goblet

cells per 10 crypts (D), as well as the cytokines, (E) IL-4, (F) IL-13, (G) IL-10, and (H) IFNγ. (I) represents the pro- to anti-inflammatory cytokine (IFNγ/IL-10) ratio. Each value represents the mean ± SEM (*n* = 3-4 mice per group). **P <0.05.*

In addition to the mucins mentioned above, several other markers of intestinal barrier function were assessed by qPCR (Figure 4.7). Both occludin (Figure 4.7A) and β-defensin 1 (Figure 4.7B) were downregulated in *A. muciniphila*-receiving groups. Similar to findings in the aforementioned DSS study, though ZO-1 had a slight upregulation in the *A. muciniphila*-receiving group compared to controls, no statistical significance was reached (Figure 4.7B).

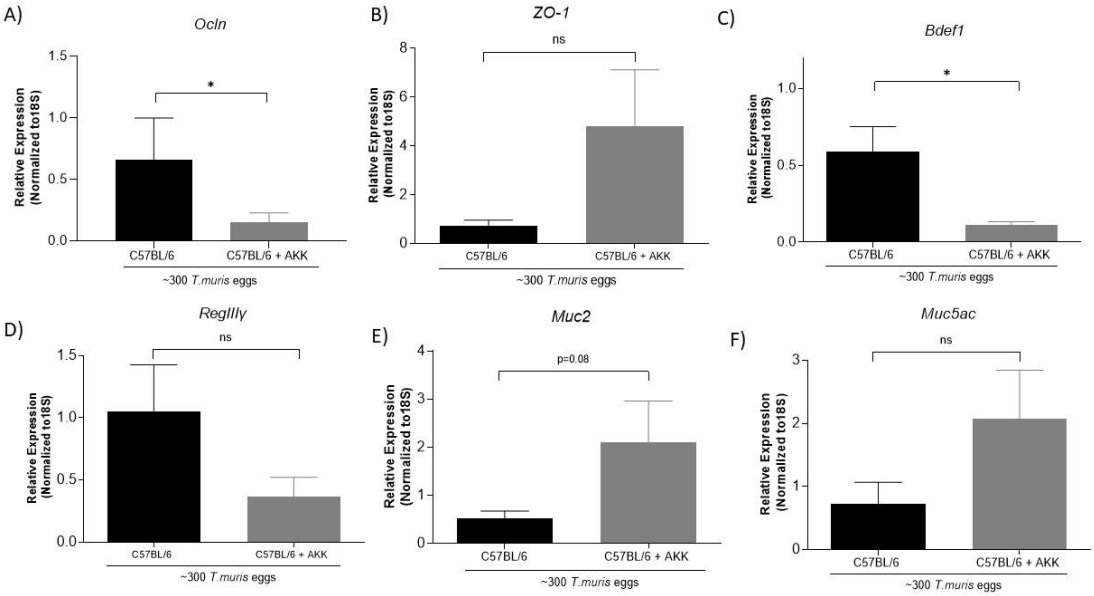

Figure 4.7. *Markers of barrier function in T. muris-infected GF mice gavaged with A. muciniphila.*

GF C57BL/6 mice received wild-type cecal content for three days, followed by a 7-day colonization period. Starting three days before administration of 2.5% DSS, mice were inoculated with *A. muciniphila* daily. Genes involved in barrier function including the tight junction proteins, (A) occludin and (B) zonula occludens-1, the antimicrobial peptides, (C) mouse β-defensin 1 and (D) *RegIIIγ,* and the mucins, (E) *Muc2* and (F) *Muc5ac*, were

assessed by qPCR. Each bar represents the mean ± SEM (*n* = 3-4 mice per group). *$P < 0.05$.

To measure the effects of *A. muciniphila* supplementation on microbial composition and to verify stable colonization of *A. muciniphila* in the gut, 16S rRNA sequencing of fecal samples was performed. Three weeks post-gavage increased levels of *A. muciniphila* (Figure 4.8) were detected in those mice receiving *A. muciniphila*-supplemented C57BL/6 cecal contents versus those receiving unaltered contents ($P = 0.06$). This increase was sustained 14 days post-*T. muris* infection ($P = 0.02$).

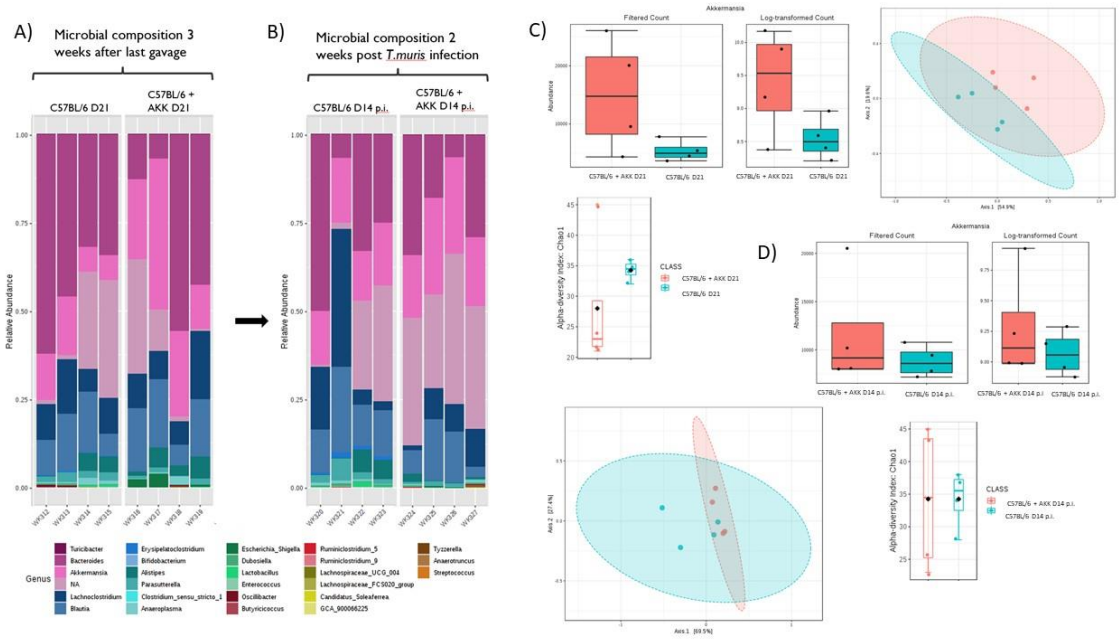

Figure 4.8. *16S rRNA analysis of A. muciniphila gavage in T. muris-infected GF mice.*

GF C57BL/6 mice were given wild-type cecal contents or wild-type cecal contents supplemented with *A. muciniphila* for three days, followed by a 21-day period of colonization. ~300 *T. muris* eggs were then administered. Mice were sacrificed 14 days

post-infection. Relative abundance of mice gavaged with wild-type (C57BL/6) cecal contents or wild-type cecal contents supplemented with *A. muciniphila* (C57BL/6 + AKK) at day 21 post-colonization with microbial contents (A) and day 14 post-infection with *T. muris* (B), represented at the genus level. Classical univariate analysis of *Akkermansia*, beta- and alpha-diversity at day 21 post-colonization with microbial contents (C) and day 14 post-infection with *T. muris* (D).

4.3 Discussion

In this chapter, the impact of *A. muciniphila*-modulated microbiota on intestinal inflammation was investigated in both chemically-induced and enteric parasitic models of colitis in GF mice. In acute DSS experiments, administration of additional *A. muciniphila* concurrently with C57BL/6 microbial content provided only minor alterations in inflammation severity as reflected by DAI (day 5) and macroscopic scoring. To investigate whether the timing of gavage impacted this result, in a separate experiment, *A. muciniphila* administration was separated from the initial C57BL/6 cecal content gavage. This subsequent administration significantly ameliorated the markers of DAI and macroscopic score and promoted a slight downward trend in the proinflammatory cytokines IL-6 and TNFα. Intriguingly, levels of anti-inflammatory IL-10 were significantly increased and the TNFα/IL-10 ratio were significantly diminished in the AKK receiving group. GF mice infected with *T. muris* also reflected this elevated level of IL-10 in AKK treated mice. As well, these mice displayed decreased worm burden along with altered levels of IL-4 on day 14 post-infection.

Contrary to what was expected, there were no statistically significant differences in the expression levels of the major structural mucin of the gut, *Muc2*, between *A. muciniphila*-gavaged mice and control mice in both the delayed DSS and *T. muris*

experiments. Despite this result, in both experiments, PAS+ staining revealed that the *A. muciniphila*-treated group displayed goblet cells with a more "filled" appearance as well as slightly increased numbers of these mucin-producing cells compared to those mice which received unsupplemented microbial contents. Though beyond the scope of this thesis, these findings may suggest that alterative mucins may be influenced by *A. muciniphila* in the context of colitis.

It should be noted that although, as mentioned, the relative "blank slate" of GF mice provides a unique opportunity to manipulate the administered microbial contents, alterations in GF biology may affect *A. muciniphila's* ability to survive and/or confer benefits in this model and is not necessarily reflective of normal pathology. Indeed, in comparison to conventional mice, GF mice have underdeveloped gut-associated lymphoid tissue (GALT) (77,177), decreased IEC turnover (13), and altered mucus properties, including enhanced permeability, a thinner inner mucus layer and diminished colonic Muc2 (178). Notably, GF mice also display altered immune function associated with lack of microbial exposure and a relatively "uneducated" immunological status (13). Thus, *A. muciniphila,* with its heavy reliance on mucin for both its own metabolic functions as well as its interaction and participation in microbial cross-feeding, suggests its ability to elicit particular immunological cascades may be affected under these conditions. To determine if this altered biology influenced *A. muciniphila*'s ability to survive and/or elicit effects and to determine if more robust immunological manipulation in the context of intestinal inflammation was present without these hindrances, similar and expanded experiments were conducted in SPF mice; these findings are described in Chapters 5-7.

CHAPTER 5: INVESTIGATING THE EFFECTS OF AKKERMANSIA MUCINIPHILA SUPPLEMENTATION IN ACUTE PREVENTATIVE AND RECOVERY MODELS OF CHEMICALLY INDUCED COLITIS

5.1 Introduction

Evidence from a wide array of studies has supported the idea that *A. muciniphila* plays a role as a keystone species of the gut (121) not only by directly supporting the health of the microbial community (120) but also by interacting either directly or indirectly with the host and altering gut physiology. This microbe's distinctive ability to break down intestinal mucins provides, via its metabolic products including the SCFAs propionate and acetate as well as oligosaccharides, substrates for other resident microbes (106). Providing the substrates for microbial cross-feeding promotes the growth and metabolic functions of other resident microbes, and thus, *A. muciniphila* can indirectly hamper the ability of pathogenic bacteria to colonize the mucus layer of the gut via competitive exclusion (106). These SCFAs have also been shown to interact via Gpr43 and Gpr41 receptors, potentially prompting changes in host expression machinery, metabolic signalling and influencing host immune activation (119).

A. muciniphila's ability to alter host physiology has been particularly well studied in recent years in the context of metabolic syndrome and obesity. Evidence suggests that this microbe is particularly adept at fortifying and maintaining gut barrier integrity (91,112), effects which include but are not limited to: increasing mucus thickness (91,103), increasing the number of goblet cells (103), stimulating mucus production (120), decreasing intestinal permeability (130–132), restoring appropriate tight junction expression (133,134), boosting levels of anti-inflammatory T_{reg} cells and subsequent production of IL-10 (91,107,127), and stimulating the production of AMPs such as RegIIIγ in the murine colon (91).

Interestingly, several of these findings have been recapitulated, or even enhanced, with the use of pasteurized bacteria or isolated bacterial components (91,112,127–129,133,134). Although the exact mechanism by which *A. muciniphila* affects the host immune response is still being examined, these findings suggest its metabolic and/or mucolytic effects may not drive these outcomes.

As discussed in Chapter 4, though GF mice provide a unique opportunity to substantially manipulate the incoming microbial contents, these mice often do not reflect normal host physiology. In the work presented in Chapter 4, over the course of DSS administration, only a minor decline in disease severity was observed in *A. muciniphila* treated mice compared to controls. Here, to offset the altered physiology of GF mice, we investigate the impact of *A. muciniphila* supplementation in both a preventative and a recovery setup of the DSS colitis model in SPF C57BL/6 mice. We also investigate whether the potential amelioratory effects of *A. muciniphila* on colitis were to its metabolic properties or simply to the presence of the bacteria itself by utilizing live (Live AKK) or pasteurized (Past. AKK) bacterial samples.

5.2 Results

Administration of either live or pasteurized *A. muciniphila* does not have preventative effects in acute DSS colitis

To determine if *A. muciniphila* had more potent effects outside of a GF model, SPF C57BL/6 mice were administered either live or pasteurized bacteria (70°C for 30 minutes) (129,143), or the equivalent volume of brain-heart infusion (BHI) media by oral gavage for 15 days. Live and pasteurized bacteria were utilized to investigate whether the potential effects of *A. muciniphila* on colitis were due to its metabolic properties or

simply to the presence of the bacteria itself. Colitis was induced in all groups by administering 3.5% DSS in autoclaved drinking water for five days. Neither live nor pasteurized *A. muciniphila* administration proved beneficial in this context (Figure 5.1). Markers including DAI (Figure 5.1C), macroscopic scoring (Figure 5.1B), and levels of the pro-inflammatory cytokines (Figure 5.1E), IL-1β, IL-6, and TNFα, proved similar between groups. Levels of IL-10 were also unaltered between groups (Figure 5.1D). In addition, histological evidence indicated that severe and consistent levels of colitis were established across all three groups (Figure 5.1F). Fecal 16S rRNA analysis indicates only minor microbial changes existed between groups gavaged with either broth, live AKK or pasteurized AKK on Day 5 of DSS administration (Figure 5.2).

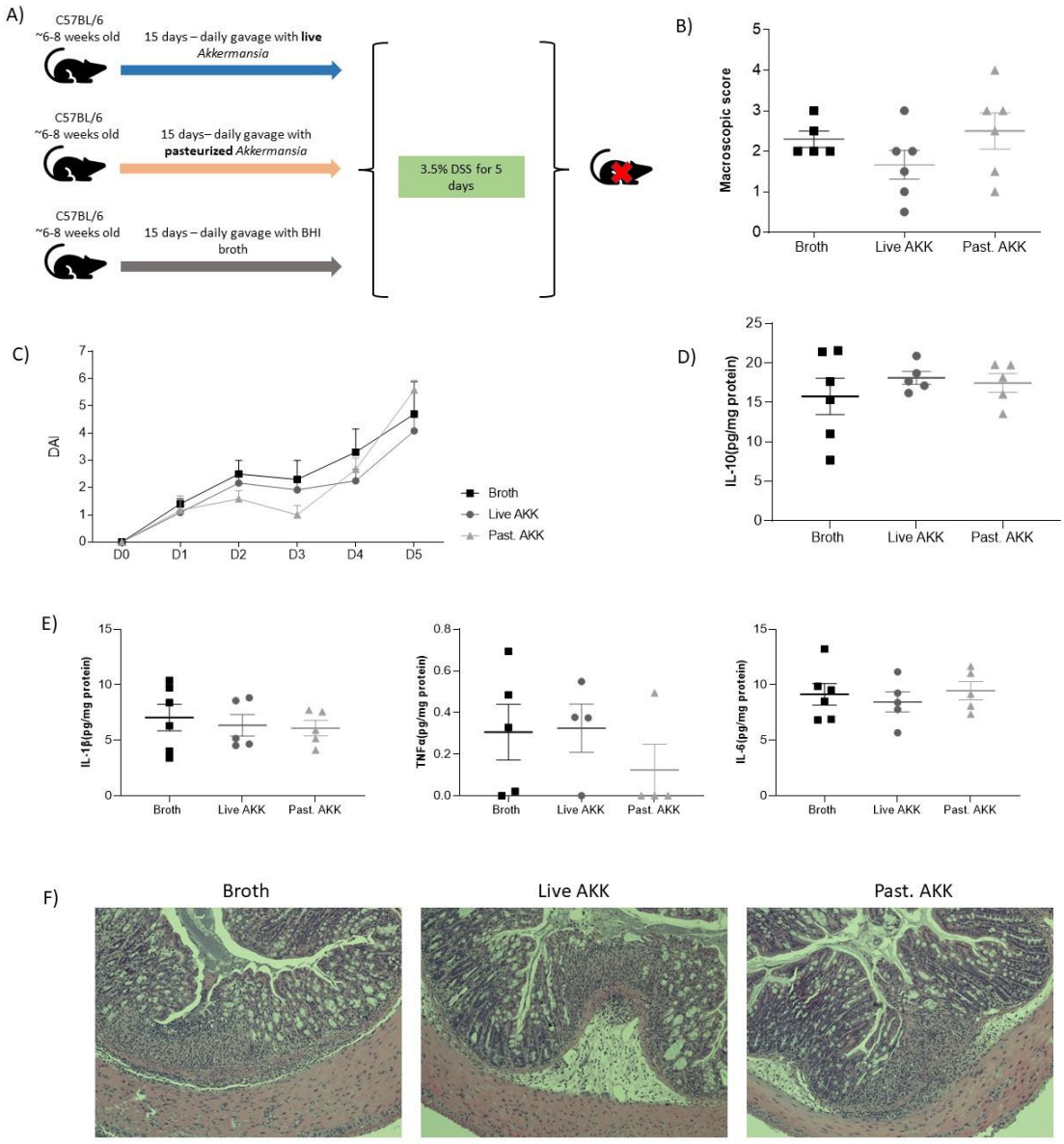

Figure 5.1. *Effect of live and pasteurized A. muciniphila on markers of inflammation in SPF mice with DSS-induced colitis.*

SPF C57BL/6 mice received either live or pasteurized *A. muciniphila*, or control broth daily for 15 days by oral gavage. Following this, mice were administered 3.5% DSS for five days. (A) shows the overall experimental layout. Disease progress and severity of inflammation were assessed by (B) macroscopic scoring, (C) DAI, (D) the anti-inflammatory cytokine, IL-10, and the pro-inflammatory cytokines (E), IL-1β, IL-6 and

TNFα. (F) shows representative H&E-stained colon tissue micrographs in broth, live and pasteurized *A. muciniphila*-treated groups. Each value represents the mean ± SEM (*n* = 5-6 mice per group). **P <0.05.*

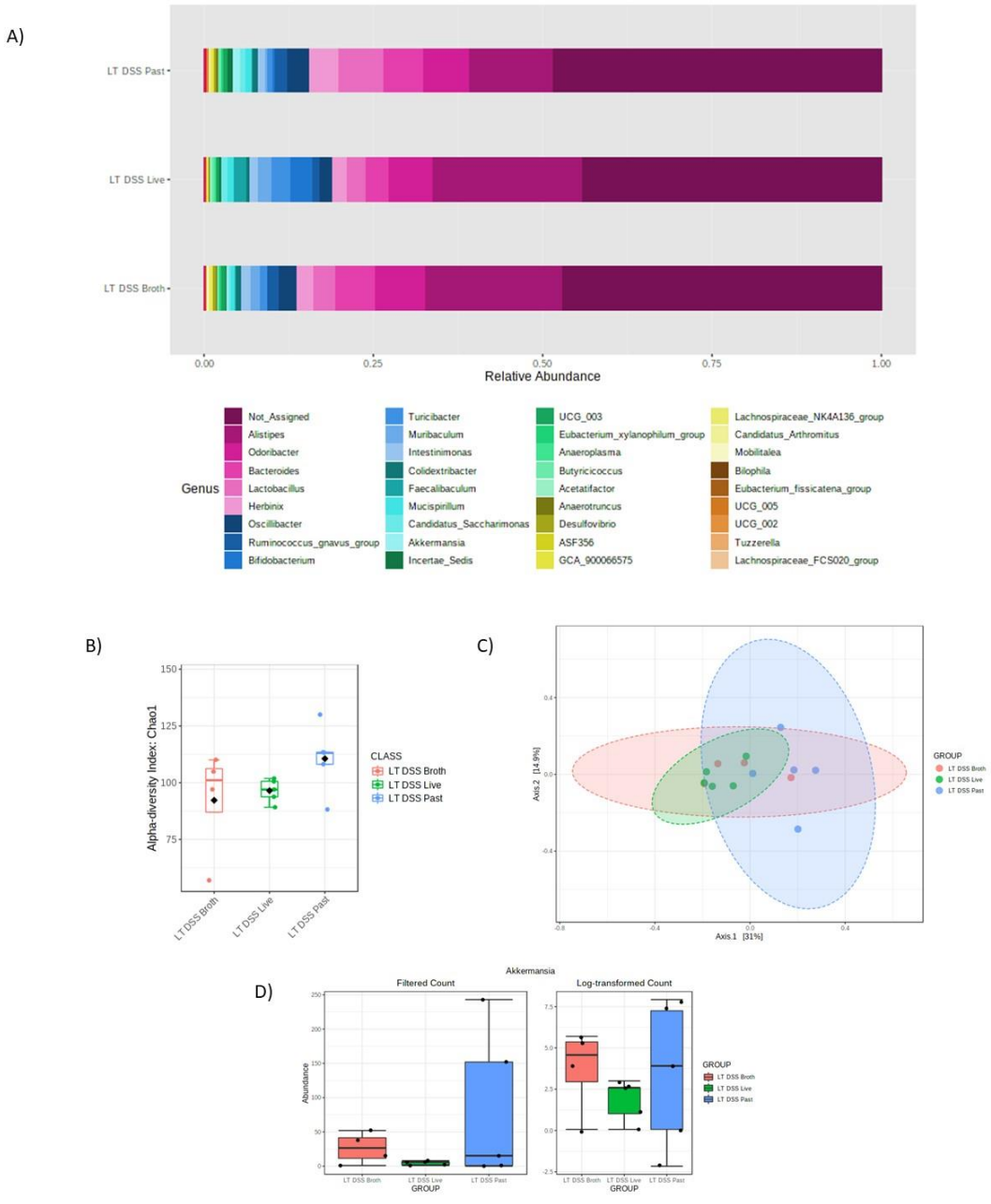

Figure 5.2. *Effect of live and pasteurized A. muciniphila on microbial composition in SPF mice with DSS-induced colitis.*

SPF C57BL/6 mice received either live or pasteurized *A. muciniphila*, or control broth daily for 15 days by oral gavage. Following this, mice were administered 3.5% DSS for five days. (A) Relative abundance, represented at the genus level, in mice gavaged with either BHI broth, live or pasteurized *A. muciniphila* on day 5 of DSS administration. (B) alpha diversity, (C) beta diversity and (D) classical univariate analysis of *Akkermansia* are also shown.

Treatment with *A. muciniphila* after establishment of colitis reduces severity of inflammation and enhances recovery in DSS-induced colitis

To elucidate the role of *A. muciniphila* in recovery from DSS-induced colitis, SPF C57BL/6 mice were given low dose (1.5%) DSS for five days. Starting on day 4 of DSS administration, mice received either BHI broth or live or pasteurized *A. muciniphila* by oral gavage (Figure 5.3A). Day 4 in an acute model of DSS-induced colitis is often a key inflection point in disease severity and, thus, was chosen as the treatment start date. This treatment continued until the end of the experiment. On day 5, DSS was removed, and mice were given access to sterile drinking water *ad libitum* for three days which constituted the "recovery" phase. Throughout the administration of DSS and in this recovery period, mice were carefully monitored; DAI over the course of the experiment indicated similar levels of colitis were established until day 4 at which time a separation in the severity of colitis was seen between groups (Figure 5.3C). In particular, stool consistency and evidence of fecal blood were greatly improved in mice receiving both live and pasteurized *A. muciniphila* after the first gavage. It should be noted that before the gavage/treatment period began, mice in the *A. muciniphila* groups, both live and pasteurized, showed increased weight loss during DSS administration. To account for this observation, an additional analysis of DAI without body weight measures was conducted

(Figure 5.3D & 5.3F). Here, distinct differences were evident, with significance being reached on all five days of gavage treatment for the pasteurized *A. muciniphila* group in comparison to the broth group. The group administered live bacteria also fared better than the control, reaching significant differences in colitis severity on days 4 and 5 and continuing this decreased severity for the remainder of the experiment though significance was not reached (Figure 5.3D & 5.3F). Analyses that took into account body weight also showed slight reductions in colitis severity in mice receiving pasteurized samples compared with broth (Figure 5.3C & 5.3E). It should be noted that colitis severity in all groups dropped once gavage began, possibly due to decreased thirst/increased hydration that may have occurred with the gavaging of additional fluids. Further analysis comparing within-group DAI scores of pre-gavage colitis (day 3) severity to the severity of colitis during gavage/off DSS (day 7 and 8) were also performed (Figure 5.3E, 5.3F).

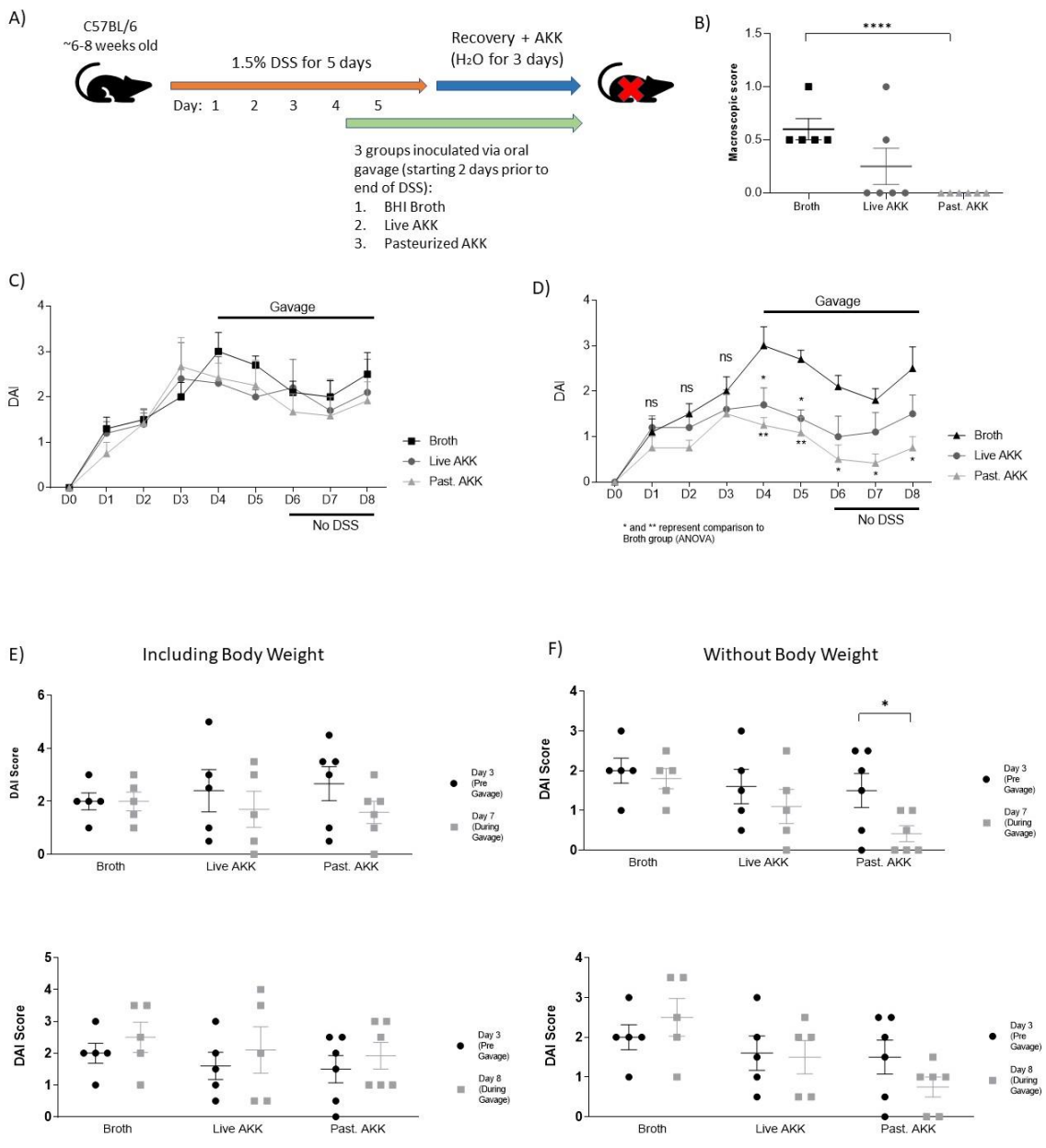

Figure 5.3. *Impact of the administration of live and pasteurized A. muciniphila in recovery from DSS-induced colitis.*

SPF C57BL/6 mice were administered low dose (1.5%) DSS for five days. Starting on day 4 of DSS administration and continuing for the remainder of the experiment, mice received daily gavage of either live or pasteurized *A. muciniphila*, or control broth. Mice were allowed a recovery period for three days at the cessation of DSS. (A) shows the

overall experimental layout and (B) represents macroscopic scoring. Several measures of DAI were recorded over the course of the DSS and recovery periods: (C) DAI including body weight fluctuations and (D) DAI without body weight. DAI scoring was also compared on day 3 (prior to treatment) and days 7 and 8 (during gavage treatment) with (E) and without (F) body weight measures. Each value represents the mean ± SEM (n = 5-6 mice per group). *$P < 0.05$.

Upon sacrifice, macroscopic scoring (Figure 5.3B) and the cytokines, IL-6 and TNFα (Figure 5.4B, 5.4C) reflected the decreased severity of colitis observed throughout the experiment with regard to the pasteurized *A. muciniphila* group; significant decreases in all these measures were detected compared to controls. Intriguingly, mice receiving pasteurized bacteria also displayed significantly lower levels of IL-6 compared to those receiving live samples (Figure 5.4C). These findings suggest that these anti-inflammatory effects may simply be due to the presence of the bacteria/bacterial components and not necessarily occur as a function of *A. muciniphila's* metabolic and/or mucus turnover contributions to the colonic microenvironment. No significant differences in IL-1β or PAS+ stained goblet cell number were detected between groups (Figure 5.4A & 5.4F). Interestingly, though no statistical significance was achieved when comparing levels of anti-inflammatory IL-10 (Figure 5.4D) between groups, the pro- to anti-inflammatory ratio as represented by TNFα/IL-10 was markedly decreased in the pasteurized AKK-receiving groups compared with those receiving broth alone (Figure 5.4E).

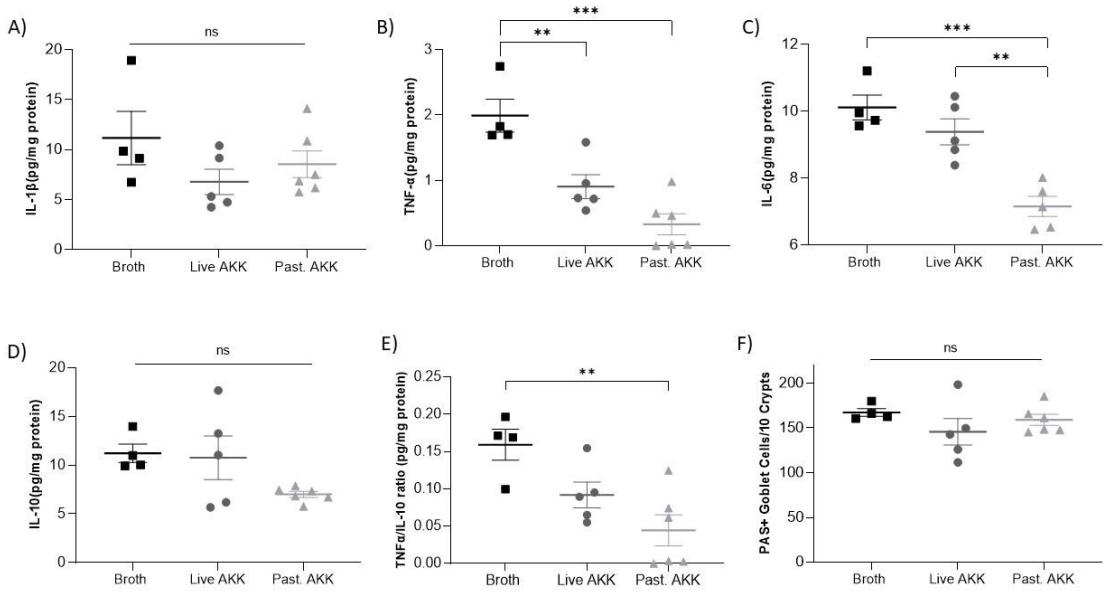

Figure 5.4. *Effect of live and pasteurized A. muciniphila on markers of inflammation in a recovery model of DSS-induced colitis.*

SPF C57BL/6 mice were administered 1.5% DSS for five days. Starting on day 4 of DSS administration and continuing for the remainder of the experiment, mice received daily gavage of either live or pasteurized *A. muciniphila*, or control broth. Mice were allowed a recovery period for three days at the cessation of DSS. Disease progress and severity of inflammation were assessed by the pro-inflammatory cytokines (A) IL-1β, (B) TNFα and (C) IL-6 and (D) the anti-inflammatory cytokine IL-10. The pro- to anti- inflammatory ratio as represented by TNFα/IL-10 is shown in (E). (F) shows the number of PAS+ stained goblet cells per 10 crypts, representative pictures of which are shown in Figure 5.6. Each value represents the mean ± SEM (n = 5-6 mice per group). *$P < 0.05$.

Evaluation of several markers of barrier function, including *Muc2*, *ZO-1*, occludin, *RegIIIγ* and mouse β-defensin 1 were also assessed (Figure 5.5). Interestingly, though significance was not reached, all of these markers showed elevated expression levels in both live- and pasteurized-receiving groups. Of these markers, *RegIIIγ* (Figure 5.5D) was particularly elevated in the pasteurized *A. muciniphila* group, suggesting that manipulation of AMPs may account, at least in part, for the aforementioned decrease in

colitis severity associated with this group. Histological evidence (Figure 5.6A) also reflected the decreased severity of colitis associated with the pasteurized *A. muciniphila*-receiving group compared with the broth-receiving group.

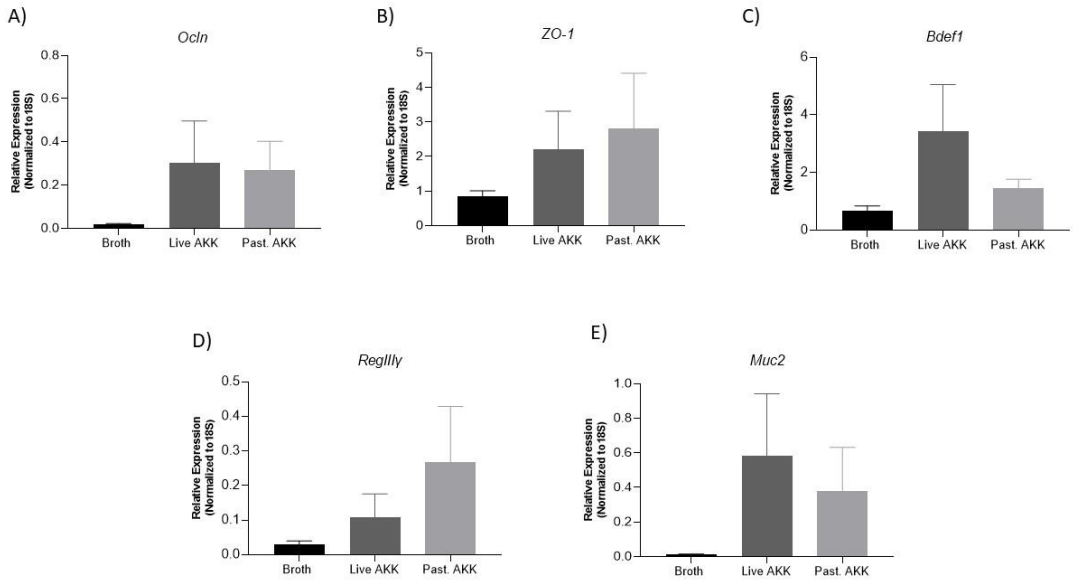

Figure 5.5. *Effect of live and pasteurized A. muciniphila on markers of barrier function in a recovery model of DSS-induced colitis.*

SPF C57BL/6 mice were administered 1.5% DSS for five days. Starting on day 4 of DSS administration and continuing for the remainder of the experiment, mice received daily gavage of either live or pasteurized *A. muciniphila*, or control broth. Mice were allowed a recovery period for three days at the cessation of DSS. Markers of barrier function, including the tight junction proteins, (A) occludin and (B) zonula occludens-1, the antimicrobial peptides, (C) mouse β-defensin 1 and (D) *RegIIIγ*, and the major structural mucin of the gut, (E) *Muc2*, were assessed by qPCR. Each value represents the mean ± SEM (n = 5-6 mice per group). *$P < 0.05$.

To visualize the effects of live and pasteurized *A. muciniphila* supplementation on microbial composition and diversity, 16S rRNA sequencing of fecal samples was performed at the cessation of the recovery period (Figure 5.7). Interestingly, the

pasteurized AKK-receiving group showed slightly diminished, though not statistically significant (P=0.069) measures of alpha diversity compared to the groups gavaged with either BHI broth or live *A. muciniphila* (Figure 5.7B). Beta diversity indicated distinct microbial composition, in particular, between the broth and pasteurized *A. muciniphila* receiving groups (P<0.004). Interestingly, classical univariate analysis (P=0.3) did not indicate different bacterial counts between groups (Figure 5.7D); however, relative abundance measures (Figure 5.7A) suggest that although the absolute number of microbes may not be enhanced in the treated groups versus controls, the overall percentage of the microbial niche occupied by *A. muciniphila* may be enhanced.

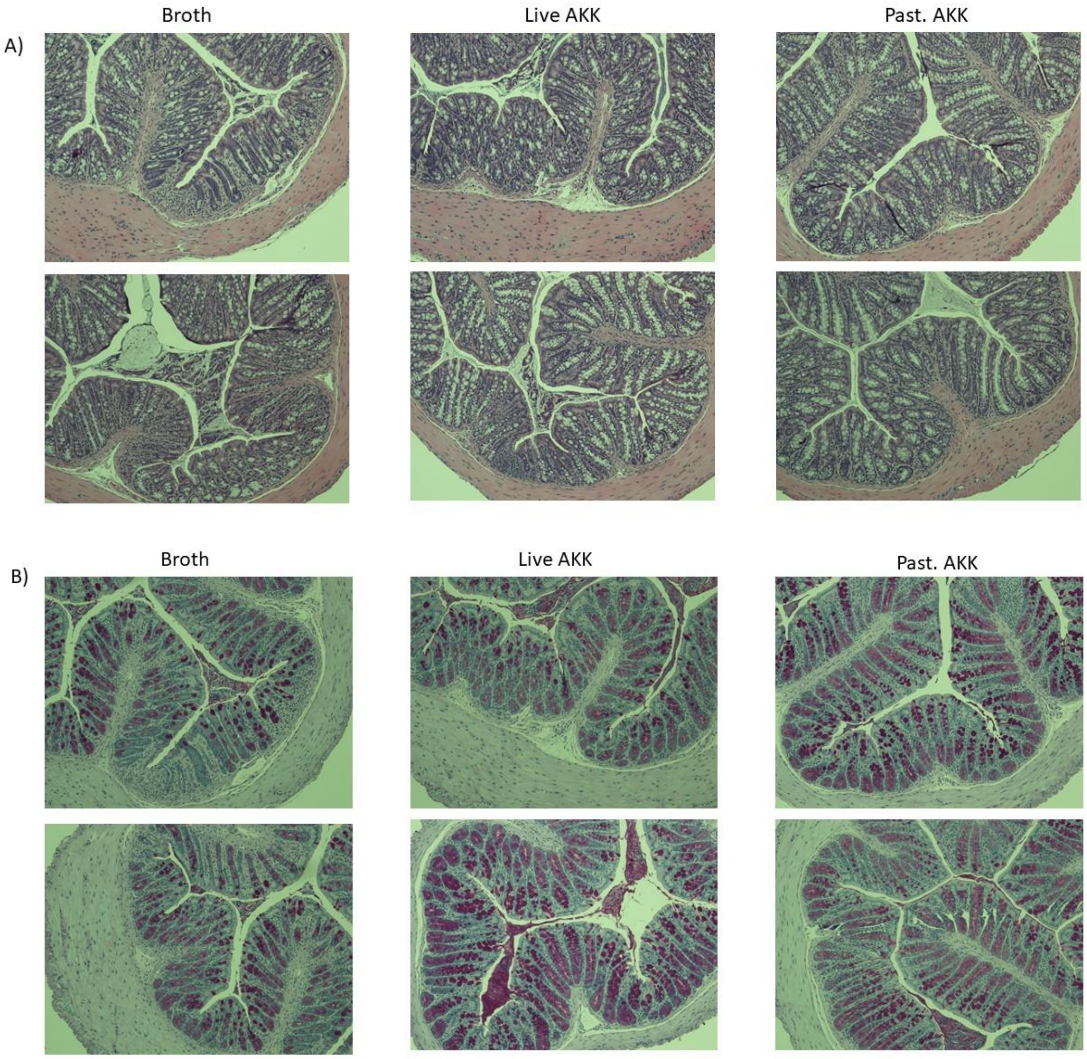

Figure 5.6. *Histological alterations in a recovery model of DSS-induced colitis in SPF mice supplemented with live or pasteurized A. muciniphila.*

SPF C57BL/6mice were administered 1.5% DSS for five days. Starting on day 4 of DSS administration and continuing for the remainder of the experiment, mice received daily gavage of either live or pasteurized *A. muciniphila*, or control broth. Mice were allowed a recovery period for three days at the cessation of DSS. (A) H&E-stained representative micrographs and (B) PAS-stained representative micrographs of colonic tissue.

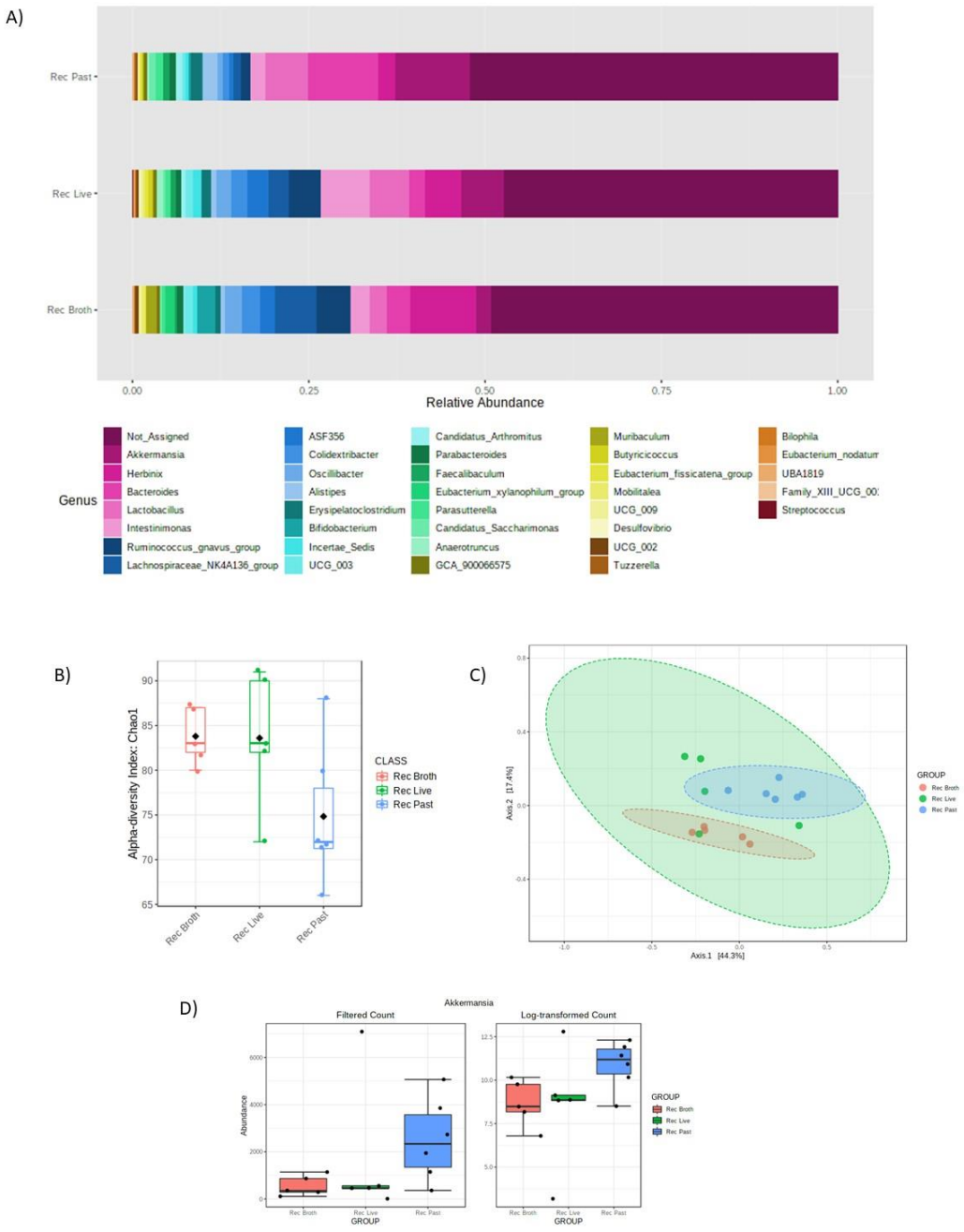

Figure 5.7. *Effect of live and pasteurized A. muciniphila on microbial composition in SPF mice in a recovery model of DSS-induced colitis.*

SPF C57BL/6 mice were administered 1.5% DSS for five days. Starting on day 4 of DSS administration and continuing for the remainder of the experiment, mice received daily gavage of either live or pasteurized *A. muciniphila*, or control broth. Mice were allowed a

recovery period for three days at the cessation of DSS. (A) Relative abundance, represented at the genus level, in mice gavaged with either BHI broth, live or pasteurized *A. muciniphila* post-recovery period. (B) alpha diversity, (C) beta diversity and (D) classical univariate analysis of *Akkermansia* are also shown.

5.3 Discussion

In this chapter, the impact of *A. muciniphila* supplementation in both a prevention and a recovery setup of the DSS colitis model was examined in SPF C57BL/6 mice. Additionally, by utilizing live and pasteurized bacterial samples, the question of whether the potential amelioratory effects of *A. muciniphila* on colitis are due to its metabolic properties or simply to the presence of the bacteria itself was also investigated.

In the prevention model, SPF mice were either administered live or pasteurized *A. muciniphila* for 15 days prior to the start of DSS administration. Unexpectedly, neither live nor pasteurized *A. muciniphila* administration proved beneficial in this setup. Despite promising histological and macroscopic data in GF mice (Figure 4.2 & 4.4), none of these effects were carried over to an SPF mouse model. In a similar study undertaken by Bian et al. (139), the authors found opposing results; *A. muciniphila* treatment over the course of 14 days significantly ameliorated the effects of DSS induced colitis improving DAI, decreasing several proinflammatory cytokines and boosting IL-10 in colonic tissue. It should be noted, however, that in the study conducted by Bian et al., unlike the one reported here, *A. muciniphila* gavage was carried out during the 5-day DSS administration period, mice were taken off DSS two days prior to sacrifice, and a lower dose of DSS (2%) was used; these are significant procedural changes which may account for the differing results.

In the recovery model, SPF mice were administered either live or pasteurized *A. muciniphila* starting day 4 of DSS administration. This treatment continued past the cessation point of DSS on day 5 and continued until sacrifice. Here, distinct separation of DAI, particularly with regard to stool consistency and fecal blood, were greatly diminished with pasteurized AKK-receiving mice showing the most robust effects. This amelioration in colitis severity was mirrored in markers of inflammation, including significantly decreased levels of IL-6, TNFα, and macroscopic score. Though no significant difference in IL-10 levels were detected, comparison of the pro- to anti-inflammatory ratio of TNFα to IL-10 revealed that in mice receiving pasteurized samples, this ratio was significantly diminished. This data suggests that it may not simply be the levels of individual cytokines but the overall inflammatory milieu that influence colitis severity, and that *A. muciniphila* has the ability to influence the overall immunological environment within the colon. It should also be noted that both live and pasteurized receiving groups displayed distinct blooms of *Lactobacillus* species compared with the broth group (Figure 5.7A). This beneficial microbe has been shown to have ameliorative effects in colitis (179–182). Thus, either directly through microbial components or indirectly through microbial cross-feeding, *A. muciniphila*'s ability to manipulate the microbiota may prove a key component in its ameliorative properties in the context of colitis. The above findings suggest that in this context, both live and pasteurized bacteria can influence microbial composition, perhaps in the case of the pasteurized samples by favourably altering the intestinal microenvironment for the niche expansion of beneficial resident microbes. This data not only provides intriguing evidence that *A. muciniphila* can

enhance recovery from established inflammation but also suggests that these effects are promoted most effectively by more stable pasteurized samples. These findings, most importantly, have clinical significance and may in the future aid the development of novel treatments for patients with established IBD and those patients experiencing an exacerbation of intestinal inflammation.

CHAPTER 6: EVALUATING THE IMPACT OF LIVE AND PASTEURIZED *AKKERMANSIA MUCINIPHILA* IN PROMOTING PROTECTIVE EFFECTS AGAINST *T. MURIS* INFECTION IN SPF C57BL/6 MICE

6.1 Introduction

Enteric pathogens, including intestinal nematodes such as *T. muris* (94,99) have, in the evolutionary arms race with their hosts, evolved the ability to manipulate host biology and resident microbes. The unique interactions of the host-parasite-microbe matrix, thus, provides an ideal model by which to study altered host defence, gut barrier physiology and microbial influence during periods of local intestinal inflammation (93,150).

As an inhabitant of the cecum and proximal colon, *T. muris* provides a local infection-induced model of colitis and does not penetrate beyond the intestinal mucosal layer (168). In *T. muris* infection, as the host mounts a defence the intestinal mucosa thickens, goblet cells hyperplasia develops, and inflammatory cells infiltrate the mucosa (26,100,101,169). Successful clearance of the infection in resistant mice is correlated with the activation of the Th2 type response (IL-4, IL-13) (102,169,171) and is highly mucin dependent (102,105). In parasitic infection, upregulation of mucins are thought to inhibit parasite motility and feeding capacity (26,100–102). Across a number of studies, we have previously demonstrated that the ability of mice to expel *T. muris* relies heavily on increased Muc2 production, particularly in the initial stages of infection and *de novo* expression of Muc5ac in the latter stages (102,104,105). We have also formerly demonstrated that treatment of live *L. rhamnosus* (JB-1) in *T. muris* infection significantly enhances worm expulsion in resistant C57BL/6 mice, *Muc2* $^{-/-}$, and susceptible AKR mice. This treatment was associated with increases in IL-10 levels, goblet cell number, and epithelial cell proliferation (105). These findings are particularly

intriguing with regard to the ability of *A. muciniphila* to stimulate mucin production, increase goblet cell number, promote mucus thickness, and enhance gut barrier integrity (5,91,103) suggesting that this microbe may be highly advantageous in the clearance of enteric infection (102,105,151,152) and may work to ameliorate local inflammation by greatly influencing intestinal barrier integrity.

Previous work in GF mice (Chapter 4) suggests that supplementation with *A. muciniphila* can, to a certain extent, alter infection severity and markers of intestinal barrier function in a *T. muris* infection-based model of colitis. Here, in SPF C57BL/6 mice, we investigate the capability of both live and pasteurized *A. muciniphila* to ameliorate infection severity and intestinal inflammation in the highly mucin-dependent *T. muris* model.

6.2 Results

Supplementation of live *A. muciniphila* promotes an environment favourable to *T. muris* expulsion in SPF C57BL/6 mice

To determine if *A. muciniphila* had more robust effects on the severity of *T. muris* infection outside of a GF model, SPF C57BL/6 mice were administered live (Live AKK) or pasteurized (Past. AKK) bacteria (129,143) by oral gavage for 15 days starting one day before infection with ~300 *T. muris* eggs (Figure 6.1A) (105). Live and pasteurized bacterial samples were utilized to determine whether the potential protective effects of *A. muciniphila* within the enteric parasitic *T. muris* model are due to its metabolic properties or simply the presence of the bacteria itself. As in the experiments outlined in Chapters 5

and 7, a portion of the bacterial samples were pasteurized at 70°C for 30 minutes prior to gavage (143). Mice were sacrificed 14 days post-*T. muris* infection.

Upon sacrifice, evaluation of infection severity and subsequent inflammatory consequences were performed. Here, mice administered live *A. muciniphila* over the course of infection had more pronounced differences in markers of infection and inflammation compared with mice which received pasteurized bacteria. Intriguingly, the group receiving live *A. muciniphila* had decreased worm burden (Figure 6.1B), increased levels of IL-4 (Figure 6.1D), and markedly increased levels of the anti-inflammatory cytokine, IL-10 (Figure 6.1G). Notably, the live *A. muciniphila* treated group, when compared to both broth and pasteurized AKK treated groups, also showed a significant upregulation in levels of *Muc5ac* (Figure 6.2F), a gene which codes for a mucin not normally expressed in the colon but key in the latter stages of *T .muris* clearance (102,104,105). Across all other measured markers, the group receiving the pasteurized microbe displayed no statistically significant differences between either the broth or live AKK treated groups.

Measures of IL-13 (Figure 6.1E) followed a similar pattern to that of IL-4, indicating an overall boost in the beneficial Th2 response in the live *A. muciniphila* supplemented group compared with controls. It should be noted that, as in experiments in Chapters 4, it may be that the overall cytokine milieu and not simply levels of individual cytokines influenced worm expulsion and the severity of *T. muris* infection. Therefore, levels of the pro-inflammatory Th1 cytokine, IFNγ, were measured. In conjunction with the above finding, the opposing Th1 proinflammatory cytokine, IFN-γ, and a pro- to anti-

inflammatory ratio of IFN-γ/IL-10 displayed a slight, though not statistically significant, decrease in the live *A. muciniphila*-treated group compared with controls (Figure 6.1F).

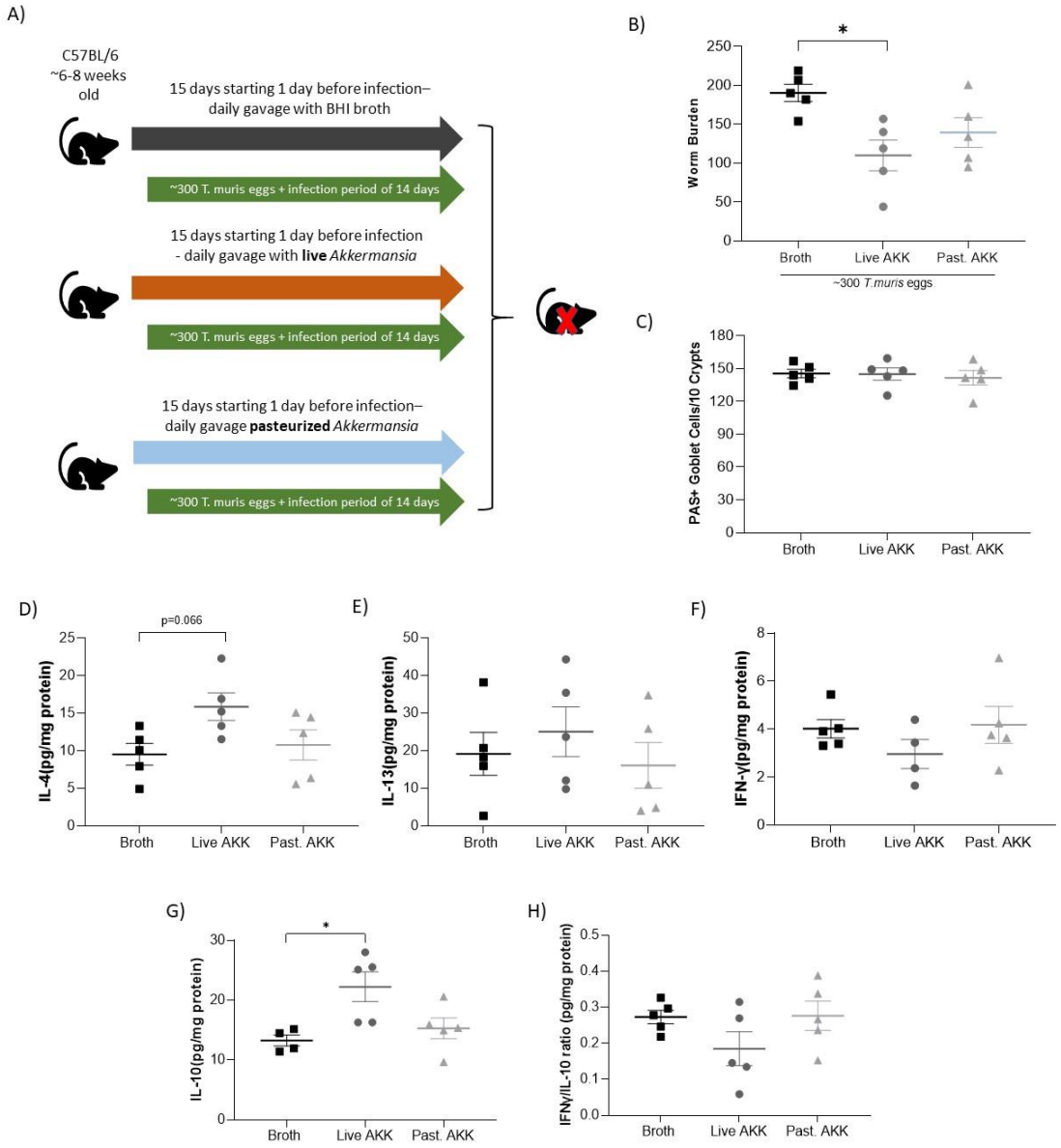

Figure 6.1. *Effect of A. muciniphila against T. muris infection-induced changes in intestinal epithelium and gut inflammation in SPF mice.*

Starting one day prior to the administration of ~300 *T. muris* eggs, SPF C57BL/6 mice received a daily gavage of either live or pasteurized *A. muciniphila*, or control broth for 15 days. Mice were sacrificed on day 14 post-*T. muris* infection. (A) represents a brief

experimental outline. Infection severity and inflammation were analyzed by (B) worm burden as well as the cytokines, (C) IL-4, (D) IL-13, and (E) IFN-γ. (F) (G) (H) Each value represents the mean ± SEM (n = 4-5 mice per group). *$P < 0.05$.

In addition to indicators of inflammation and infection severity, markers of barrier function in broth, live, and pasteurized *A. muciniphila*-treated mice were also investigated (Figure 6.2). Gene expression of occludin proved substantially lower in the live *A. muciniphila* group compared to the controls (Figure 6.2A). Expression of the AMPs, β-defensin-1 and *RegIIIγ* (Figure 6.2C & D), did not prove substantially different between the broth and live *A. muciniphila* treated groups. A significant downregulation of β-defensin-1 was observed between the live, and pasteurized *A. muciniphila* treated groups. *ZO-1* did not prove significantly different between groups.

Little difference in goblet cell hyperplasia was observed between the groups in PAS+-stained colonic tissue; live *A. muciniphila*-gavaged group displayed only minor signs of increased "fullness" (Figure 6.1C & Figure 6.3). Parallel with these findings, *Muc2* gene expression did not prove significantly different between the groups.

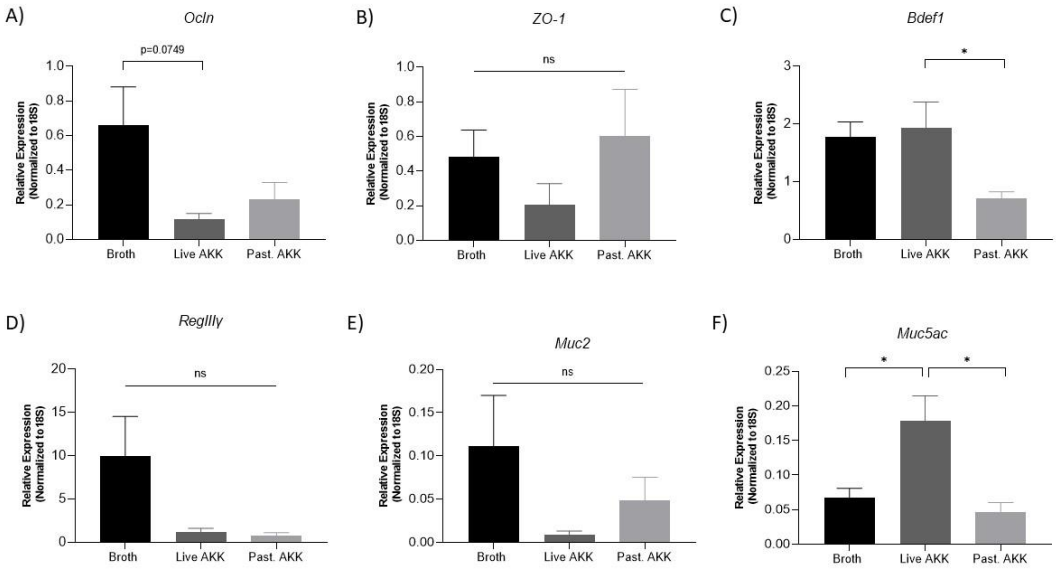

***Figure 6.2**. Effect of A. muciniphila against T. muris infection-induced changes in intestinal epithelium and gut inflammation in SPF mice.*

Starting one day prior to the administration of ~300 *T. muris* eggs, SPF C57BL/6 mice received a daily gavage of either live or pasteurized *A. muciniphila* or control broth for 15 days. Mice were sacrificed on day 14 post-*T. muris* infection. Genes involved in barrier function including the tight junction proteins, (A) occludin and (B) zonula occludens-1, the antimicrobial peptides, (C) mouse β-defensin 1 and (D) *RegIIIγ,* and the mucins, (E) *Muc2* and (F) *Muc5ac*, were assessed by qPCR. Each bar represents the mean ± SEM (*n* = 4-5 mice per group). **P <0.05.*

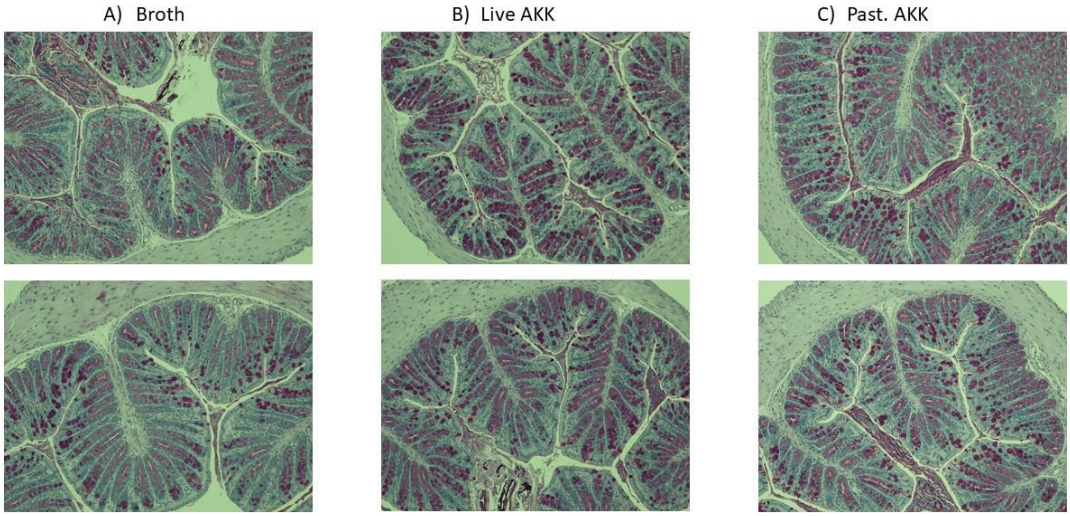

***Figure 6.3**. Effect of A. muciniphila against T. muris infection-induced changes in intestinal epithelium and gut inflammation in SPF mice.*

Starting one day prior to the administration of ~300 *T. muris* eggs, SPF C57BL/6 mice received a daily gavage of either live or pasteurized *A. muciniphila* or control broth for 15 days. Mice were sacrificed on day 14 post-*T. muris* infection. PAS-stained representative micrographs of colonic tissue from mice receiving (A) broth, (B) live *A. muciniphila* or (C) pasteurized *A. muciniphila.*

To measure the effects of live and pasteurized *A. muciniphila* supplementation on microbial composition and diversity, 16S rRNA sequencing of fecal samples was performed 14 days post-infection (Figure 6.4). No differences in alpha diversity were observed in mice gavaged with either BHI broth, live, or pasteurized *A. muciniphila* at day 14 post-infection with *T. muris* (Figure 6.4B). Beta diversity indicates different though not distinct microbial composition between groups ($P<0.001$). Both relative abundance (Figure 6.4A) and classical univariate analysis ($P=0.017$) (Figure 6.4D) indicate live *A. muciniphila* was able to establish a foothold in the microbial milieu.

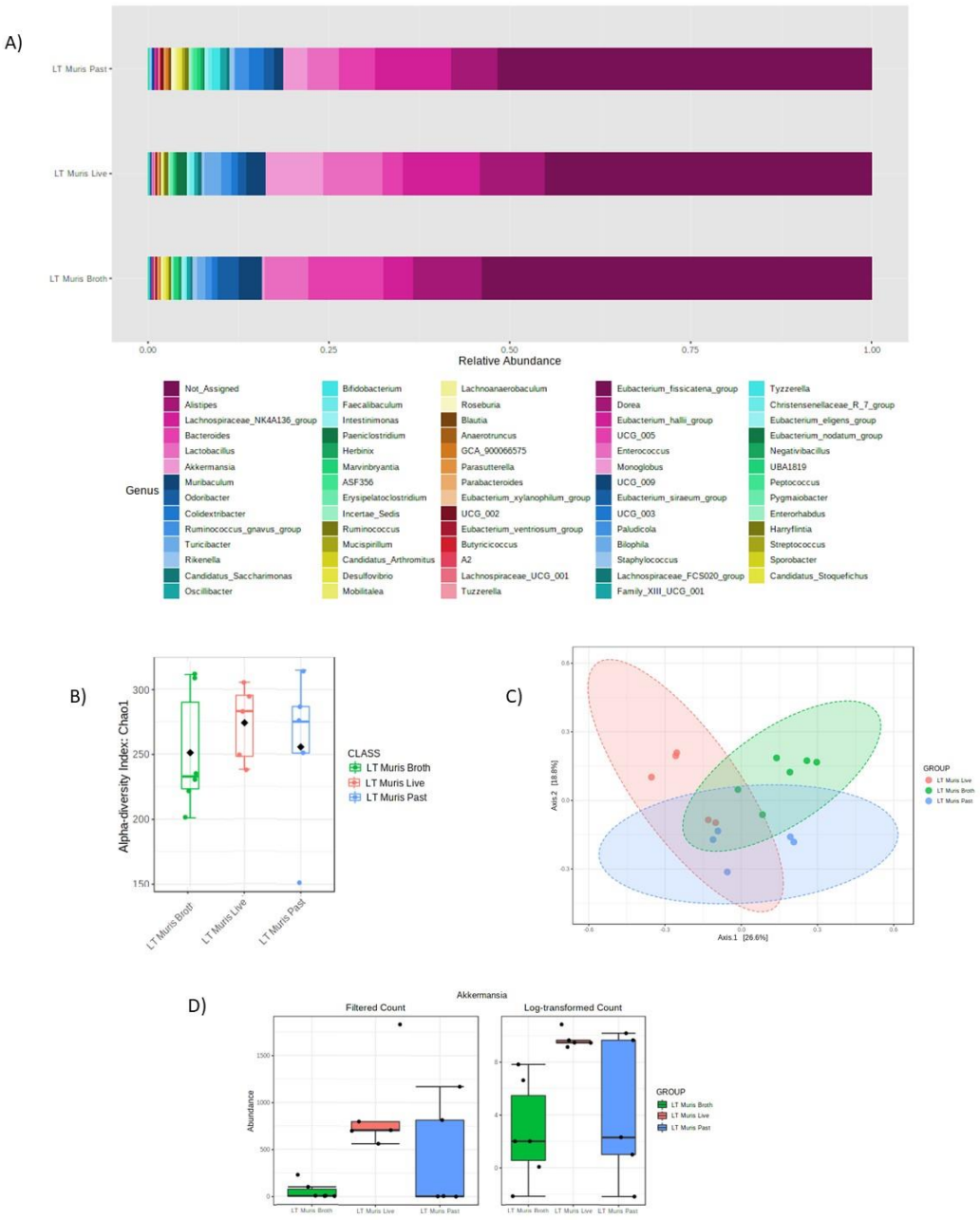

Figure 6.4. *Effect of live and pasteurized A. muciniphila on microbial composition in SPF mice infected with T. muris.*

Starting one day prior to the administration of ~300 *T. muris* eggs, SPF C57BL/6 mice received a daily gavage of either live or pasteurized *A. muciniphila* or control broth for 15 days. Mice were sacrificed on day 14 post-*T. muris* infection. (A) Relative abundance,

represented at the genus level, in mice gavaged with either BHI broth, live or pasteurized *A. muciniphila* at day 14 post-infection with *T. muris*. (B) alpha diversity, (C) beta diversity and (D) classical univariate analysis of *Akkermansia* are also shown.

6.3 Discussion

In this chapter, the capability of both live and pasteurized *A. muciniphila* to ameliorate infection severity and intestinal inflammation in the *T. muris* model was investigated. Mice were gavaged with either live or pasteurized samples of *A. muciniphila* or control broth in parallel with the establishment of *T. muris* infection. In comparison to the broth group, mice receiving live bacteria displayed significantly decreased worm burden as well as increased IL-10, IL-4, and *Muc5ac* expression, indicative of diminished infection severity.

As with work in GF mice (Chapter 4), in the context of *T. muris* infection, gene expression of occludin proved substantially lower in the live *A. muciniphila* group compared to the controls (Figure 6.2A). In both instances, as indicated by diminished worm burden in those groups treated with *A. muciniphila,* earlier worm expulsion and the stage of defensive posturing by the host may prompt a relative decrease in the demand for occludin production at this timepoint.

Contrary to our observations in GF mice (Chapter 4), PAS-stained colonic tissue in SPF mice displayed minor signs of goblet cell hyperplasia in live *A. muciniphila*-receiving groups compared to both broth receiving and pasteurized *A. muciniphila*-receiving groups. Interestingly, in both SPF and GF mice (Chapter 4), *Muc2* gene expression did not prove significantly different between the groups in either experiment,

suggesting that a *Muc2* independent mechanism may be contributing to *A. muciniphila's* beneficial effects in this model.

Taken together, the above findings indicate early expulsion and diminished infection severity of the parasite in live *A. muciniphila* treated mice compared to the broth group. These findings are also suggestive that the live bacterium has immunomodulatory properties independent of its ability to influence goblet cell number and the mucus layer.

CHAPTER 7: INVESTIGATING THE IMPACTS OF *AKKERMANSIA MUCINIPHILA* ADMINISTRATION IN THE PROGRESSION OF SPONTANEOUS COLITIS IN *MUC2*$^{-/-}$ MICE

7.1 Introduction

The mucus layer of the GI tract is an essential component for the physical and chemical protection of underlying host cells (18). Largely produced by goblet cells within the colon, mucins compose the backbone of this protective layer (26). Of these high molecular weight glycoproteins, MUC2 is the major structural component of the colonic mucus layer, providing rigidity, viscosity, and water-retaining properties to this protective layer overlying IECs (18,118).

Proper functioning and maintenance of this layer are crucial in maintaining intestinal barrier function and integrity; disruption of this layer can allow the penetration of bacteria across the mucosal barrier, a major factor in the inflammation associated with colitis (3,5). Indeed, alterations in mucin quantity, quantity, and mucus layer thickness are associated with IBD; in CD and UC, both goblet cells and the structural mucin, MUC2, are depleted (30–33). Alterations in the mucus layer are also heavily intertwined with outcomes in both the *T. muris* (102,104,105) and DSS models of colitis (149,163). Given *A. muciniphila's* known mucolytic properties as well as its influence on goblet cell number, mucus production, and mucus thickness (91,103), it is not a far-reaching hypothesis that these represent a potential mechanism underlying its protective effects *in vivo.* Interestingly, however, in our work both GF mice (Chapter 4) and SPF (Chapter 5 & 6) and across DSS and *T. muris* models, we observed a distinct lack of influence of either live or pasteurized *A. muciniphila* on *Muc2* gene expression suggesting a Muc2 independent mechanism may contribute to *A. muciniphila's* beneficial effects. Here, we set out to explore if the changes observed in the aforementioned experiments are indeed

independent of *A. muciniphila*'s known influence on mucin production and turnover by utilizing the *Muc2*$^{-/-}$ model of spontaneous colitis.

7.2 Results

Live and pasteurized *A. muciniphila* ameliorates colitis progression and severity in spontaneous colitis developed in *Muc2*$^{-/-}$ mice

Due to the mixed findings across GF and SPF experiments regarding *A. muciniphila*'s influence on *Muc2*, as well as histological alterations in "fullness" and number of goblet cells (Chapters 4-6), we set out to investigate if the changes observed in the aforementioned experiments were independent of *A. muciniphila's* known influence on mucin production and turnover. To do so, mice deficient in Muc2 were utilized. These mice have not only severely diminished goblet cell numbers along with inhibited mucin production but also develop spontaneous colitis as early as five weeks of age (153,183). To observe if *A. muciniphila* could ameliorate colitis severity independent of its influence on Muc2 production (103,112,119,132), both live and pasteurized *A. muciniphila* were administered to *Muc2*$^{-/-}$ mice via daily gavage for 15 days (Figure 7.1A) (105). Knockout (KO) mice and *Muc2*$^{+/+}$ receiving BHI broth, as well as *Muc2*$^{+/+}$ mice receiving live *A. muciniphila,* served as controls. It should be noted that at the start of the experiment, all *Muc2*$^{-/-}$ mice (~14 weeks of age) had established colitis, the progress of which was monitored over the course of the experiment (Figure 7.1B). Though no significant differences were observed in the measure of DAI between groups (Figure 7.1B), the pro-inflammatory cytokines IL-6, and TNFα, showed distinct differences. Mice gavaged with live or pasteurized *A. muciniphila* had greatly reduced levels of both IL-6 and TNFα compared to their broth-receiving KO counterparts (Figure 7.1C & 7.1 F). In fact, TNFα

levels in these mice were comparable to those found in wild-type mice. Between the KO groups, IL-1β showed no remarkable difference. Here, though not statistically significant, measures of IL-10 in both live AKK and pasteurized AKK-gavaged groups displayed a slight decline compared to those mice receiving the control broth suggesting an overall "tamping down" of the inflammatory response may be in effect in *A. muciniphila* treated groups (Figure 7.1E). Comparative analysis of the pro- to anti- inflammatory cytokines (TNFα/IL-10) reflect this possibility (Figure 7.1G) and suggest in particular that mice treated with live *A. muciniphila*, at least in this context, had the lowest comparative inflammatory potential among the KO mice. Intriguingly, this expression pattern in both IL-10 and the subsequent TNFα/IL-10 ratio was reflective of findings in the SPF recovery DSS experiments in Chapter 5.

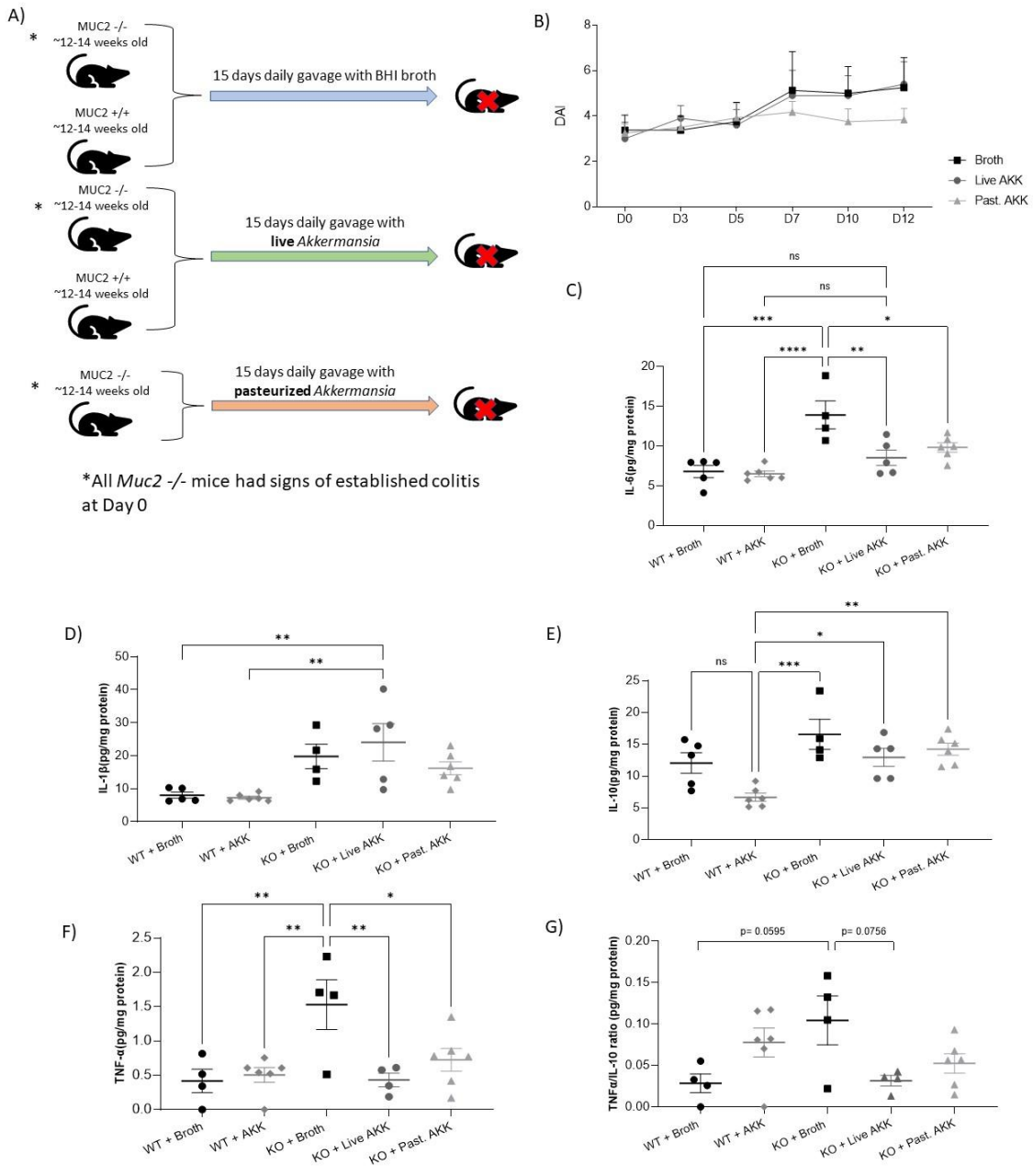

Figure 7.1. *Effect of live and pasteurized A. muciniphila on markers of inflammation in the Muc2 $^{-/-}$ model of spontaneous colitis.*

Muc2$^{-/-}$ mice develop spontaneous colitis as early as five weeks of age. (A) *Muc2*$^{+/+}$ and *Muc2*$^{-/-}$ (with established colitis) were administered a daily gavage of either live or pasteurized *A. muciniphila*, or control broth for 15 days. Over the course of the experiment, (B) DAI was recorded in *Muc2*$^{-/-}$ mice to visualize disease progression over time. Upon sacrifice, the severity of inflammation was assessed by the cytokines (C) IL-6,

(D) IL-1β, (E) IL-10 and (F) TNFα. The pro- to anti- inflammatory ratio as represented by TNFα/IL-10 is shown in (G). Each value represents the mean ± SEM (*n* = 4-6 mice per group). **P <0.05.*

Markers of barrier function, occludin, ZO-1, mouse β-defensin 1, and *RegIIIγ* were analyzed in KO constituents. Intriguingly, levels of both *RegIIIγ* and occludin (Figure 7.2A & 7.2D) were elevated in both *A. muciniphila*-receiving groups, similar to the DSS recovery experiments in Chapter 5. Though both live- and pasteurized-receiving groups had elevated levels of occludin, only the pasteurized *A. muciniphila* group showed a significant increase in this tight junction protein in comparison to the broth group (Figure 7.2A). No differences in ZO-1 were found between the groups. Live AKK treated mice showed a moderate decline in β-defensin 1, in comparison with the pasteurized AKK receiving mice.

Between the groups of KO mice, H&E-stained colonic tissue micrographs revealed only minor histological differences in crypt disruption and inflammatory cell infiltration (Figure 7.3A). Notably, however, distinct differences in both PAS+ stained goblet cell number and "fullness" were observed in the live *A. muciniphila* receiving group compared with the broth-receiving group (Figure 7.3B & 7.3C).

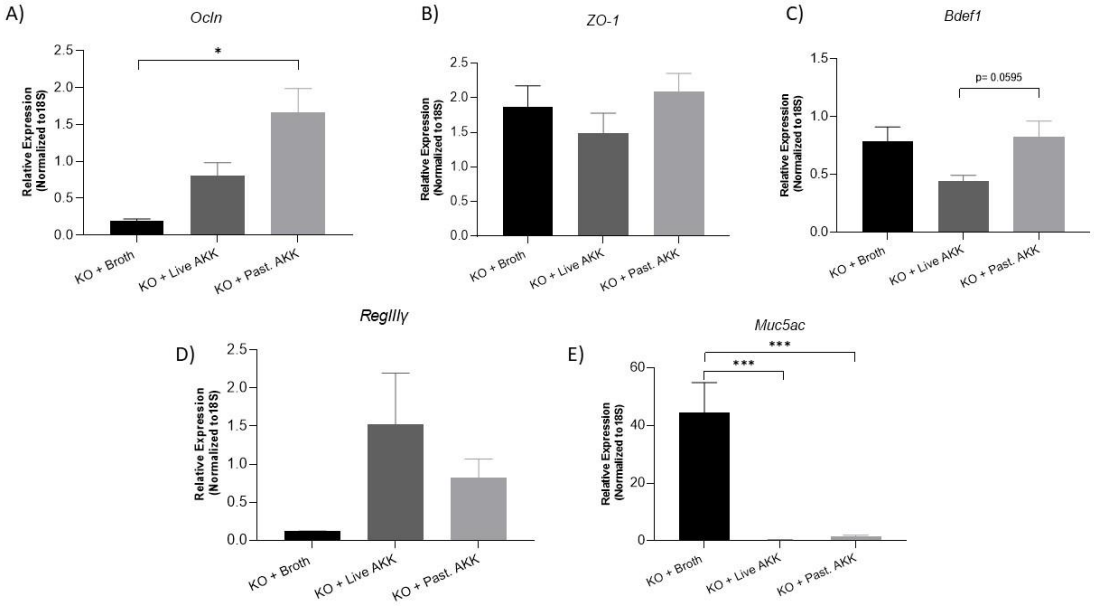

Figure 7.2. *Effect of live and pasteurized A. muciniphila on markers of intestinal barrier function in the Muc2 $^{-/-}$ model of spontaneous colitis.*

Muc2$^{-/-}$ mice develop spontaneous colitis as early as five weeks of age. *Muc2*$^{-/-}$ (with established colitis) were administered a daily gavage of either live or pasteurized *A. muciniphila,* or control broth for 15 days. Markers of barrier function, including the tight junction proteins, (A) occludin and (B) zonula occludens-1, and the antimicrobial peptides, (C) mouse β-defensin 1 and (D) *RegIIIγ,* were assessed by qPCR. De novo expression of the mucin, (E) *Muc5ac*, was also investigated. Each bar represents the mean ± SEM (*n* = 4-6 mice per group). **P <0.05.*

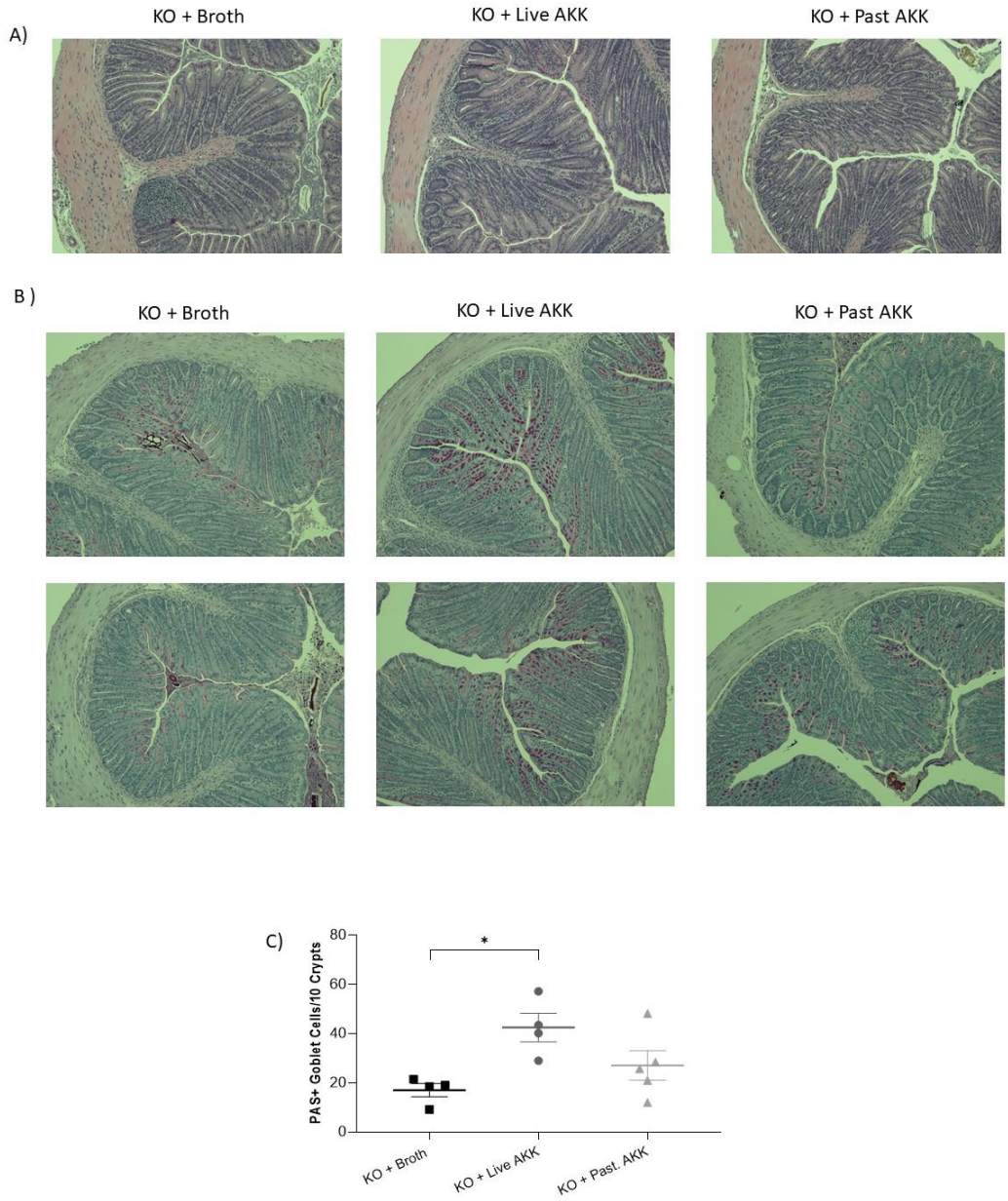

***Figure 7.3**. Histological alterations in the Muc2 $^{-/-}$ model of spontaneous colitis in mice supplemented with live or pasteurized A. muciniphila.*

Muc2$^{-/-}$ mice develop spontaneous colitis as early as five weeks of age. *Muc2*$^{-/-}$ (with established colitis) were administered a daily gavage of either live or pasteurized *A. muciniphila*, or control broth for 15 days. (A) H&E-stained representative micrographs and (B) PAS-stained representative micrographs of colonic tissue. (C) shows the number

of PAS+ stained goblet cells per 10 crypts. Each value represents the mean ± SEM (*n* = 4-6 mice per group). **P <0.05.*

To visualize the effects of live and pasteurized *A. muciniphila* supplementation on microbial composition and diversity, 16S rRNA sequencing of fecal samples was performed after 15 days of gavage treatments (Figure 7.4). Beta diversity indicates a particular dissimilarity in microbial composition in the pasteurized AKK group compared to the broth and live AKK group (P< 0.047) (Figure 7.4C). The groups receiving pasteurized samples also showed diminished alpha diversity (P=0.021364) compared with those mice gavaged with either BHI broth or live *A. muciniphila* (Figure 7.4B). Classical univariate analysis (P=0.4) did not indicate different bacterial counts between groups (Figure 7.4D); however, relative abundance measures (Figure 7.4A) suggest that in the live-treated mice the overall percentage of the microbial niche occupied by *A. muciniphila* is enhanced.

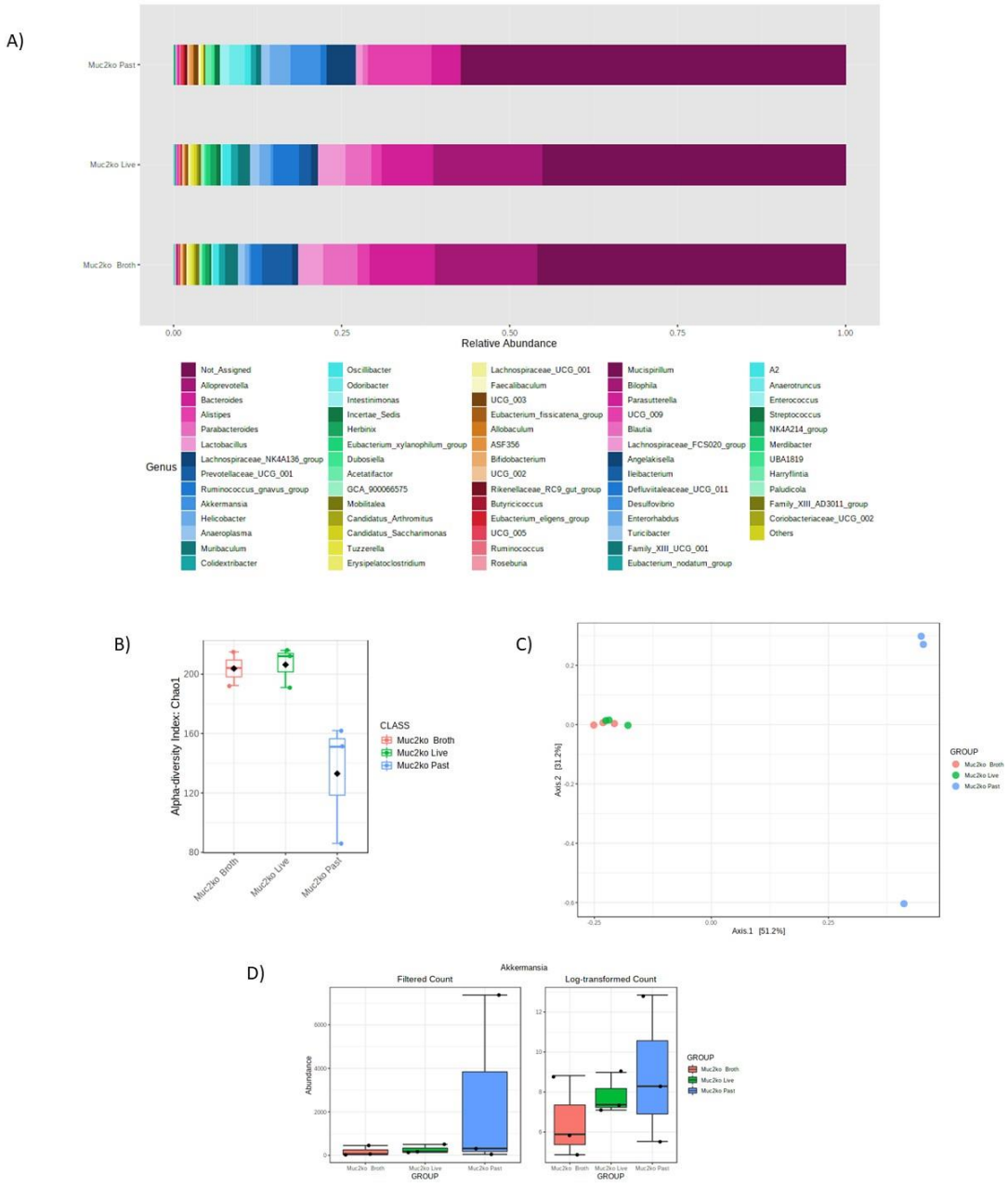

Figure 7.4. *Effect of live and pasteurized A. muciniphila on microbial composition in SPF mice in the Muc2 $^{-/-}$ model of spontaneous colitis.*

Muc2$^{-/-}$ mice develop spontaneous colitis as early as five weeks of age. *Muc2*$^{-/-}$ (with established colitis) were administered a daily gavage of either live or pasteurized *A. muciniphila*, or control broth for 15 days. (A) Relative abundance, represented at the genus level, in mice gavaged with either BHI broth, live or pasteurized *A. muciniphila*.

(B) alpha diversity, (C) beta diversity and (D) classical univariate analysis of *Akkermansia* are also shown.

7.3 Discussion

In this chapter, the ability of *A. muciniphila* to modulate the severity and progression of spontaneous colitis independent of the mucin, Muc2, was examined. Here, by utilizing *Muc2*$^{-/-}$ mice, we assessed the effects of both live and pasteurized samples of this microbe within established colitis and investigated its impact on barrier function in this context.

As the name implies, *A. muciniphila* is intimately connected with the mucus layer of the gut. This microbe not only relies heavily on mucins as its main source of carbon, nitrogen and energy but also, through its breakdown, provides substrates for neighbouring commensal microbes. Here, we established that supplementation with both live and pasteurized *A. muciniphila* ameliorated markers of intestinal inflammation, in particular significantly decreased levels of the pro-inflammatory cytokines IL-6 and TNFα, in established spontaneous colitis developed in *Muc2*$^{-/-}$ mice. Though statistical significance was not reached, it should also be noted that supplementation with live AKK also conferred a marked decrease in the TNFα/IL-10 ratio suggesting a shift to a more anti-inflammatory environment in the presence of this microbe. Live bacteria were also associated with an elevated level of *RegIIIγ*. Intriguingly, in both this study and the DSS recovery study (Chapter 5), increased levels of *RegIIIγ* were associated with those groups which had the greatest decreases in cytokines levels and inflammation severity (live and pasteurized groups, respectively).

In KO mice, though only minor differences in H&E-stained tissues were observed between groups, notable differences in both PAS+ stained goblet cell number and "fullness" were discovered in the live *A. muciniphila* receiving group compared with the broth-receiving group. Intriguingly, this finding is reflective of the observations in the GF experiments presented in Chapter 4, where, despite unchanged *Muc2* expression levels between groups, mice supplemented with live *A. muciniphila* had similar goblet cell hyperplasia. These findings suggest *A. muciniphila* may have influence over the production of other mucins in addition to its known influence on Muc2. Based on our work in the *T. muris* model, we speculated that this occurrence may reflect influence over *de novo* expression of *Muc5ac*; however, both live and pasteurized AKK treated groups displayed significantly diminished levels of *Muc5ac* in comparison to the broth group (Figure 7.2E). Though within the context of *T. muris* infection *Muc5ac* has proven a key component in infection clearance, this diminished *Muc5ac* expression may be promising in the setting of spontaneous colitis; *de novo* expression of *Muc5ac* is often associated with GI pathology, including IBD and colorectal cancer (18). These findings spur an intriguing line of inquiry into additional mucin-related influence of *A. muciniphila;* however, further investigation is beyond the scope of this thesis.

Collectively, these findings suggest that *A. muciniphila* can confer anti-colitic benefits via a Muc2 independent mechanism. In conjunction with our previous work, this study also provides further evidence that live and/or pasteurized supplementation of this microbe may prove beneficial in lessening the inflammatory load. These findings are particularly promising given the altered mucus environment and depleted levels of MUC2

in patients with IBD and suggest that, in the future, supplementation with this microbe may provide an additional tool for ameliorating intestinal inflammation.

CHAPTER 8: OVERALL DISCUSSION & CONCLUSIONS

In this thesis, we explored the role of the bacterium, *A. muciniphila*, in intestinal inflammation, its potentially protective impact within this state and whether these effects are modulated by this microbe's ability to strengthen intestinal barrier integrity (5,91,103,112,113,129–132). Research from both our lab and others have found diminished levels of *Akkermansia* under more severe colitis conditions in both animal models and patients with IBD (113,135,144). Though it has previously been demonstrated in the context of metabolic syndrome (103,129,131,132,135,136), *A. muciniphila*'s ability to ameliorate intestinal inflammation in the context of colitis is not well studied. With that being said, *A. muciniphila's* influential host interactions, including its ability to manipulate the production of AMPs, increase goblet cell number and thickness of the mucus layer, enhance barrier function and the expression of several TJPs (145,146) and increase the number of anti-inflammatory T_{reg} cells (103), suggests that it may ameliorate colitis and play a protective role in IBD.

In Chapter 4, across several experiments, we explored the effects of *A. muciniphila* -modulated microbiota in both DSS and *T. muris* models of colitis in GF mice. Though we did not see particularly robust results in GF mice, administration of live *A. muciniphila* under these conditions did result in diminished DAI and macroscopic score, and increased levels of IL-10 in the DSS model as well as a reduced TNFα to IL-10 ratio suggestive of an overall shift to a more anti-inflammatory microenvironment. In the *T. muris* model, GF mice with *A. muciniphila* modulated microbiota also displayed altered cecal worm burden, IL-4, PAS+ stained cells and notably, increased IL-10.

In Chapter 5, to offset the altered physiology of GF mice, we investigated the impact of *A. muciniphila* supplementation in both a preventative and a recovery setup of the DSS colitis model in SPF C57BL/6 mice. In the prevention experiments, neither live nor pasteurized *A. muciniphila* treated mice showed signs of improved colitis either histologically or via cytokine analysis. However, in the recovery experiments, mice administered pasteurized *A. muciniphila* showed distinct differences compared to the control group in terms of DAI, the proinflammatory cytokines, IL-6 and TNFα, as well as the TNFα/IL-10 ratio, along with histological improvements. Though not statistically significant, it should be noted that across markers of barrier function, both live- and pasteurized-receiving groups displayed increases in nearly all markers examined. Distinctively, those mice receiving pasteurized microbes had increased expression levels of *RegIIIγ* compared to controls.

In Chapter 6, we expanded upon our findings in GF mice and explored the effects of both live and pasteurized *A. muciniphila* on infection severity and intestinal inflammation in the highly mucin-dependent *T. muris* model in SPF C57BL/6 mice. Mice supplemented with live *A. muciniphila* exhibited an environment favourable to *T. muris* expulsion; decreased worm burden, increased IL-4, IL-10, upregulated *Muc5ac* and a slight decrease in the IFN-γ/IL-10 ratio compared to those mice receiving only broth.

A summary of the key findings from experiments discussed in Chapters 4-7 is found in Figure 8.1.

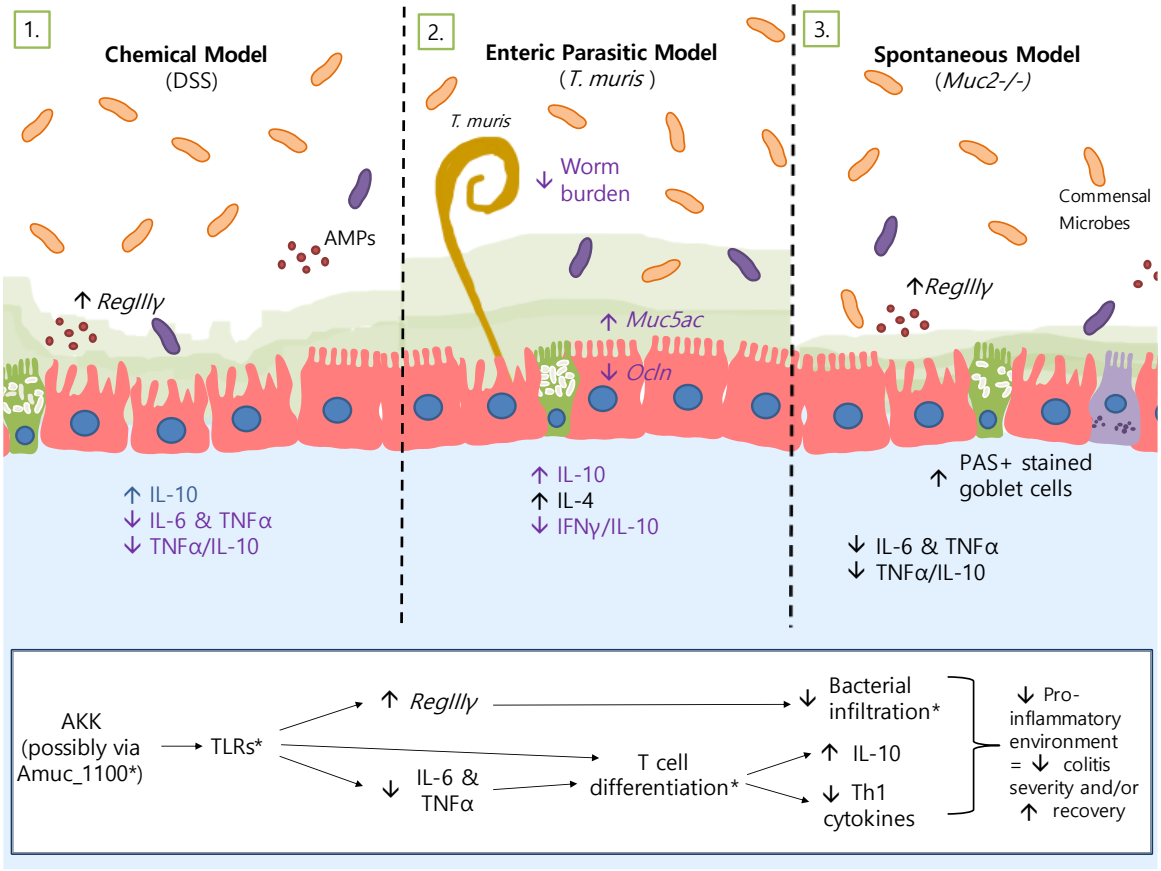

Figure 8.1. *Summation of the effects of live or pasteurized A. muciniphila across chemical, enteric parasitic, and spontaneous models of colitis.*

A summary of the key findings in Chapters 4-7. *A. muciniphila* (represented in purple) can promote beneficial immunomodulatory effects in: (1.) DSS colitis (2.) an infection-based model of colitis and (3.) a *Muc2*$^{-/-}$ spontaneous model of colitis. Black text represents findings from SPF experiments, blue text represents findings from GF experiments, and purple text represents findings from both GF and SPF studies. The boxed area below the diagram shows a potential mechanism by which *A. muciniphila* affects the host. Asterisks in the figure represent sections of the mechanism inferred from other areas of research regarding *A. muciniphila* and where further exploration of its role in the context of intestinal inflammation is warranted.

Due to the mixed findings across all forementioned experiments regarding *A. muciniphila*'s influence on *Muc2* as well as histological alterations in "fullness" and number of goblet cells (Chapters 4-6), as outlined in Chapter 7, we set out to investigate if

the changes observed were independent of *A. muciniphila's* known influence on mucin production and turnover by utilizing a *Muc2*$^{-/-}$ model of spontaneous colitis. Intriguingly, in established colitis mice gavaged with live or pasteurized *A. muciniphila* had considerably reduced levels of both IL-6 and TNFα compared to their broth-receiving KO counterparts. The TNFα/IL-10 ratio chiefly in the live AKK group was also diminished compared to the KO broth group. Rather unexpectedly, mice in the live AKK treated group also had an observable and statistically significant increase in PAS+ stained goblet cells compared to KO broth counterparts. As discussed in Chapter 7, examining *A. muciniphila's* ability to influence mucin production other than Muc2 will provide an interesting line of inquiry for future work. Similar to the DSS recovery experiments discussed in Chapter 5, though not statistically significant, the *Muc2*$^{-/-}$ mice that had the greatest reduction in inflammation also showed increased expression levels of *RegIIIγ* compared to controls.

Taken together, these experiments shed light on *A. muciniphila's* protective role in intestinal inflammation and suggest that underlying these effects is a Muc2-independent mechanism. When examining the results as a whole, several key findings and noteworthy patterns arise. For instance, supplementation of *A. muciniphila* elicited a particular immunomodulatory pattern fairly consistent across several experiments; IL-1β was largely unaffected, whereas IL-6 and TNFα were diminished. In several instances, the diminishment in these proinflammatory cytokines were found in both live and pasteurized *A. muciniphila* receiving group compared to controls. Interestingly, this pattern in cytokine expression was reflected across studies discussed in Chapters 4, 5 and 7,

suggesting *A. muciniphila* has consistently stronger influence on ameliorating levels of IL-6 and TNFα, and little influence over IL-1β. Though these proinflammatory cytokines all play a key role in innate response and colitis progression, both IL-6 and TNFα are highly involved in modulating T cell proliferation and balancing out effector functions (184,185) suggesting that in conjunction with changes in IL-10, *A. muciniphila* moderates the adaptive immunological balance needed to ameliorate inflammation in the colon. With that being said, elevated levels of the anti-inflammatory cytokine, IL-10, and/or altered TNFα/IL-10 ratios were present across experiments suggesting that *A. muciniphila* has direct modulatory effects on the host immune response. Similar results were also found in the aforementioned DSS study conducted by Bian et al. (139). The alterations in both pro- and anti-inflammatory cytokines may be reflective of *A. muciniphila* known ability to upregulate the proliferation of T_{reg} cells (103), crucial components in the suppression and regulation of inflammatory states. Drawing from both *T. muris* and DSS experiments, though further work needs to be done to elucidate these claims, *A. muciniphila's* protective effects do not seem to be limited by the inciting factors of inflammation (i.e. more Th1 driven in the DSS model whereas the *T. muris* model is Th2 dominant), again supporting the idea that this microbe's mode of action in colitogenic environments is one of modulating and balancing the inflammatory milieu of the colon.

Because all models used disrupt intestinal barrier integrity to a certain extent, and because of *A. muciniphila's* ability to seemingly reverse these features within the context of metabolic syndrome, several markers of intestinal barrier function were measured. Contrary to our hypothesis, the measures of barrier function, including the tight junction

proteins, AMPs and mucins were overall largely unaltered between groups though with some noteworthy exceptions. Notably, though significance was not reached, the "best" performing groups in both the DSS recovery and *Muc2*$^{-/-}$ had elevated levels of the AMP, *RegIIIγ*. Intriguingly, RegIIIγ, with its broad-spectrum antibiotic properties, particularly targets Gram-positive bacteria indicating that *A. muciniphila*, a Gram-negative bacterium, can induce host immunity in favour of its survival. Thus, this microbe not only perpetuates its own immunomodulatory effects and promotes competitive exclusion but also, by promoting this AMP, make it less likely for mucosal invasion of either commensal or pathogen bacteria (186). Though future work is needed, including protein expression analysis and work in *RegIIIγ*$^{-/-}$ and/or *MyD88*$^{-/-}$ (186), these findings suggest alterations in RegIIIγ may be a potential contributing candidate for *A. muciniphila's* anti-inflammatory properties in the context of DSS colitis. Unexpectedly, in both the GF and SPF *T. muris* experiments (Chapter 4 and Chapter 6), occludin was substantially decreased in live AKK treated groups compared to broth-receiving mice. It could well be that with the enhanced anti-inflammatory properties promoted in *A. muciniphila*-treated mice, the tight junction proteins, for example, do not "need" to be upregulated in order to protect the host from colitis since penetrative bacteria are less able to assault the epithelia directly with these additional immunological protective measures in place. Thus, in opposition to the initial hypothesis, the beneficial effects that *A. muciniphila* has on intestinal epithelial integrity within the context of metabolic syndrome (112,129–132) do not seem to transfer to the context of colitis. These findings suggest that though *A. muciniphila* may not be able to directly impact the "leakiness" of the gut via junctional

proteins, it may be able to manipulate host defence and adaptive immune response in a favourable way. Future research to explore and parse out the effect and influence of AMPs and IL-10 within this context is highly warranted.

Further, in both the SPF *T. muris* and *Muc2*$^{-/-}$ model of colitis experiments, mice administered live *A. muciniphila* performed "best" in comparison to both the pasteurized receiving groups and the broth receiving groups. Interestingly, both of these models have altered mucus environments. Together these studies support the idea that despite an altered mucus environment, *A. muciniphila* is still able to invoke anti-colitic effects and, most intriguingly, be able to do so in the absence of the major structural mucin, Muc2. It should be mentioned that no overarching pattern of mucus-effecting properties prompted by *A. muciniphila* was detected between experiments. Though some slight upregulation was detected in GF experiments, no other experiments showed significant differences in the number of PAS+ stained goblet cells, curiously with the exception of the *Muc2*$^{-/-}$ mice treated with live AKK, which displayed distinct changes in this regard. Mixed findings across the experiments with regards to *Muc2* and *Muc5ac* gene expression suggest that *A. muciniphila*'s ability to influence mucin expression is highly contextual; however, further work detecting other gut mucins and protein analysis is decidedly necessary and may provide more concrete and biologically relevant information in this respect.

In contrast to the results in both the SPF *T. muris* and *Muc2*$^{-/-}$ model of colitis experiments discussed above, pasteurized AKK treated mice showed the greatest improvement in this context of recovery. *A. muciniphila* and, indeed, several other next-generation probiotics are oxygen sensitive (110,112); therefore, finding ways to preserve

this bacteria's beneficial immunomodulatory properties while maintaining stability "off the bench" is crucial (79). Pasteurization here presents a solution by adding much sought after stability, greater ease of administration, and potentially exposing the host to a wider array of intracellular components that may not be accessible in high concentrations with live bacteria including, in the case of *A. muciniphila*, the outer membrane protein, Amuc_1100 (128,154–157). Pasteurization may also curtail adverse effects associated with a particular microbe while still delivering the benefit (157). A plethora of studies have demonstrated in the context of metabolic syndrome and obesity, that pasteurized *A. muciniphila* has elicited similar or, in some cases, greater effects than live bacteria (112,128–132,154–157). This finding is not exclusive to this microbe. Heat-killed *Lactobacillus casei* has also been shown to improve inflammatory markers and prevent increased intestinal permeability associated with DSS colitis by increasing ZO-1 expression and altering the balance between pro- and anti-inflammatory cytokines in the gut (187). Similar findings have also been reported in *L. rhamnosus GG* in mice lacking MyD88 (157). Thus, the work presented here in the DSS recovery model suggests that pasteurized *A. muciniphila* may be a beneficial supplemental therapy for patients in IBD flares, helping to diminish the severity of established inflammation. Further work in a chronic DSS model and/or the CD4+ T cell transfer model of colitis may elucidate these findings.

It should be noted that for microbial analysis with regards to *A. muciniphila*, fecal pellets, which largely reflect luminal contents, are an imperfect measure of colonization and abundance. Because this microbe is largely mucosally associated (119,120),

extracting samples directly from the colon or using fluorescence in situ hybridization (FISH) in conjunction with fecal analysis may prove more efficacious. Due to experimental constraints, multiple timepoints for 16S rRNA sequencing were not used. However, obtaining and analysing samples from, for example, pre- and post-DSS conditions, would greatly enrich the current data.

Lastly, comprehensive analysis of the evidence procured in work throughout this thesis suggests that although supplementation with live or pasteurized *A. muciniphila* cannot prevent the onset of colitis, this microbe has the potential to ameliorate established or concurrently derived intestinal inflammation as seen in the DSS recovery set up (Chapter 5), the SPF *T. muris* experiment (Chapter 6) and the *Muc2*$^{-/-}$ experiments (Chapter 7). These findings, most importantly, have clinical significance and may, in the future, aid the development of novel treatments for patients with established IBD and those patients experiencing flares of intestinal inflammation.

Rising incidence worldwide, particularly in westernized countries, has drawn attention to the role that microbial and environmental factors play in IBD. According to the Crohn's and Colitis Foundation of Canada, the prevalence of IBD in Canada is one of the highest in the world, and over 270,000 Canadians deal with the symptoms and ramifications of Crohn's disease and ulcerative colitis on a daily basis (188). These diseases not only profoundly impact the quality of life of patients but also impose a significant financial burden, particularly regarding medications. Further, biologics, such as TNF inhibitors or anti-TNFs, often considered the "gold standard" of treatment for severe IBD, are accompanied by adverse effects and risks (189). Taken together,

however, the research outlined in this thesis suggests that *A. muciniphila* acts as a protective microbe in the context of inflammation. Evidence across the studies presented propose that *A. muciniphila* works in a Muc2 independent manner to confer anti-inflammatory effects. This research also indicates that potential candidates the underlying mechanisms of action may include altered AMPs such as RegIIIγ and adaptive immune modulation via IL-10 rather than direct physical strengthening of the intestinal epithelial barrier. In addition, these findings suggest that *A. muciniphila* supplementation may be most effective in lending an anti-inflammatory boost in established colitis rather than acting as a preventative strategy. That being said, though the mechanism of action remains unclear, this research has helped elucidate several promising pathways by which *A. muciniphila* may confer beneficial effects to the host in the context of colitis. Moreover, this research suggests a promising future for *Akkermansia muciniphila* as a next-generation probiotic that may help ameliorate the severity of inflammation and symptomatology in inflammatory bowel disease.

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