

UNIVERSITY OF CALGARY

Distinct Roles of the Mucus Layer and Microbiota in Conferring Innate Host Defense and
Susceptibility to Disease

by

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A THESIS

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Abstract

The intestinal epithelium is covered with a thick viscous MUC2 mucin bilayer that acts as a protective barrier and is the habitat for 10^{14} microorganisms that form the microbiota. Even though disruption of colonic mucus and alterations in microbial composition are intimately linked to gastrointestinal health and disease, their distinct contribution in the pathogenesis of colonic injury is not well understood. In this thesis, I interrogated the impact of alterations in the microbial composition in animals with and without Muc2 mucus in colonic epithelial barrier dysfunction and inflammation, using chemical and pathogen-induced colitis models. For all studies *Muc2*^{+/+} and *Muc2*^{+/+} littermates were used to normalize the microbiota. To induce dysbiosis (alterations in microbial composition) and reduce bacterial numbers, mice were treated with a broad-spectrum cocktail of antibiotics. Basally, *Muc2*^{-/-} littermates showed low-grade ongoing inflammation, gastrointestinal permeability and alterations in tight junction proteins that were restored after antibiotic treatment. *Muc2*^{+/+} microbiota had a protective innate role against DSS-induced colitis, while microbiota from Muc2 deficient animals did not. *Muc2*^{+/+} littermates that received *Muc2*^{-/-} microbiota exhibited a shift in bacteria families and increased susceptibility to DSS and *Citrobacter rodentium*-induced colitis, demonstrating a critical role for Muc2 mucus in shaping a healthy microbiota in intestinal homeostasis. I also investigated the interdependent relationship between the mucus layer and colonic microbiota in *Entamoeba histolytica* (*Eh*)-induced secretory and inflammatory responses, using *Muc2*^{-/-} and *Muc2*^{+/+} littermates and germ-free mice. Following microbial disruption with antibiotics, *Eh* elicited increased water and mucus secretions and enhanced pro-

inflammatory responses. *Eh* also dysregulated the transcription factor for secretory goblet cells, *Math1*, which was microbiota dependent. Germ-free mice showed decrease pro-inflammatory and mucus secretagogue responses towards *Eh*, demonstrating that commensal microbiota plays an important role in mediating host protection against *Eh* and that dysbiosis can render the colonic epithelium susceptible to *Eh*-induced pro-inflammatory responses and tissue injury. These findings unravel the interconnected role of a healthy Muc2 barrier and commensal microbiota in maintaining intestinal innate host defense against colonic injury.

Preface

This thesis consists of a literature review and two manuscripts.

CHAPTER 1 provides a brief overview of the studies presented in the thesis. It also constitutes an introduction to MUC2 tissue barrier and colonic microbiota and their roles in maintaining intestinal homeostasis.

CHAPTER 2 is an extensive literature review on intestinal barrier, MUC2 mucin, microbiota, and innate host defense in the gastrointestinal tract, fecal microbiota transplantation and *Entamoeba histolytica*.

CHAPTER 3 is a manuscript, which is being prepared for submission to *Cell Host and Microbe*. I designed and conducted the experiments, analyzed data and prepared the manuscript for publication. Dr. Manish Kumar assisted with the flow cytometry experiments and helped with manuscript preparation. Dr. Matthew Workentine did all the bioinformatics analysis. Dr. Michael Surette performed the 16s sequencing and bioinformatics analysis. France Moreau provided technical advice.

CHAPTER 4 is a manuscript that is being prepared for submission to *PLoS Pathogens*. I designed and conducted the experiments, analyzed the data and prepared the manuscript for publication. Dr. Manish Kumar assisted with some of the experiments. France Moreau assisted with technical advice and in the colonic loops surgery for the study.

CHAPTER 5 provides a general discussion on the findings during my PhD research, conclusions that summarize the key findings of the research, and future directions.

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Dedication

I would like to dedicate this thesis to my parents José Luis León de la Luz and Rocío

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List of Symbols, Abbreviations, Nomenclatures

<i>Symbol</i>	<i>Definition</i>
Abx	antibiotics
AU	arbitrary units
CD	Chron's disease
DAI	disease activity index
DSS	dextran sulphate sodium
<i>Eh</i>	<i>Entamoeba histolytica</i>
FITC	fluorescein isothiocyanate
FMT	fecal microbiota transplantation
GC	goblet cell
GF	germ free
GFP	green fluorescent protein
GI	gastrointestinal
gr	grams
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
IL	interleukin
L	litre
M	molar
OTU	operational taxonomic units
SPF	specific-pathogen free
UC	ulcerative colitis
Wt	Wildtype
η	nano
μ	micro

Chapter One: **Introduction**

The human intestine is one of the most densely populated ecosystems, being inhabited by an extensive, diverse and dynamic group of microorganisms called the microbiota that have evolved and adapted to live either on the surface of the mucus layer or in the intestinal lumen¹. This microbiota is shaped mainly by approximately 1×10^{14} bacteria as well as by protozoa and fungal species, which together play a key role in promoting digestive health as well as in the development of a balanced immune system². The immune cells and tissues of the gastrointestinal tract are considered the largest and most complex part of the human immune system³.

MUC2 mucin is produced, stored and secreted by specialized secretory cells in the epithelium, called goblet cells. Mucins are heavily glycosylated, high molecular weight proteins that are the main component of the mucus layer that covers the mucosal epithelium⁴. MUC2 is the major secretory mucin in the gastrointestinal tract^{4,5}. The intestinal MUC2 mucus layer is a great surface lining the epithelial cells and forms a barrier between the cells and luminal contents, which not only contains nutrients but also is loaded with potential hostile microorganisms^{5,6}. The MUC2 layer also acts as a lubricant that protects against enzymatic, chemical and mechanical damage, serving as a shield against non-pathogenic and pathogenic antigens⁷. Mucus secretion into the lumen is a constitutive process, and is required for proper functioning of the gastrointestinal tract⁸. Mucus is also essential for microbiota since bacteria readily uses mucin as energy source by degrading mucin O-glycans and utilising the released monosaccharides to produce short-chain fatty acids. Likewise, mucus provides binding sites to commensal bacteria, allowing them to

colonize and establish and to occupy niches that otherwise could be taken by pathogens or opportunistic microorganisms^{5,9}. Alterations in microbiota composition, a condition known as dysbiosis, as well as disruption of the mucus barrier are both associated with a number of gastrointestinal pathological conditions such as irritable bowel disease (IBS), inflammatory bowel disease (IBD) and colorectal cancer¹⁰⁻¹³.

IBD encompasses two pathologies, ulcerative colitis (UC) and Crohn's disease (CD). Both are immunomodulated inflammatory and chronic disorders of the intestine, which evolve into outbreaks and remission periods. Both diseases alter the body's ability to digest food and absorb nutrients and share clinical and pathological features. Some common symptoms are: diarrhea, blood in the stool, fatigue, abdominal pain, and loss of appetite, weight loss and fever. UC is characterized by chronic inflammatory lesions in the wall of the colon, while CD can appear in any part of the digestive system, from the mouth to the anus^{13,14}. The cause of IBD is unknown, although it is believed that it may be due to the interaction of genetic and environmental factors, and changes in the intestinal microbiota¹⁵. There is no single cause for Crohn's or UC. They appear in genetically predisposed individuals affected by certain environmental factors which cause an alteration of the immune system triggering an anomalous inflammatory response that perpetuates and ends up causing the disorder¹⁶.

It is known that microbiota-host interactions play an important role in the development and regulation of the immune system¹⁵. A major disruption within this equilibrium could lead to a failure of the homeostatic state, thereby favouring the development of immune dysregulation-

linked pathologies^{2,15}. In IBD, commensal bacteria are the target to the host immune aggression, thus triggering an inflammatory phenomena and perpetuating mucosal destruction¹⁷. Data suggest that the bacterial population of these individuals holds anomalous features in terms of its composition: unusual bacterial groups, low diversity and high ecosystem instability^{11,18,19}. Experiments with germ free mice support these findings as these animals possess a thin adherent mucus layer that is restored when challenged with bacterial products²⁰, have increased susceptibility to chemical induced injury²¹ and their nutritional and physical development are affected²². They have atrophic lymph nodes and a decrease in lymphocytes in the intestinal mucosa as well as low levels of plasma immunoglobulins²³. These findings clearly show that the presence of bacteria is necessary for the proper functioning of the host and the immune system.

In the first experimental part of the thesis, I interrogated the role of the Muc2 mucus barrier and microbiota in susceptibility and recovery to colonic injury. Since the mucus layer is composed of MUC2 mucin, mice deficient in the Muc2 gene (*Muc2*^{-/-}) were used to investigate the biological functions of secretory Muc2 in the gastrointestinal tract. Interestingly, *Muc2*^{-/-} mice share several phenotypes typically seen in UC patients²⁴. To examine the role of microbiota, mice received broad-spectrum antibiotics to induce a dysbiotic environment. Results show that the lack of a protective Muc2 barrier alters the colonic microbiota, which in turn, promoted inflammation and intestinal permeability. As predicted, mucus deficient microbiota increased susceptibility to colonic injury when transplanted to mucus-sufficient littermates.

In the second experimental part of the thesis, I explored the distinct roles of the colonic microbiota and the mucus layers in colonic injury and host defense against the human colonic parasite *E. histolytica*. Our laboratory has vast experience using *E. histolytica* as a model for pathogen-induced colonic injury. Therefore, a short-term infection model was used on mice to study the acute response to the parasite.

I used *E. histolytica* in this study, as it is a human protozoa parasite and the causative agent of amebiasis presented as amebic colitis and amebic liver abscess. Of every 10 people to whom the parasite is detected, only one of them will develop symptoms²⁵. In symptomatic cases, the intensity of the symptomatology varies from mild diarrhea to a more intense manifestation characterized by anorexia, asthenia, abdominal pain, alterations in the intestinal transit and mucous diarrhea. *E. histolytica* has two stages in its life cycle: the trophozoite and the cyst. In the trophozoite form, *E. histolytica* inhabits the colon, where it interacts with the resident microbiota as well as with the Muc2 mucus layer. It is known that *E. histolytica* requires commensal bacteria presence for its pathogenicity²⁶. Amebae could also feed on the microbiota or on bacteria-produced glycans. Moreover, *E. histolytica* can degrade the mucus layer using cysteine proteases to break down the macromolecular structure of Muc2 mucin. To counteract this effect, goblet cells increase mucus release, keeping the parasite away from the epithelial barrier^{27,28}. This increase in mucus secretion could potentially promote differential bacterial colonization²⁹, making evident the relevance of the interaction microbiota-ameba-mucus for the onset and progression of this disease.

A major focus of my study was to establish the distinct roles that an intact mucus layer and microbiota play in susceptibility, onset, progression and/or recovery of colonic injury. As the events responsible for the initiation/course of IBD are still unknown, this study highlights the impact of loss of MUC2 and alterations in the microbiota. Either of these factors can act separately or together to aggravate the colonic epithelial barrier dysfunction and inflammation that predispose the colon to further inflammatory insults. At present, it is still not known how microbiota exert their beneficial effects and/or if an intact mucus barrier is required for this to occur. As disruption of intestinal barrier functions, as well as dysbiosis have been associated with IBD, colorectal cancer and other important gastrointestinal diseases it is critical we understand the role of microbiota and an intact mucus layer in epithelial barrier function and disease. This was the focus of this thesis.

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Chapter Two: **Literature Review**

2.1 Intestinal barrier

The intestinal epithelium constitutes a barrier between the luminal content and the lamina propria. This barrier is multilayered and regulates the flow of cells, gases, nutrients and other molecules between both compartments. It has a double function: on the one hand, it is responsible for absorption of nutrients necessary for proper functioning of the organism, and on the other hand, it acts as a barrier that prevents entry of harmful substances¹. In the colon, the intestinal barrier is composed of three layers: a single layer of intestinal epithelial cells (IEC) on top of the lamina propria, the mucus layer in the middle and the microbiota and secreted products at the luminal side² (Fig 2-1).

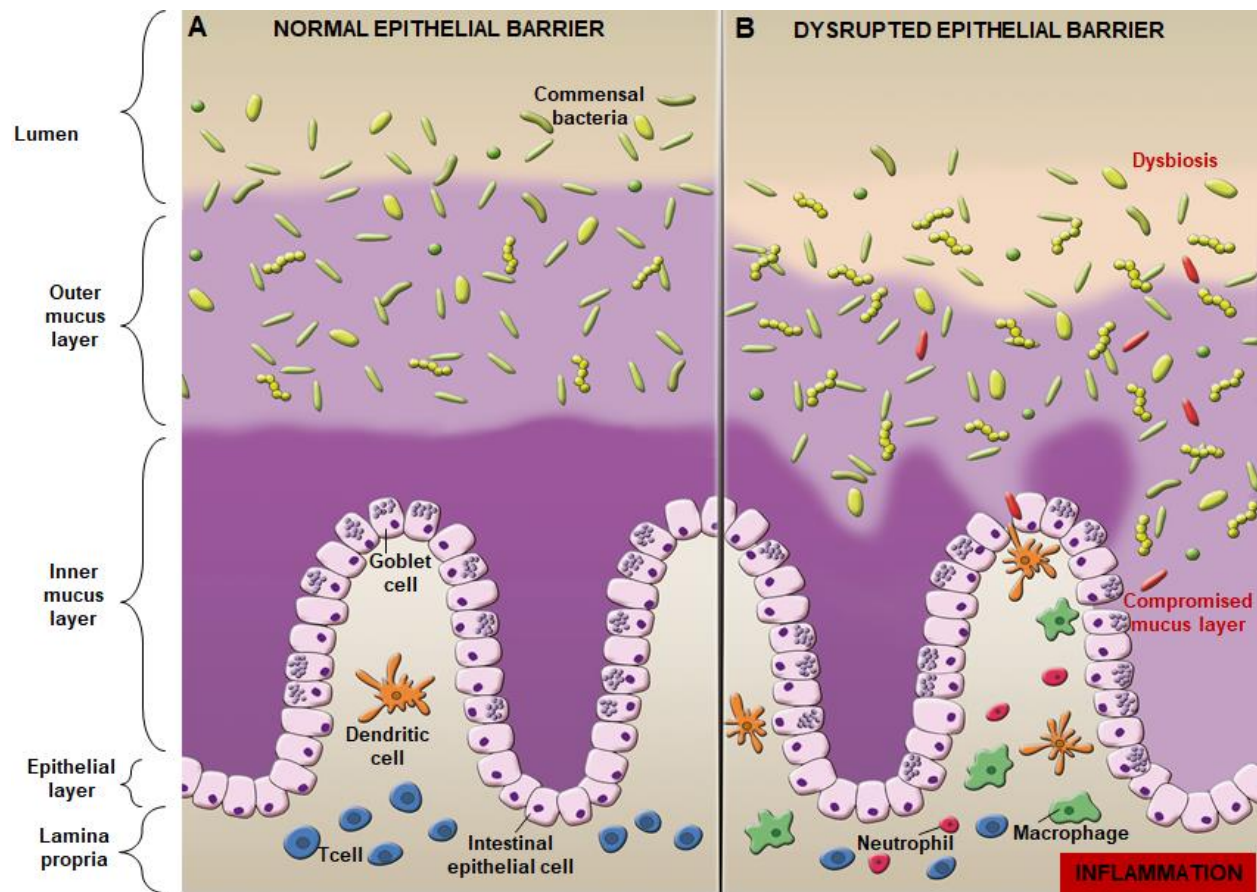


Figure 2- 1 The intestinal epithelial barrier under normal and disrupted conditions. The colonic epithelial barrier is composed by the MUC2 bilayer, and a monolayer of epithelial cells. **A.** Under normal conditions, the resident microbiota is present in the lumen as well as in the outer mucus layer, while the inner mucus layer is devoid of bacteria. The epithelial layer is composed of intestinal epithelial cells, goblet cells and enteroendocrine cells. Within the lamina propria, resident dendritic cells and T cells are present. **B.** Whenever there is a disruption of the mucus barrier, this allows commensal and pathogenic bacteria to come in close contact with the epithelial cells, which in turn, evokes a pro-inflammatory cytokines and chemokines response, immune cells recruitment and inflammation. Adapted from Cornick S. and Chadee K. (2017) *Tissue Barriers*, 5:1.

2.1.1 Intestinal epithelial cells

The colonic intestinal epithelium consists of a monolayer of different specialized cell types that derive from the intestinal stem cells located deep in the crypts. These specialized cells are: absorptive enterocytes, hormone producing enteroendocrine cells and mucus producing goblet cells³. The most abundant epithelial cells are the absorptive enterocytes, whose main functions are the absorption of nutrients and water and acting as a physical barrier between luminal content and the mucosal immune system¹. Goblet cells are located between absorptive enterocytes and are responsible for the production and secretion of mucus, which contributes to the lubrication and protection of the intestinal mucosa⁴. Enteroendocrine cells produce and secrete different hormones and neurotransmitters, and can be found scattered among these cells⁵. Underlying the epithelial cells is the lamina propria, formed by connective tissues, blood and lymphatic vessels, nerve endings and cells of the immune system such as dendritic cells and macrophages¹.

Epithelial barrier integrity depends on various types of junctions that regulate the permeability of ions and molecules. They are composed of tight junctions, desmosomes and adherent unions⁶. These junctions play a key role in cell permeability, forming binding sites as well as enabling communication between adjacent cells⁷. Despite this complex barrier and its diversity of mechanisms, there is no complete exclusion for the passage of molecules from the lumen to the lamina propria. On the contrary, there is a bidirectional, selective and constant movement of particles between both compartments, subject to the regulation of permeability and the transport of different molecules through the intestinal epithelium⁸.

2.1.2 Goblet cells and MUC2

2.1.2.1 Goblet cells and the mucus barrier

With the purpose of maintaining intestinal barrier integrity, goblet cells produce mucin that produces the thick mucus layer lining the colonic epithelial cells⁹. Goblet cells are specialized columnar calyx-shaped cells with a wider apical part, where the secretion vesicles accumulate, and a narrow basal part where the nucleus, Golgi apparatus, endoplasmic reticulum, and other organelles are located. The major function of goblet cells is to synthesize and secrete mucins⁴.

The colonic mucus layer can be divided into two compartments: right above the apical side of epithelial cells there is a condensed inner mucus layer which is devoid of bacteria¹⁰, while the superficial loose outer mucus layer is colonized by commensal microorganisms¹¹. Besides mucin, this protective layer is also composed of water, ions, immunoglobulins as well as host and microbial secreted products¹⁰. In the colon, mucus layer thickness ranges from 800 to 900 μm . MUC2 N-glycosylation and dimerization, via disulfide bonds in the cysteines, take place in the endoplasmic reticulum, whilst O-glycosylation and packing into granules as large net-like structures occur in the Golgi apparatus. The intestinal mucus layer forms a barrier between the body and the luminal environment which not only contains nutrients, but it is also loaded with potentially hostile microorganisms and toxins^{9,12}. Its main function is to allow the efficient transport of nutrients through the single layer of epithelial cells while dangerous molecules and microorganisms are rigorously excluded⁹.

2.1.2.2 MUC2 mucin biochemical structure

Mucins are high molecular weight glycoproteins with tandem repetitions of amino acids rich in proline, threonine and serine (PTS) that are heavily O-glycosylated⁹. MUC2 is the predominant gel forming mucin in the colon¹⁰. MUC2 is a large glycoprotein with a central protein core of approximately 5,179 amino acids¹³. The protein core is formed by the irregular repeat (IR) and the variable number tandem repeat (VNTR) regions, which are highly O-glycosylated. N-acetylglucosamine, galactose and N-acetylgalactosamine are the main sugars present on MUC2, representing more than 60% of the carbohydrates in the molecule¹⁴. The side chains contain sialic acid and sulfosaccharides residues at their terminal end, which provides the molecule with a negative charge¹⁵. The N-terminal region has three complete von Willebrand domains (D1, D2, D3) and one incomplete domain (D'). The C- terminal part has one von Willebrand domain (D4-B-C) and a cysteine knot domain (CK). The N- and C- terminal ends are poorly glycosylated, which renders them susceptible to proteolytic degradation, and are rich in cysteine domains for dimer and oligomer formation via sulfide bonds (SH)¹⁶. The structure of MUC2 is shown in Fig 2-2.

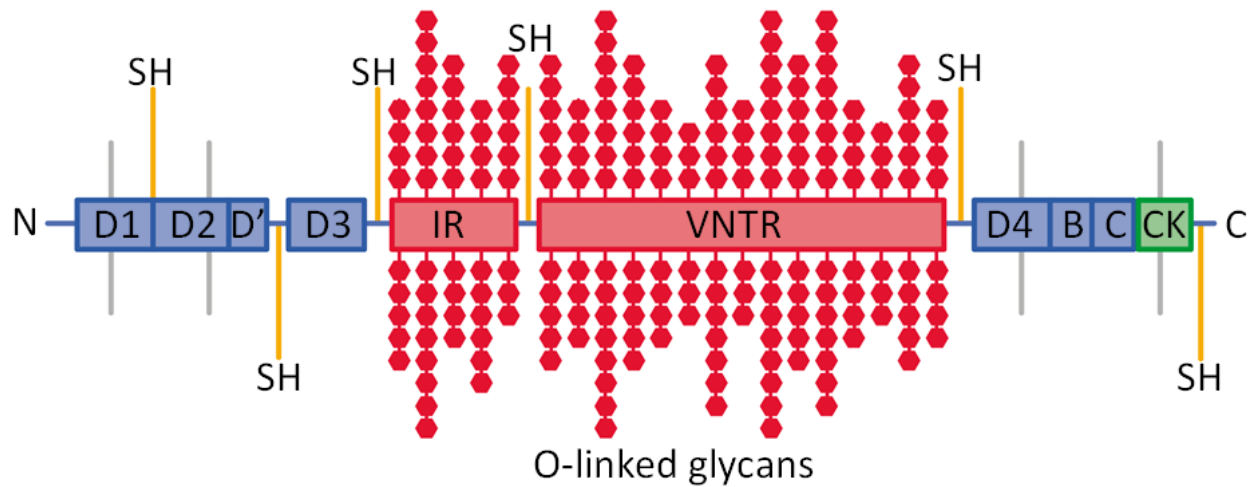


Figure 2- 2 The monomeric structure of MUC2. The MUC2 monomer is conformed by a large central protein backbone that is heavily glycosylated. The N- and C-terminal domains have four von Willebrand D domains (D), three at the N-terminus (D1, D2, D', D3) and one at the C-terminus (D4) with multiple sites to form disulphide bonds (SH). The protein backbone is made up of mostly serine, threonine and proline residues which conform the irregular repeat (IR) and the variable number tandem repeat (VNTR) regions and both these regions are heavily O-glycosylated. The C- terminal end contains a cysteine knot (CK).

As a polymer, the mesh-like structure of MUC2 is key for its protective function since it impedes the diffusion of undesired molecules, but maintains permeable for gas and nutrients exchange⁹. Once polymerized, MUC2 is condensed, packed and stored in secretory granules within the goblet cells. These granules are released constitutively or in response to a stimuli, and once released they hydrate in the lumen resulting in the polymer expanding up to 1,000-fold their size¹⁷.

2.1.3 Gastrointestinal commensal microbiota

Microbiota refers to the community of microorganisms gathered together in a determined ecological niche. The human digestive tract harbours a numerous, diverse and dynamic population of microorganisms, mostly bacteria, but also important numbers of protozoa, fungi and viruses. These microorganisms have been adapted to live on/in the mucus surface and in the intestinal lumen¹⁸. Much of the microorganisms that inhabit the human intestine are natives or indigenous since they are permanently present in the gastrointestinal tract, while others are considered transient microbes¹⁹, altogether they conform the microbial ecosystem found in the intestine. It is estimated that the human gastrointestinal microbial population is formed by approximately 100 billion of bacteria of around 500 to 1,000 species^{20,21}. The stomach and duodenum have a reduced number of bacteria, normally less than 10^3 colony-forming units (CFU) per gram of content. Stomach, biliary and pancreatic secretions destroy most of the ingested microorganisms²², and the propulsion motor activity prevents a stable colonization in the small intestine^{22,23}. The number of bacteria progressively increases along the small intestine from approximately 10^4 in the jejunum to 10^7 CFU in the ileum with a predominance of facultative anaerobes and some obligated anaerobes^{20,24}. In the colon, transit time is slower than in the small intestine, providing microorganisms with the opportunity to proliferate fermenting dietary substrates²⁴. The colon is densely populated with anaerobes and the bacterial counts are approximately 10^{11} CFU¹⁹.

2.1.3.1 Intestinal microbiota functions

At present, germ-free animals and controlled intestinal colonization through fecal microbiota transplantation (FMT) experiments have helped to investigate the main functions of the intestinal microbiota.

2.1.3.1.1 Nutrition and metabolism

A major function of gut microbiota is the breakdown of non-digestible carbohydrates into short-chain fatty acids (SCFA) that serves as an important energy source for intestinal epithelial cells (IEC)²⁰ as well as in the regulation of inflammatory processes²⁵. Microbiota also synthesize nutrients and vitamins such as B₁₂, K, biotin as well as folic acid and amino acids from urea and ammonia²⁰. Around 10 to 20% of the dietary carbohydrates are resistant to small intestine digestion, thus, microbiota is capable of fermenting soluble fibers such as: pectin, hemicellulose, resistant starch and other oligosaccharides. Soluble fibers are broken down into acetyl-CoA that is converted into acetate (2 carbons), propionate (3 carbons) and butyrate (4 carbons). Succinate and lactate are also produced²⁶. Primary degraders such as *Bifidobacterium* spp, *Bacteroides* spp and *Ruminococcus* produce acetate and propionate. Butyrate is produced by secondary degraders, such as members of the *Firmicutes* phylum, either from directly breaking down the glucose molecule or by modification of acetate²⁷. Around 95% of the short chain fatty acids (SCFA) are rapidly absorbed by epithelial cells. SCFA have proven to have multiple beneficial effects for the host's colonic environment, such as: stimulation of mucus production, decrease of cell permeability with a corresponding increase in barrier function, and stimulation of Tregs cell population^{26,28}. Dietary vitamins are absorbed in the small intestine, whilst microbial-produced vitamins are produced and

absorbed in the colon. *Lactobacillus spp*, *Bifidobacterium spp*, *Bacillus subtilis* and other anaerobes are involved in producing important vitamins like folate, riboflavin (B2), vitamin B12, vitamin K and biotin^{27,28}.

2.1.3.1.2 Protection and immune modulation

Within the colonic environment, commensal microbiota competes with pathogens by occupying ecological niches as well as by producing bacteriocins, preventing the invasion of infectious organisms and the overgrowth of resident species with pathogenic potential^{20,29}. Bacteriocins are bacteria-produced peptides that have bacteriostatic or bactericidal effects on other bacteria, and have a key role in maintaining a stable and diverse microbial population within the colon³⁰. The constant competitive metabolic interactions between microbiota also stimulate the host immune response. The relationship between the microbiota and the immune system, and the effect it has on many diseases, could itself be the subject of an extensive review. For the purposes of this thesis, a summary of the topic is presented here.

2.1.3.1.2.1 Pattern recognition receptors (PRRs)

It is impossible for our body to recognize every possible antigen that it is exposed to. Instead, our cells recognize certain molecular patterns that are expressed by different microorganisms (bacteria, protozoa, fungi and viruses) that are highly evolutionary conserved. These conserved motifs are called pathogen-associated molecular patterns (PAMPs) and include certain lipids, LPS, lipoproteins and nucleic acids. They are recognized by pattern recognition receptor (PRRs) in the host cells. The interactions between PRRs and PAMPs result in the activation of common

pathways to regulate inflammatory genes and the production of inflammatory cytokines and/or chemokines, which alert the host to the presence of an external agent and the possibility of an infection³¹. Among others, PRRs include family members of Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NOD-like receptors, NLRs).³²

TLRs are a family of evolutionarily conserved transmembrane receptors. Thirteen of those have been identified in mammals: 10 in humans (TLR1 to 10) and 12 murine (TLR1 to 9 and TLR11 to 13), some are homologous³¹. The corresponding PAMPs for TLRs comprise bacterial cell wall components such as: lipoproteins, peptidoglycan and lipoteichoic acid as well as zymosan from the fungal cell wall. They also recognize double and single stranded-stranded RNA, bacterial lipopolysaccharide and flagellin. TLRs recruit the signal adaptors myeloid differentiation primary response 88 (MYD88) and TIR-domain-containing adapter-inducing interferon- β (TRIF) to signal molecules via NF κ B p50 and p65 subunits activation³³. NLRs are a family of intracellular sensors for PAMPs and danger-associated molecular patterns (DAMPs). The NOD subfamily recognizes N-acetylglucosamine, N-acetylmuramic acid and other peptidoglycans from bacterial cell wall. The NLRP subfamily is involved in the formation of inflammasomes, which can recognize PAMPs and DAMPs, and activate an inflammatory cascade with the production of pro-inflammatory cytokines IL-1 β and IL-18³². These pro-inflammatory responses are necessary for the correct functioning and maintenance of the host immune response.

2.1.3.1.2.2 Secretory immunoglobulin A (sIgA)

Another mechanism of extreme relevance is the specific blocking of certain bacterial epitopes by the binding of sIgA to the antigen by the variable region. This prevents adhesion of commensals to epithelial cell and prevents bacterial overgrowth. sIgA is formed by an IgA dimer joined by a J chain, to which a polypeptide called secretory piece is attached. sIgA limits microbial mobility of pathogenic bacteria by binding to flagellin. In addition to neutralizing pathogens in the intestinal lumen, sIgA can also intercept intracellular bacteria and toxins within intestinal cells³⁴.

The intestinal immune system uses various strategies to respond to microbes in a way that does not compromise homeostasis. These strategies are diverse, multifunctional and interconnected, and function to avoid incorrect activation of the mucosal immune system.

2.1.3.2 Intestinal microbiota composition

For a long time, the knowledge we had about the composition of intestinal microbiota was based on information obtained by culturing intestinal biopsies or stool samples. Unfortunately, less than 50% of bacterial cells observed in a fecal microscopic exam can be recovered in culture media²¹. Over the last two decades diverse molecular techniques have been developed to identify and characterize microbiota. One of the most utilized is 16s rRNA sequencing. This technique analyze the diversity of sequences of the ribosomal 16s rRNA gene³⁵, which has permitted the development of phylogenetic and taxonomic studies offering a much more complete picture of human intestinal bacterial ecosystem. Based on this, it has been determined that 7-9 phyla³⁶ are present in the

human gastrointestinal tract and about 90% of these bacteria belong to the phyla Firmicutes and Bacteroidetes followed by Actinobacteria, Proteobacteria, Verrucomicrobia and Fusobacteria^{37,38}.

Different factors such as age, diet, drugs treatment (especially antibiotics), stress, illness and geographic location determine variability in bacterial composition over time^{20,29}. However, the comparison of bacterial genes associated with biological functions is quite stable in healthy individuals^{20,36}. Among these genes are those related to important metabolic pathways and genes related to survival in the gastrointestinal tract³⁹. It is noteworthy that even though each individual has a unique bacterial composition, widespread distribution of bacterial phyla and families remain constant between healthy individuals^{37,40}. Alteration in the microbial composition, condition known as dysbiosis, has been related to intestinal and extra-intestinal conditions⁴⁰⁻⁴³. A schema of bacterial distribution in the adult gastrointestinal tract is shown in Fig 2-3.

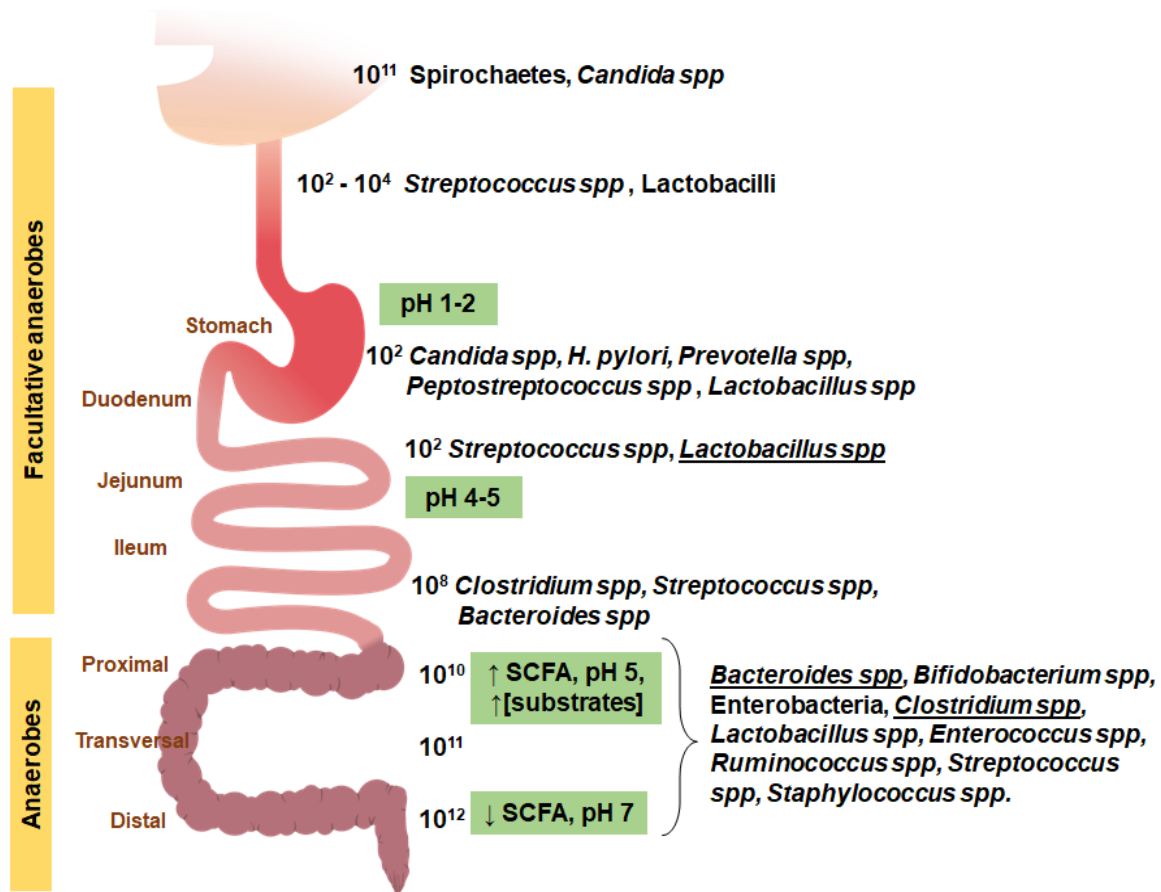


Figure 2- 3 Schema of the bacterial distribution in the human gastrointestinal tract. Within the mouth, the number of bacteria is as high as 1×10^{11} CFU/g. Bacterial numbers decrease in the trachea and in the stomach, where it reaches 1×10^2 CFU/g due to the acid pH. In the small intestine (SI), pH starts to increase as well as bacterial numbers, with the duodenum having 1×10^2 CFU/g and the ileum reaching 1×10^8 CFU/g. Within the proximal colon the bacterial numbers reach 1×10^{10} CFU/g, there is a high concentration of substrates that are utilised by bacteria for production of short chain fatty acids (SCFA), mainly butyrate since the pH is slightly acidic (pH 5). At the distal portion of the colon, the bacterial numbers reach as high as 1×10^{12} CFU/g, there is less production of SCFA and the pH is neutral. The upper gastrointestinal tract and the SI are mostly populated by facultative anaerobes, whereas the colon hosts anaerobes microorganisms. Representative bacteria content in each portion of the gastrointestinal tract are indicated on the right, underlined point out at dominant bacteria. Adapted from: Microbial interactions with humans. Chapter 21: 700-725. In: Madigan MT, Martinko JM, Brock, Biology of Microorganisms. Pearson Prentice Hall. 2005.

2.1.4 Mucin-bacteria interactions

The host-microbe interaction starts in the mucus. The MUC2 O-glycosylated side-chains are rich in N-acetylglucosamine, galactose, N-acetylgalactosamine and fucose¹⁷. These saccharides residues are key for commensals and pathogenic organisms binding through their lectins. Pathogens and opportunistic organisms can be expelled by binding to the outer mucus layer. Commensal bacteria harness this bond to have a niche protected by mucus. Mucus plays a role in maintaining a healthy and stable microbial community, since commensals and pathogens are in constant competition for binding sites to the mucus layer¹⁴.

Recent studies have showed that the O-glycan complex is highly conserved between individuals, suggesting a role in the selection of the commensal microbiota providing binding sites to certain sugars⁴⁴⁻⁴⁶. This way, intestinal mucin influences the composition of the microbiota community. Bacteria carry out proteolytic degradation of mucus and use mucin glycans as an energy source, simultaneously, the released monosaccharides are turned into SCFA and used as an energy source for intestinal epithelial cells⁴⁷. Butyrate and propionate induce MUC2 transcription via AP1 binding and histones acetylation and methylation on the MUC2 promoter⁴⁸. Mucin is an important carbohydrate source, mainly in the distal colon where the abundance of fermentable carbohydrate substrates is low⁴⁵. Thereby, bacterial stimulation of mucus secretion, as well as mucin degradation is part of the normal process of mucus turnover and contributes to maintain a functional protective barrier to potential hazards.

2.2 Innate-host defense in the gastrointestinal tract

The intestinal immune system is the most extensive and complex part of the immune system. The innate immune system of the intestinal mucosa, through a set of receptors, cells and mediators, is capable of sensing, processing and generating an inflammatory or an immunotolerance response^{49,50}. The intestine is exposed to a huge antigenic load and its main challenge is to distinguish between invasive pathogens and harmless antigens from food and commensal bacteria. The sensitivity and specificity of this important function are maintained, at least in part, by the ability to recognize these antigens via pattern recognition receptors (PRR) like TLRs and NLRs³¹. TLRs are expressed both in lymphoid and non-lymphoid tissue and their expression pattern varies according to the cell type and the tissue studied. Different types of TLR are expressed in monocytes, neutrophils, NK cells, B cells, dendritic cells, endothelial cells and intestinal epithelial cells^{31,33}. The bacteria-TLRs interaction can be reviewed in the "Pathogen recognition receptors (PRRs)" section of this thesis.

The cellular component of the innate immune system is formed by dendritic cells, macrophages, enterocytes, and B cells. Within the lamina propria, there are B-cells that secrete IgA⁵⁰ (the role of sIgA in protection and immune modulation is mentioned in the section "Secretory immunoglobulin A (sIgA)"). The large intestine also houses resident macrophages, eosinophils and mast cells. These cells can phagocytose pathogens and release toxic and inflammatory mediators such as reactive nitrogen species and histamine⁵¹. Neutrophils are normally the first cells recruited at the site of inflammation, phagocytosing and destroying pathogens through toxic enzymes such as

lysozymes and peroxidase. Macrophages, dendritic cells and mast cells are in a continuous state of migration, differentiation and renewal within the intestinal mucosa^{49,51}.

2.2.1 Dendritic cells and oral tolerance

Within the gastrointestinal tract, dendritic cells (DCs) constantly sample the intestinal microenvironment to maintain a balanced and immunological tolerance to harmless antigens during the immune response against pathogens. DCs are differently stimulated depending on the components of the bacterial strain, leading to secretion of IL-12 and a Th1-type response, or the secretion of IL-4 and a Th2-type response. The production of IL-6, IL-21 or IL-23 stimulates a Th17-type response and TGF- β 1 a Treg response^{49,50}.

In the intestinal mucosa, CD103⁺ DC is specialized in the generation of Tregs, which limit the immunological responses to commensal bacteria and antigens of the diet. CD103⁺ DCs produce all-trans retinoic acid (ATRA) and TGF- β , which act together to stimulate the generation of Tregs. DCs generate activated TGF- β after integrin-mediated cleavage of the inactive precursor. ATRA also induces tropism in T cells activated by intestinal DCs by induction, in Treg cells, of the α 4 β 7 integrin and the chemokine receptor CCR9. This, in turn, promotes FOXP3 induction and differentiation of Tregs, which produces IL-10 and TGF- β and the induction of tolerance and immune suppression^{49,52}.

2.3 Role of microbes in gastrointestinal diseases

The interaction between the mucosal immune system and the intestinal lumen microbiota seems to be a primary mechanism in the conformation of a Treg cell-mediated immunotolerant state⁵³. According to the hygiene hypothesis, in westernized societies the increasing incidence of atopic (eczema, asthma, rhinitis, allergies), irritable bowel syndrome (IBS) and autoimmune disorders (multiple sclerosis, type 1 diabetes) could be explained by a decrease in bacterial load in the first months of life^{19,54,55}.

2.3.1 Bacteria role in inflammatory bowel disease (IBD)

IBD is a disorder of the gastrointestinal tract from the mouth to the anus, characterized by chronic inflammation resulting on an imbalance of the intestinal immune response in genetically susceptible subjects⁵⁶. In Crohn's disease (CD) and ulcerative colitis (UC), the two forms of IBD, there is aberrant activation of the mucosal immune system against enteric microbiota elements^{24,57-59}. In patients, increased mucus secretion and IgA antibodies against commensal bacteria is detected, suggesting the abolition of local tolerance mechanisms⁶⁰. In UC, treatment with broad-spectrum antibiotics quickly reduce inflammatory activity⁶¹. Antibiotics induce intestinal dysbiosis in children under 1 year and can triplicate the prevalence of pediatric IBD⁵⁴. There is abundant information indicating that IBD patients' intestinal bacteria population differs from that of healthy individuals^{59,62}. Studies have shown that 30-40% of dominant bacteria in samples from patients with IBD belong to phylogenetic groups, rare in healthy individuals^{62,63}, but it is unclear if this is a primary or secondary phenomenon to inflammation. In general, there is no direct

association between specific pathogens and the development of IBD, although a less diversity of species than healthy individuals is commonly reported^{62,64}, with a very large tendency to have a reduction in Firmicutes and an increase in Proteobacteria and Bacteroidetes^{62,65}. Reduction of Firmicutes *Clostridium leptum* and *Faecalibacterium prausnitzii* is also common in IBD samples^{40,66}. In CD, there is a reduction in the abundance of members of the Firmicutes phyla and species of the genus *Bifidobacterium*, with a corresponding increase in Proteobacteria⁶⁵. Another important factor is the alteration of bacterial metabolic functions that are affected by dysbiosis^{29,67}. UC patients showed an increase in sulfate-reducing bacteria (SRB); SRB produce hydrogen sulfide, which is toxic to IEC and induces inflammation. SRB bacteria include species of *Desulfovibrio spp* ad *Desulfobacter spp*⁶⁸. Similarly, the reduction of *F. prausnitzii* (a butyrate-producing bacteria) could induce a reduction of butyrate in the intestine and induce an inflammatory state⁶⁶. An increase in mucus degrading bacteria is frequently seen in patients with IBD⁶⁹. Despite advances in molecular identification of bacterial communities related with this pathology, it is currently not possible to define an associated or causal IBD microbiota.

2.3.2 Dysbiosis effect in other diseases

Other important gastrointestinal diseases have also been related with alterations in bacterial composition. A dysbiotic state has been found in patients with IBS. Patients with celiac disease have a decreased in bacterial diversity and in species richness⁷⁰. A general trend found in obese people and in patients with type-2 diabetes is an increase in Firmicutes and a corresponding decrease in the Bacteroidetes phylum; this characteristic microbial diversity impacts through their ability to harvest energy from ingested food⁷¹. Interestingly, a dysbiotic state in colonic microbiota

also seems to affect non-GI diseases. An appropriate microbial colonization in early stages of life seems to be important for the correct development of the enteric nervous system²⁴. This process is suggested to affect patients with asthma, presenting an increase in serum IgE and an exacerbated Th2 response⁷², as well as patients with autism, where a trend to present increased intestinal permeability has been observed⁷³.

2.4 *Entamoeba histolytica* and amebiasis

2.4.1 *E. histolytica* biology and life cycle

E. histolytica is a unicellular eukaryotic parasite that during its life cycle presents two morphologies: the trophozoites and the cyst. Trophozoites contain a single nucleus, with a diameter of 20-40 µm. Cysts are smaller than trophozoites, measuring about 10-16 µm in diameter, they have a single nucleus when they are immature and four nuclei when they are mature and they also contain a glycogen vacuole and are protected by a chitin cystic wall. The nucleus is vesicular, spherical, with a membrane coated with small granules of chromatin and with a spherical and central karyosome⁷⁴. Infection starts with the ingestion of cysts from fecally contaminated food or water. The cyst is resistant to the gastric environment and descends in the digestive tract until arriving to the small intestine where, under contact with digestive juices, the process of excystation begins. During excystation the cystic wall softens, the nuclei duplicate and small trophozoites will form to later grow into mature trophozoites that migrate to the colon. In the colon, trophozoites multiply and feed on resident bacteria. In most cases, trophozoites remain in the intestinal lumen (non-invasive disease) of individuals who are asymptomatic carriers. In a small percentage of

infected individuals, for unknown reasons, the parasite breaches innate and epithelial host defenses invading and attaching to mucosal tissues (intestinal disease). After penetrating the intestinal mucosa, trophozoites can enter the bloodstream through the portal system, to reach the liver and other sites such as brain and lungs (extra-intestinal disease), resulting in serious pathological manifestations. Upon reaching the liver, amoebas initially produce an inflammatory reaction with subsequent tissue necrosis and formation of one or more abscesses⁷⁵. Other forms of extra-intestinal amoebiasis are usually originated from hepatic abscesses, which will lead to trophozoites spreading to the lung, pleura or pericardium or –via blood– to the lung and brain. In non-invasive colonization, trophozoites encystment occurs when the parasite begins to form reserve material in the form of glycogen vacuoles and chromatoid bars, stops emitting pseudopods and takes on a spherical shape and a thick cystic wall appears. The differentiation of the trophozoite ends with the formation of a tetranucleated cyst. Cysts are eliminated through the stool. Due to their protective chitin cell walls, cysts can survive days and even weeks in the environment and are responsible for the transmission of the infection. The trophozoites can also be expelled through diarrhea, but they are quickly destroyed once they leave the host, and if they are ingested they do not survive exposure to the gastric environment (reviewed in ^{74,76,77}).

2.4.2 Epidemiology

The infection by the protozoan *E. histolytica* can be found all over the world, from very cold climates to tropical zones. In 2013, *E. histolytica* was ranked as the fourth leading cause of death by parasites⁷⁸. The transmission of *E. histolytica* by contaminated water is common in developing countries where most of the water for human consumption is not treated. The use of human feces

as a fertilizer is also a major source of infection. Its incidence is highly influenced by hygienic and economic status of the population⁷⁶.

2.4.3 Pathogenesis of amebiasis

E. histolytica's ability to invade and destroy tissue is determined by the expression and action of several proteins and mechanisms that regulate its virulence. *E. histolytica* is characterized by its extraordinary ability to invade and destroy host tissues⁷⁹. The pathogenic capacity of the trophozoites has been attributed to their ability to phagocytose, resistance to complement and expression of virulence factors, such as: adhesins, cysteine proteases, amebapores, collagenases and phospholipases, which provides amoeba with high cytotoxic activity⁸⁰. Trophozoites can also ingest red blood by erythrophagocytosis⁸¹.

2.4.3.1 Virulence factors

2.4.3.1.1 Gal/GalNAc lectin

One of the virulence factors is the membrane (Gal/GalNAc)-specific lectin, which recognizes and binds to exposed galactose/N-acetyl-D-galactosamine (Gal/GalNAc) residues in glycoproteins of target cell membrane, as well as mediates adhesion of amoeba to Gal and GalNAc residues of colonic mucus for colonization. Cells that do not express Gal or GalNAc residues are resistant to *E. histolytica* cytotoxic activity. Inhibition of lectin binding with exogenous Gal or GalNAC sugars abrogates amoeba killing of target host cells⁸² (reviewed in ^{83,84}).

2.4.3.1.2 Amebapores

Tissue lysis occurs through pore-forming proteins called amebapores, which are polypeptides that facilitate tissue invasion, permeabilize the membrane and insert themselves into the lipid bilayer of target cell membranes. This also allows channels formation through which there is diffusion of water and ions (Na^+ and K^+ leave cells, and Ca^{2+} enters the cell). Consequently, the internal cell medium changes and cell lysis occurs by osmotic shock⁸⁵ (reviewed in ⁸⁶).

2.4.3.1.3 Cysteine proteases

Another *E. histolytica* virulence factor are cysteine proteases, which are proteolytic enzymes that are essential in the ability to destroy host tissues. Additionally, they have an important role in invasion and digestion of phagocytosed material, as they degrade different components of the extracellular matrix (fibronectin, laminin and collagen, among others; reviewed in⁸⁷). At present, more than 50 cysteine proteases genes have been identified in *E. histolytica*⁸⁸. Cysteine protease 5 (*EhCP5*) is perhaps one of the best characterized and one of the most important virulent factor by ameba. *EhCP5* proteolytically cleaves MUC2 in the carboxy-terminal domain depolymerizing the MUC2 polymer facilitating parasite invasion to the underlying epithelium⁸⁹. Cysteine protease 5 role in mucus degradation is reviewed in the "*E. histolytica* interaction with mucus layer" section of this thesis.

2.4.3.2 Phagocytosis

E. histolytica phagocytosis is characterized by the ingestion of bacteria, living cells, dead cells and cell detritus. Phagocytosis of red blood cells (erythrophagocytosis) is a very common feature by

invasive amebas, and its presence in the cytoplasm was one of the biological criteria used in the past to recognize them in dysenteric stools and liver lesions⁷⁷. Phagocytosis is a complex and dynamic event involving adhesins and enzymes. It is a highly ordered and orchestrated process in which, once the parasite joins and adheres to the target cells and kills it, it is phagocytosed and processed into specific vacuoles called lysosomes and phagosomes, where the cell fragments are degraded⁹⁰.

2.4.3.3 Trogocytosis

A pathogenic mechanism called trogocytosis has been recently described in *E. histolytica* where the parasite ingests pieces of living cells. During trogocytosis (trego: to nibble), trophozoites destroy human cells by "biting" and internalizing different fragments of the cellular membrane, causing an elevation of intracellular calcium and subsequent cell death. Once the cell dies, the ingestion of fragments ceases⁹¹. This mechanism likely plays a key role in intestinal damage and perhaps in the pathogenesis of amebiasis. Despite the identification of numerous virulence factors that are important in disease pathogenesis, the cellular and molecular mechanisms central in invasive disease remain unknown. A schema of invasive amebiasis is shown in Fig 2.4.

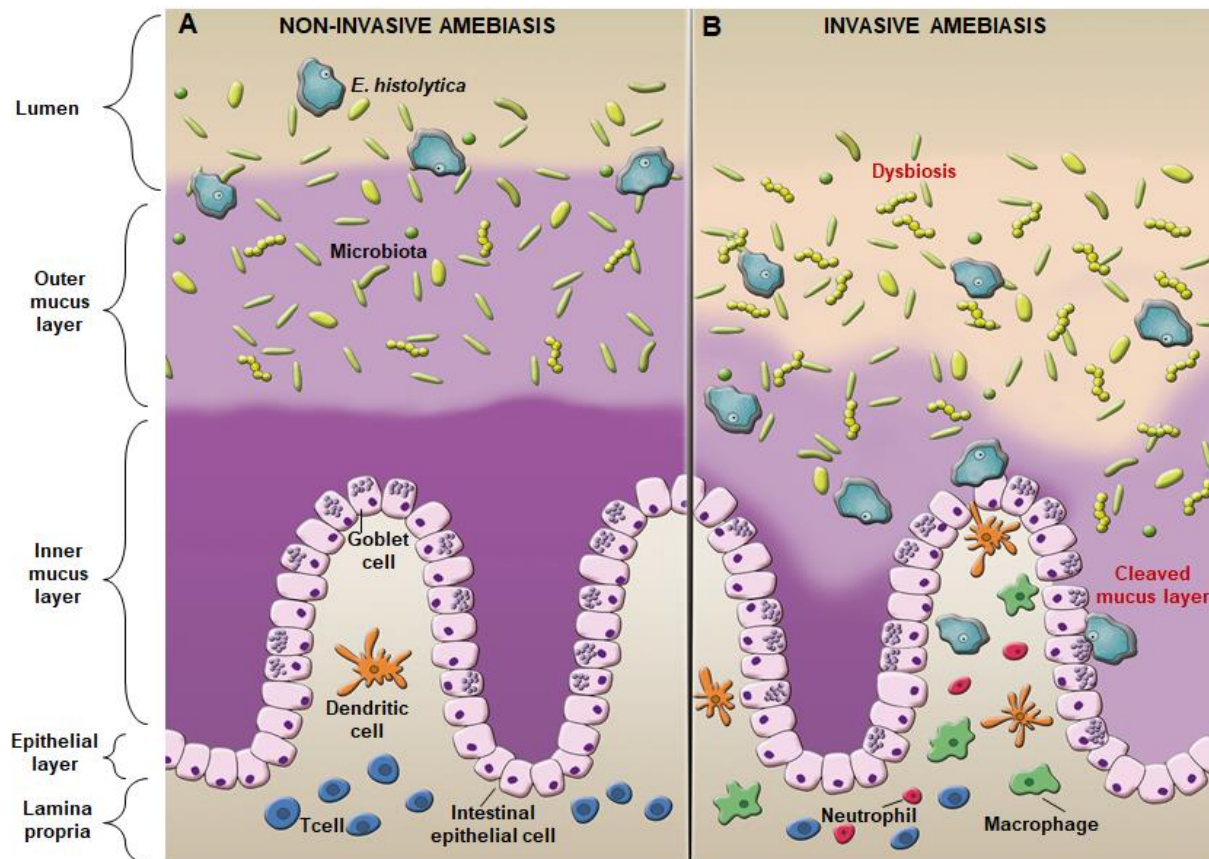


Figure 2- 4 *Entamoeba histolytica* disrupts the mucus layer to cause invasive disease.

A. In asymptomatic infections *E. histolytica* colonizes in/on the loose outer mucus layer and away from epithelial cells. Here, ameba feed on bacteria, cell detritus and sugars from digested mucus. **B.** Under unknown circumstances, *E. histolytica* degrades the mucus layers by cysteine proteases. The degradation of mucus compromises the epithelial barrier and alters the microbial composition. This allows amoebae to contact epithelial cells, disrupts tight-junction proteins and the extracellular matrix, inducing an inflammatory cell infiltrate and causing intestinal amebiasis.

2.4.4 *E. histolytica* interaction with mucus layer

The first barrier *E. histolytica* must overcome to invade into the intestinal mucosa is the MUC2 mucin layer. Trophozoites gain access to the intestinal cells by proteolysis of the mucin layer that covers the intestinal epithelium. This process is fundamental for the parasite to exert its pathogenic potential on host cells. *E. histolytica* strongly binds to mucin via the Gal/GalNAc-lectin, which has high affinity for galactose and N-acetyl-D-galactosamine glycans present on the O-linked sugar side chains of the MUC2 molecule⁹².

2.4.4.1 Ameba glycosidases

Once ameba is bound to the mucus layer, it can break down the polysaccharides present in the outer structure of the MUC2 molecule utilising various glycosidases, such as: N-acetylgalactosamidase, N-acetylglucosaminidase⁸³, β -galactosidase, β -N-acetylhexosaminidase⁹³, among others. β -amylase brought special attention as the absence of this enzyme abrogates mucus depletion and consequent tissue invasion. Only virulent amebae up-regulate genes involved in carbohydrate metabolism⁹³.

2.4.4.2 Ameba proteases

Simultaneously, proteases can target the peptides in the central protein core. A genome study of *E. histolytica* have identified numerous genes that code for enzymes with proteolytic activity, among them: cysteine proteases, serine proteases, metalloproteases and aspartic proteases⁹⁴.

Cysteine proteases are present in *E. histolytica*-secreted products and have a crucial role in mucin degradation⁹⁵.

2.4.4.3 *EhCP5* and mucus degradation and hypersecretion

Cysteine protease 5 (*EhCP5*) cleaves MUC2 mucin by targeting and cleaving mucin C-terminal cysteine rich domain, leading to disassembling of the MUC2 macromolecular structure, and eventual parasite penetration of the mucus layer⁸⁹. Trophozoites deficient in *EhCP5* failed to exert their cytopathic effect on cell lines with mucin secretory phenotype, but remain virulent to cell lines without a mucus barrier⁹⁶.

In addition to degrading MUC2 mucin, the parasite can also evoke a mucus hyper secretion response by goblet cells. It is known that *E. histolytica* is a potent mucus secretagogue, which leads to goblet cells cavitation and mucus depletion. *EhCP5* carries out this phenomenon by interacting with $\alpha_v\beta_3$ integrin on goblet cells. This contact induces a signalling cascade with proto-oncogene tyrosine-protein kinase Src (SRC) family kinases and phosphatidylinositol-3-kinases (PI3K), which activates protein kinase C delta (PKC δ) and a further phosphorylation of the vesicle trafficking protein myristoylated alanine-rich C-kinase substrate (MARCKS), driving mucin secretion from colonic goblet cells⁹⁷. A recent study from our laboratory has showed that in response to *E. histolytica*, mucus secretion from goblet cells is modulated, in a classical exocytosis manner, by vesicle SNARE vesicle-associated membrane protein 8 (VAMP8) present in mucin granules⁹⁸.

2.4.5 *E. histolytica* and microbiota

In the colon, trophozoites interact with multiple components that can modulate the course of tissue invasion and destruction. The contact between the trophozoites and resident microbiota constitutes the beginning of the first host-parasite interactions that could initiate the disease.

2.4.5.1 Microbiota modulates ameba pathogenicity

E. histolytica colonizes the outer mucus layer⁸⁹ and as such, shares a niche rich in a diverse community of microbiota directly interacting with them and probably benefiting from their presence. Colonic microbiota breaks down complex carbohydrates into glycans that can serve as a nutrient source for ameba. At the same time, *E. histolytica* can also feed from the resident microbiota. It is known that the presence of enteric bacteria is required for ameba to express virulence associated genes. Infection of *E. histolytica* in germ-free animals failed to induce disease, but ameba pathogenicity was re-established after inoculation of bacteria⁹⁹. Similarly, *in vitro* cultures of trophozoites in axenic conditions exhibit decreased ameba virulence that could be restored after inoculation and incubation with live bacteria¹⁰⁰. The exact mechanism whereby this happens remains elusive.

2.4.5.2 Microbiota alteration by *E. histolytica*

Recent studies from endemic areas have shown evidence that ameba infection alters resident bacterial composition. A study in Northern India linked ameba-positive patients with a dysbiotic state, characterized by decrease in *Bacteroides*, *Clostridium coccoides*, *C. leptum*, *Lactobacillus*,

Campylobacter and *Eubacterium* species, with a corresponding increase in *Bifidobacterium*¹⁰¹. Another study performed in Cameroon showed that *E. histolytica* infection correlated with an augmented number of species (alpha diversity) and a significant decrease in inter-individual variation (beta diversity). This study also linked ameba presence with an increase in Clostridiales and Ruminococcaceae and a corresponding decrease in *Prevotella copri* and Fusobacteria¹⁰². A longitudinal study performed in children from a rural zone in Bangladesh associated parasite-induced diarrhea with an expansion of *Prevotella copri*¹⁰³. Another study in patients with amebic liver abscess (ALA) could not relate specific bacteria to ALA incidence, however, most of the ALA patients were co-infected with bacteria and *Klebsiella* in high abundance⁷⁵.

The fact that *E. histolytica* induces the production of antimicrobial peptides, but are resistant to their cytopathic effect¹⁰⁴ could explain the alteration in microbial composition observed in amebae infections. All these association studies come to practically the same conclusion: it is not known for sure if the observed dysbiosis is a cause or an effect of the infection by *E. histolytica*. Numerous factors need to be considered to clarify this complex relationship and how they influence the outcome of the infection, namely:

- The host immune responses against amebic intestinal infection and how it affects the microbial composition.
- The possibility that the environment created by the dysbiosis is favourable for opportunistic pathogens.
- The microbiota breakdown of carbohydrates (glycobiome) required by *E. histolytica*.

Relevant to this, it has been shown that intestinal bacteria can also affect infection by parasitic helminths (reviewed in¹⁰⁵) as well as with other parasitic protozoa (reviewed in¹⁰⁶)

2.5 Fecal microbiota transplantation therapy

Fecal microbiota transplantation (FMT) consists of obtaining intestinal microorganisms from the stool of a healthy individual (donor) and introducing them into the gastrointestinal tract of a person with a pathology to restore the damaged intestinal microbiota¹⁰⁷. FMT has been shown to be effective in the treatment of recurrent *Clostridium difficile* infection, with a cure rate of up to 90%. Most patients with a *C. difficile* infection respond well to first line treatment that includes the administration of antibiotics such as metronidazole and/or vancomycin. However, up to 10 to 20% of cases will have recurrence of symptoms after the treatment has been completed. After a first recurrence, subsequent recurrence rates increase to 40 to 65%¹⁰⁸. Recurrent *C. difficile* infection can become a chronic disease, with repeated episodes of infection, repeated use of antibiotics, multiple hospitalizations, the need for intestinal surgery and could even cause death. The management of recurrent infection is especially problematic, since strategies of repeated antibiotics cycles are often unsuccessful. Different studies have shown that FMT is highly effective for the treatment of recurrent *C. difficile* infection, and is considered the treatment of choice in this clinical context^{108,109}.

The success of FMT in treating *C. difficile* infection has inspired studies to evaluate this treatment in other gastrointestinal conditions such as IBD, IBS and metabolic disorders including obesity and type 2 diabetes mellitus (T2DM). In the case of CD treatments, the efficacy of treatments

could be as high as 70% within the first 3 months of FMT. However treatment loses its efficacy with time¹⁰⁷. Also, other complications (vomiting, small bowel perforations, fever, among others) have appeared in CD patients after receiving FMT. These adverse effects could be related to the disease itself and not with treatment, though (reviewed in¹⁰⁸). In UC, FMT treatments in randomized controlled trials have come up with results with high variability and different percentages in both remission and responsive patients. In contrast, FMT in IBS patients reverses the dysbiosis and reduces IBS symptoms in about 70% of patients, without presenting any severe adverse effect (reviewed in¹⁰⁸). FMT have also been used in trials for treating metabolic syndrome, with lean people as donors. Results from this study showed that FMT in patients with metabolic syndrome reduced their triglyceride levels and improved the peripheral and hepatic insulin sensitivity. These changes correlated with an increase abundance of butyrate-producing bacteria¹¹⁰. Recently, it has been observed that after the administration of FMT to treat *C. difficile*, this treatment also helped in decolonizing antibiotic-resistant bacteria in patients. Studies have demonstrated decolonization of these bacteria in 60 to 93% of the study subjects¹¹¹. Other studies have shown the reduction of antibiotic resistance genes in the gastrointestinal tract of patients treated with FMT for *C. difficile*¹¹².

Given the association of the intestinal microbiome with several conditions including autoimmune disease and metabolic diseases, there is a concern that the transfer of fecal matter might also transfer the propensity towards such disorders. Long-term complementary studies are necessary to investigate this potential risk. Although FMT is a safe procedure and without any known severe adverse effect attributable so far, there is a theoretical risk of transmission of infectious agents that

are now unknown and not detectable by current techniques, and that may cause disease to the recipient. As dysbiosis is associated with several diseases, research towards using FMT as a therapy promises favorable results to alleviate the above-mentioned conditions.

2.6 *Muc2*^{-/-} mice as a model for gastrointestinal mucin barrier functions

In 2002, Velcich *et al.*, created a mouse deficient in Muc2 mucin. These mice were generated in a C57BL/6 x 129s background by a targeted inactivation of the Muc2 gene and replacement of a genomic fragment with a phosphoglycerate kinase-neomycin (PKG-Neo) cassette. The authors performed a characterization of the mice, finding that animals developed adenocarcinomas and rectal tumors. The intestinal crypts showed aberrant morphologies and the processes of cellular maturation and migration were altered¹¹³. Another study on these mice showed that as early as at 5 weeks of age, Muc2 deficient mice showed signs of colitis with mucosal thickening and superficial erosions. Goblet cells were negative for Muc2 but positive for intestinal trefoil peptide (Tff3). No compensatory up regulation of other mucin was observed, with exception of Muc6 expression in the distal colon in animals 5 weeks old, although expression was lost as the mice aged¹¹⁴.

From the generation of these mice, different studies have been carried out to elucidate the role of Muc2 in intestinal homeostasis. Our lab have shown that *Muc2*^{-/-} mice are highly susceptible to intestinal pathogens such as *Citrobacter rodentium*¹¹⁵ and *E. histolytica*¹¹⁶ with increased intestinal permeability¹¹⁶. *Muc2*^{-/-} mice also presented delayed expulsion of the parasite worm *Trichuris*

*muris*¹¹⁷. Similarly, *Muc2* deficient mice have an exacerbated response to dextran sodium sulphate (DSS) model of induced colitis¹¹⁴.

The fact that colitis and colorectal cancer develops spontaneously in these mice, have given research groups the opportunity to associate molecules of interest, and their link with *Muc2* and the development of these conditions. These mice have up regulated expression of *Reg3β* and *Reg3γ*, which suggests that there is an altered bacterial-epithelial signalling in absence of *Muc2*¹¹⁸. These mice also have reduced production of antimicrobial peptides^{119,120}, which not only affects their response to inflammatory insults, but could also dictate the conformation of the microbial communities present in the lumen. A study of microRNA in *Muc2*^{-/-} suggested that microRNA related with cytokines is implicated in the malignant transformation of colitis to colorectal cancer¹²¹. Recently, it was shown that probiotics could reduce low-grade inflammation and improve intestinal barrier function in these mice¹²². Taken together, these results demonstrate that *Muc2* plays a major role in intestinal homeostasis.

2.7 Hypothesis and specific aims

As established earlier, both the MUC2 mucus layer and the microbiota are key elements in maintaining intestinal health. Microbiota and mucus are in constant interaction with each other. Mucus feeds, houses and protects bacteria, while bacteria stimulate mucus production and degradation as part of the normal mucus turnover process. To date, several studies have separately characterized the role of these processes in different pathological conditions. Despite the information we have of both these components of the intestinal barrier, little is known about how

the absence of a mucus barrier affects microbial population. Similarly, it is still unclear if an altered bacterial composition could affect the protective role of a functional mucus barrier. This thesis is based on *in vivo* experiments using *Muc2^{-/-}* and *Muc2^{+/+}* littermates, to normalize the microbiota and, at the same time, to look at the functional consequence the absence/presence of a functional mucus barrier has. The primary goal of this study was to characterize the role of both components (mucus and microbiota) in regard to intestinal injury, by using chemical and pathogen-induced colitis models.

HYPOTHESIS

The loss of Muc2 alters colonic microbiota and epithelial barrier function in innate host defense against colonic injury.

SPECIFIC AIMS

1. To delineate the distinct functional roles that an intact Muc2 barrier and microbiota play in onset, progression and recovery of colonic injury using dextran sulphate sodium (DSS) - induced colitis.
2. To investigate if *Muc2^{-/-}* mice possess a different colonic bacterial composition than *Muc2^{+/+}* littermates and to determine if this microbiota is responsible for *Muc2^{-/-}* constitutive inflammatory conditions and/or is colitogenic to *Muc2^{+/+}* littermates.

3. To determine the distinct roles of the colonic microbiota and the mucus layers in host defense against the protozoan parasite *E. histolytica*

2.8 References

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Chapter Three: **Manuscript I**

Muc2 mucin and non-mucin associated microbiota confer distinct innate host defenses in disease
susceptibility and intestinal homeostasis

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3.1 Abstract

Alterations in intestinal MUC2 mucus and microbial diversity are closely linked with IBD. However, their impact on each other and on IBD pathogenesis is vaguely characterized. To investigate these alterations, we used Muc2 mucin deficient and sufficient littermates as a model system. Although, *Muc2*^{+/+} and *Muc2*^{-/-} littermates share similar phyla distribution as evidenced by 16S sequencing they maintain their distinctive gastrointestinal phenotypes. Basally, *Muc2*^{-/-} showed low-grade colonic inflammation with higher populations of inflammatory and tolerogenic immune cells that became comparable to *Muc2*^{+/+} littermates following antibiotic treatment. Antibiotics treatment rendered *Muc2*^{+/+} but not *Muc2*^{-/-} highly susceptibility to DSS-induced colitis that was ILC3 dependent. *Muc2*^{+/+} receiving *Muc2*^{-/-} fecal microbial transplants exhibited a shift in unique bacteria families with worse outcomes to DSS and *Citrobacter rodentium*-induced colitis. These results highlight distinct roles for Muc2 mucus and microbiota in shaping innate host defenses and intestinal homeostasis.

3.2 Keywords

Muc2, microbiota, colitis, dysbiosis, mucus

3.3 Introduction

A diverse population of microorganisms, collectively called the “gut microbiota” have adapted to live symbiotically in/on the intestinal mucus surface ¹. Gut microbiota, as revealed by fecal microbiota transplantation (FMT) and experiments with germ-free (GF) animals, play a critical role in host nutrition ^{2,3} and immune-modulation ⁴⁻⁶. Their effects are largely dependent on

complex interactions with host immune cells and any imbalance in those interactions result in the development of disease ⁷.

It has been estimated that approximately 100 billion bacteria of around 500 to 1,000 species ^{2,8}, encompassing 7-9 phyla ⁹ are present in the human gut. This huge diversity of gut microbiota largely dictates the outcome of host-microbiota interactions, which has been revealed by 16S rRNA sequencing and metagenomic analysis of fecal samples from healthy and IBD patients ^{10,11}. Although, genetic and environmental factors largely determine gut microbial diversity ^{2,4}, 90% of gut bacteria belong to the phyla *Firmicutes* and *Bacteroidetes* in healthy individuals ^{12,13}. However, among IBD patients, reduced biodiversity (alpha diversity) with concomitant rise in pathobionts have been found, resulting in impaired activation of host immune system ¹⁴⁻¹⁸. Even though a reduction in *Firmicutes* and rise in *Proteobacteria* is consistently seen in IBD patients ^{19,20}, there is no direct causal association between specific pathogens and the development of IBD ^{19,21}.

The intestinal mucus layer is a polymeric sheet of densely glycosylated MUC2 mucin that spatially separates microbial population from underlying intestinal epithelial cells (IECs) ^{22,23}. Depletion of the mucus layer is one of the most common characteristics in IBD patients allowing gut microbial components to come in close contact with host cells and to elicit unregulated inflammation ²⁴. However, it is uncertain whether depletion of the mucus layer is a risk factor or a result of colonic inflammation in IBD patients ²⁵. Findings by others and us have reaffirmed the importance of the mucus layer in maintaining homeostasis between gut microbiota and the host immune system ^{24,26,27}.

Dysbiotic microbiota in IBD, have been found to be associated with an increased population of sulfate-reducing bacteria (SRB) that cause colonic inflammation by producing toxic hydrogen sulfide ²⁸. Detailed microbial profiling using healthy and IBD patients have revealed the involvement of several bacterial species in colonic inflammation; e.g. *Campylobacter* ²⁹, *Mycobacterium* ³⁰, *C. difficile* ³¹, *Helicobacter* ³² and *Bacteroides fragilis* ³³. Furthermore, a higher number of adherent-invasive *E. coli* isolates have been observed in rectal biopsies from IBD patients ^{34,35}, suggesting involvement of mucus layer depletion in IBD pathogenesis. Recent studies have suggested specific groups of bacteria and their metabolites may have potent immunomodulatory effects on the host immune system ³⁶⁻³⁸. Reportedly, *Lactobacillus reuteri* can induce development of gut epithelial CD4⁺CD8 α ⁺ T-cells ³⁹, *Clostridium* species induce Tregs induction ^{40,41} and robust Th17 induction by c(SFB) ⁴². Short chain fatty acids (SCFAs) are the main metabolite derived from gut microbiota which can modulate various immune function ⁴³, Tregs development ⁴⁴ and induction of anti-inflammatory IL-10 secreting Th1 and Th17 T-cells ⁴⁵. Indole, a bacterial metabolite and tryptophan derivative, can attenuate inflammation and improve intestinal permeability ^{46,47}. Thus, gut microbiota plays an immense role in the development of host immune system and any alteration in microbial dynamics can predispose the host towards aggravated disease ⁷.

Even though there is ample evidence to verify the close association of microbiota and the mucus layer to IBD pathogenesis, the exact role of each other on events responsible for the initiation and course of IBD is not clear. We hypothesised that these two factors separately or together, can aggravate colonic inflammation to predispose the host towards IBD. Here we used Muc2 mucin

deficient and sufficient littermates to highlight the impact of the mucus layer on gut microbial dynamics and their collective influence on disease susceptibility, onset, progression and recovery during colonic injury.

3.4 Results

3.4.1 Muc2 mucin deficient animals exhibit differential gut bacterial composition that drives constitutive colonic inflammation

Since the mucus layer supports growth and maintenance of gut microbiota we hypothesized that *Muc2*^{-/-} will have an altered gut bacterial composition than *Muc2*^{+/+} animals. To investigate this, we first characterized and compared the microbial compositions from fecal samples from commercially bought WT C57BL/6 mice (Charles River) and in-house bred *Muc2*^{-/-} on the same genetic background. 16s rRNA sequencing analysis demonstrated distinct bacterial community in WT C57BL/6 compared to in-house bred *Muc2*^{-/-} (Supplementary Fig 3-1 A). Expectedly, Firmicutes and Bacteroidetes phyla represented around 90% of bacterial population in both genotypes, however *Muc2*^{-/-} showed higher abundance in Bacteroidetes occupying ~50% of bacterial population in comparison to the ~20% present in WT C57BL/6 (Supplementary Fig 3-1 A). In contrast, concomitant reduction in Firmicutes (almost 50%) and higher levels of Tenericutes (three-fold) was noted in *Muc2*^{-/-} compared to WT. Treatment with a broad-spectrum antibiotic cocktail ⁴⁸ decreased bacterial numbers up to 80-fold (Supplementary Fig 3-1 C). Following antibiotics treatment, disruption and a shift in bacterial populations was observed, with decrease Firmicutes and absence of Bacteroidetes along with a slight increase in Proteobacteria

(Supplementary Fig 3-1 A). Bray-Curtis PCoA analysis showed differences in β -diversity among the treatments in both genotypes, with a clear separation in diversity between control and antibiotic treated, demonstrating a shift in bacterial diversity (Supplementary Fig 3-1 B).

To rule out effects of genetic changes to the phenotype of *Muc2*^{-/-}, we generated *Muc2*^{+/+} and *Muc2*^{-/-} littermates using C57BL/6 (Charles River) and in-house *Muc2*^{-/-}. Expectedly, 16s rRNA sequencing data revealed a normalized bacterial phyla distribution among *Muc2*^{+/+} and *Muc2*^{-/-} littermates, with no differences at the phyla level (Fig 3-1A, lanes a & c). Both genotypes included the phyla Firmicutes, Bacteroidetes, Tenericutes, Proteobacteria and Actinobacteria in similar proportions. Furthermore, alpha and beta diversity analysis among the littermates showed similar species richness, as measured by Shannon's index and non-metric multidimensional scaling (NMDS) (Fig 3-1 B). However, we observed significantly distinct species diversity (Fig 3-1 C) that suggests in the absence of a functional mucus layer, certain bacterial species can thrive more than others and could have detrimental effect on colon health ⁴⁹. Similar to the parental groups (C57BL/6 and in-house bred *Muc2*^{-/-}), antibiotic treatment in *Muc2*^{+/+} and *Muc2*^{-/-} littermates led to a shift in bacterial population and diversity, characterized by a decrease in Firmicutes, absence of Bacteroidetes and an increase in Proteobacteria phyla (Fig 3-1 A, lanes b & d).

In house bred *Muc2*^{-/-} consistently develop mild colitis that may culminate in rectal prolapse around five months of age and are highly susceptible towards chemical, bacterial and parasite-induced colitis ⁵⁰⁻⁵². Thus, to investigate the role of microbiota in disease pathogenesis, *Muc2*^{+/+} and *Muc2*^{-/-} littermates were treated with antibiotics and colonic pro-inflammatory cytokines

transcript levels were analyzed. Interestingly, under basal conditions, *Muc2*^{-/-} expressed significantly higher colonic expression of TNF- α and IL-1 β as compared to *Muc2*^{+/+} littermates that were normalized following antibiotic treatment (Fig 3-1 D). To quantify whether the pro-inflammatory cytokines were altering epithelial barrier function, intestinal permeability was assessed with fluorescein isothiocyanate (FITC) dextran. As predicted, *Muc2*^{-/-} showed increased intestinal permeability by the presence of increased FITC levels in peripheral blood as compared to *Muc2*^{+/+} littermates. However, following antibiotics, intestinal permeability was restored almost to the levels of *Muc2*^{+/+} littermates (Fig 3-1 E). Based on these results and with the knowledge that pro-inflammatory cytokines can alter tight junction (TJ) permeability⁵³, we measured TJ expression in untreated and antibiotic treated mice. Not surprising, *Muc2*^{-/-} showed decreased occludin and claudin 2 basal expressions that were significantly increased following antibiotic treatment (Fig 3-1 F). These results suggest that *Muc2*^{-/-} microbiota plays an important role in promoting/driving basal pro-inflammatory responses.

Accordingly, we next determined if colonic bacteria was directly involved in eliciting colonic pro-inflammatory responses and alterations in TJ by performing fluorescent *in situ* hybridization (FISH) to visualize bacterial localization. As expected, *Muc2*^{+/+} with an intact mucus layer physically separated epithelial cells and colonic microbiota, whereas in *Muc2*^{-/-} littermate's bacteria were in direct contact with the colonic epithelium (arrows, Fig 3-1 G). To quantify if gut bacteria in contact with the epithelium caused the recruitment of immune cells to clear the infection, immunophenotyping was done using lamina propria cells (LPLS) to quantify different immune cells populations (Supplementary Fig 3-1 D). We found significantly higher CD3⁺

T-cells populations and lower CD19⁺ B-cells populations in the colon of *Muc2*^{-/-} as compared to *Muc2*^{+/+} littermates (Fig 3-1 H). Taken together, these results show that depletion of the mucus barrier supports the growth of unique bacterial species that cause colonic inflammation in contact with the surface epithelium recruiting immune cells at the site of inflammation.

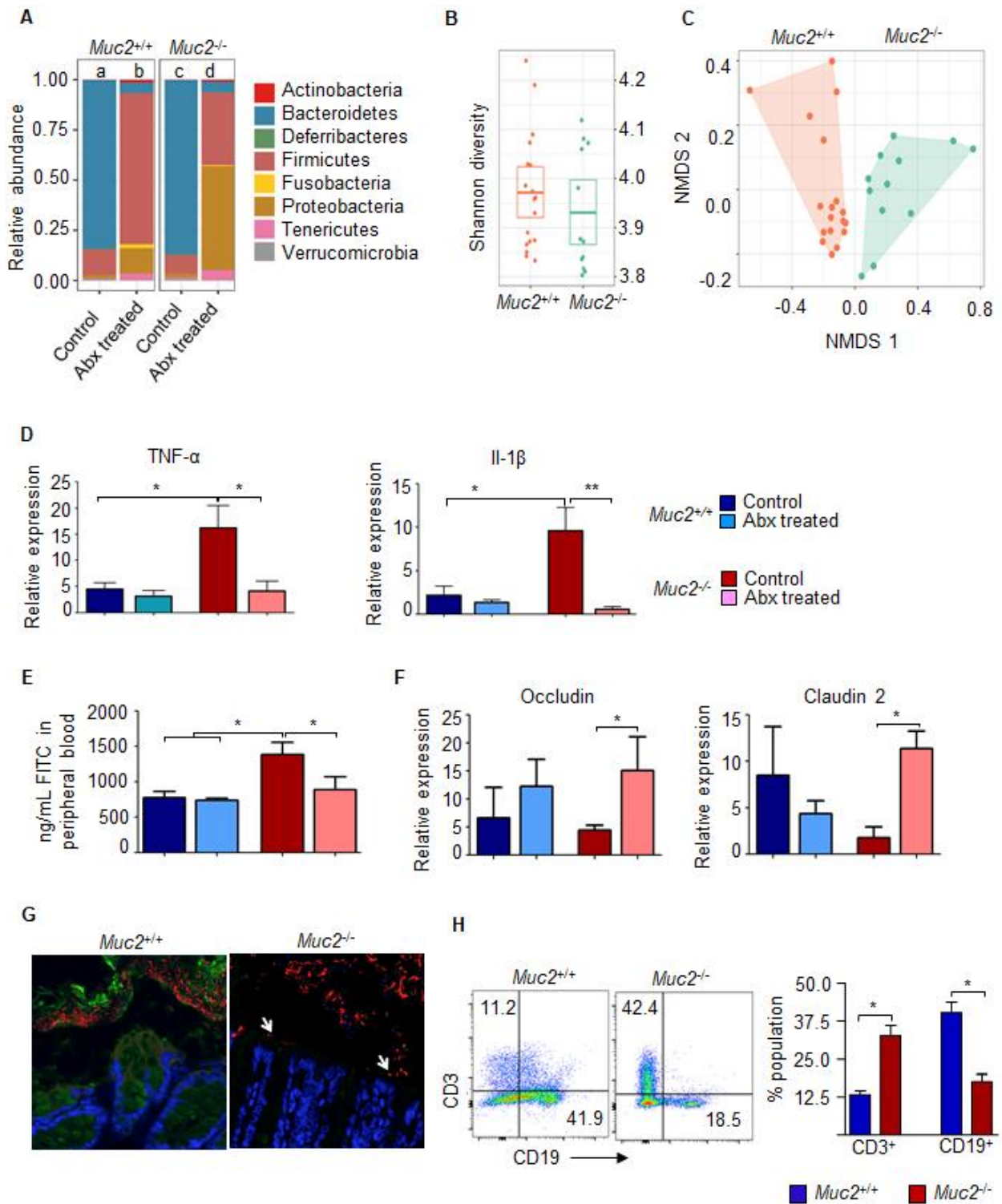


Figure 3- 1 *Muc2*^{-/-} microbiota drives inflammation and increased gut permeability that becomes normalized following antibiotic treatment. **A.** 16s rRNA Illumina sequencing of *Muc2*^{-/-} and *Muc2*^{+/+} littermates stool microbiota before and after treatment for 17 days with an antibiotic (Abx) cocktail. Bacterial phyla abundance of untreated *Muc2*^{+/+} (a) and *Muc2*^{-/-} (c) are very similar, with a predominance of Bacteroidetes and Firmicutes phyla. Antibiotics treatment disrupted bacterial population (b, d), with a decrease in Firmicutes and disappearance of Bacteroidetes and an increase in Proteobacteria. **B.** Alpha diversity as measured by Shannon's index showed equivalent species richness between *Muc2*^{+/+} and *Muc2*^{-/-} littermates, while a significant difference in beta diversity between the two untreated genotypes was evident (**C**), demonstrating that *Muc2*^{+/+} and *Muc2*^{-/-} have similar bacterial richness but different bacterial diversity. Permutational multivariate analysis of variance (PERMANOVA), NMDS: non-metric dimensional scale. Colonic tissues samples from *Muc2*^{-/-} and *Muc2*^{+/+} mice were analyzed by qPCR for a comparison of pro-inflammatory cytokines and tight-junction protein expression before and after antibiotics. **D.** *Muc2*^{-/-} mice show low-grade inflammation with significantly higher TNF- α and Il-1 β as compared to untreated *Muc2*^{+/+}. Antibiotic treatment significantly decreased the pro-inflammatory cytokines in *Muc2*^{-/-}. Antibiotic treated *Muc2*^{-/-} showed reduced constitutive intestinal permeability as measured by FITC dextran (**E**), with a correspondent increase in tight junction proteins occludin and claudin 2 (**F**). Abx: antibiotics. **G.** Representative images of fluorescence *in situ* hybridization (FISH) using the universal bacterial EUB338-Quasar 670 probe in mouse colonic tissue showing bacteria localization in the luminal side in both mice, UEA lectin-FITC conjugated was used to visualize mucus. The section was counterstained with DAPI to visualize epithelial cells nuclei. In *Muc2*^{+/+} bacteria (in red) is present within the mucus layer (green), whereas, in *Muc2*^{-/-} mice the absence of the mucus layer allowed bacteria to come in close contact with the intestinal epithelium (white arrows). 60x magnification. **H.** Comparison of colonic lamina propria CD3⁺ T cells and CD19⁺ B cells at basal level with *Muc2*^{-/-} mice presenting higher basal levels of T cells and lower levels of B cells in the lamina propria as compared to *Muc2*^{+/+} littermates. n=4-6, *P< 0.05, **P< 0.01.

3.4.2 Muc2^{+/+} but not Muc2^{-/-} microbiota is required for protection against DSS-induced colitis

To investigate whether the microbiota from *Muc2^{+/+}* and *Muc2^{-/-}* have differential protective effects against colonic damage, animals were treated with an antibiotic cocktail and challenged with DSS (Fig 3-2 A). Surprisingly, *Muc2^{+/+}* but not *Muc2^{-/-}* mice became highly susceptible to DSS-induced colitis following antibiotics treatment. Antibiotic treated *Muc2^{+/+}* mice lost 10-15% of body weight as early as day 4, and by day 6 mortality rate reached 100% (Fig 3-2 B). In contrast, antibiotic treated *Muc2^{-/-}* mice loss about 10% body weights between days 7 and 9 (Fig 3-2 C). These results suggest that *Muc2^{+/+}* littermates with an intact mucus layer and a healthy microbiota confer a protective role against colonic injury. Paradoxically, for mice lacking a mucus barrier and with dysbiotic microbiota that drives low-grade inflammation, alteration of microbiota was protective against colonic injury. We hypothesized that the low-grade inflammation in *Muc2^{-/-}* littermates driven by dysbiotic microbiota induces immuno-tolerance at colonic site that could be protective against further colonic injury. IL-22 cytokine, produced by intestinal innate lymphoid cells (ILCs), is implicated to induce immuno-tolerance in the gut and might be involved in conferring a protective role to *Muc2^{-/-}* littermates. To test this hypothesis, we isolated colonic LPLs from *Muc2^{+/+}* and *Muc2^{-/-}* and determined IL-22 production by ILCs using flow cytometry. Basally, *Muc2^{-/-}* mice had higher populations of ILC3 positive for IL-22 as compared to *Muc2^{+/+}* littermates (Fig 3-2 D). Moreover, antibiotics treatment showed an increased in IL-22⁺ ILC3 populations in *Muc2^{-/-}* but not in *Muc2^{+/+}* littermates (Fig 3-2 D). ILC3 are IL-22 producing cells involved in maintaining intestinal epithelial cells homeostasis through the induction of cellular proliferation and the production of antimicrobial peptides⁵⁴. The significant increase of IL-22⁺ ILC3 population

in antibiotics treated *Muc2*^{-/-} animals could explain why these mice have higher survival following DSS induced colonic injury.

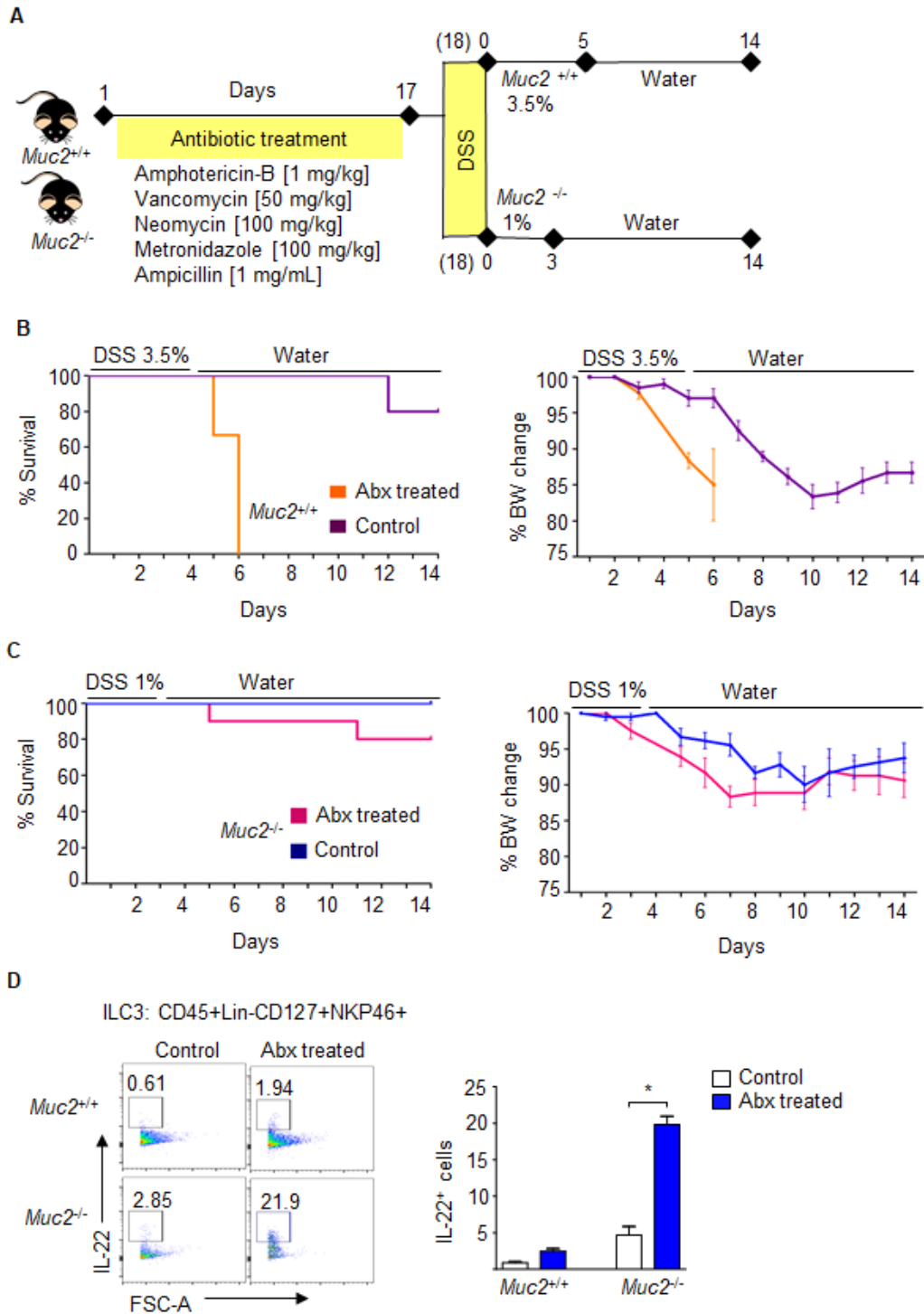


Figure 3- 2 DSS is lethal to antibiotic treated *Muc2*^{+/+} but not *Muc2*^{-/-} mice. **A.** Diagrammatic work plan, briefly, *Muc2*^{+/+} and *Muc2*^{-/-} littermates received an antibiotic cocktail for 17 days and 1 day after were given DSS in drinking water. *Muc2*^{+/+} received 3.5% DSS for 5 days while *Muc2*^{-/-} received 1% DSS for 3 days, followed by *ad libitum* water. Mice were sacrificed on day 14 following DSS treatment. Control non-antibiotic treated mice received the same % of DSS. **B.** Antibiotic treated *Muc2*^{+/+} were highly susceptible to DSS; these mice lost weight faster and reached 100% mortality by day 6 as compared with untreated *Muc2*^{+/+} subject to the same DSS regime. **C.** In *Muc2*^{-/-} littermates no significant changes in DSS susceptibility were observed between animals that received antibiotics or not. **D.** Isolated lamina propria cells from *Muc2*^{+/+} and *Muc2*^{-/-} littermates were characterized by flow cytometry. IL-22 antibody was used to characterize ILC3 cell population. n=4-6, *P< 0.05. BW: body weight, DSS: dextran sulfate sodium, Abx: antibiotics.

3.4.3 Gut microbiota from *Muc2*^{-/-} littermates aggravates DSS-induced colitis and mortality in mice with an intact mucus layer

Intestinal microbiota play an important role in the development of IBD and could be the trigger for aberrant immune responses in the gut. In support of this, gut microbiota from ulcerative colitis (UC) patients was recently shown to induce inflammation and increase susceptibility to colitis in naïve animals⁵⁵. To investigate if *Muc2*^{-/-} microbiota could affect colonic health and increase susceptibility towards colitis in *Muc2*^{+/+} littermates, fecal microbiota transplants (FMT) was given to antibiotic treated *Muc2*^{+/+} or *Muc2*^{-/-} (homologous and heterologous) littermates using stool from untreated age matched and sex matched controls. Subsequently, FMT animals were given DSS to determine susceptibility towards chemically induced colitis (experimental plan, Fig 3-3

A). As revealed by 16s rRNA sequencing, gut bacterial dynamics was restored by FMT in both genotypes (Fig 3-3 B) induced by antibiotics administration (Fig 3-1 B). Expectedly, all FMT groups showed an increase in *Bacteroidetes* abundance but slightly lower than the untreated groups (Fig 3-1 B). Interestingly, in both genotypes receiving *Muc2*^{-/-} FMT, Proteobacteria abundance was increased compared with those that received *Muc2*^{+/+} FMT (Fig 3-3 B). This was confirmed by non-metric multidimensional scaling (NMDS) plot, which showed a clear shift in bacterial populations in both genotypes after antibiotic treatment as well as after receiving FMT (both homologous and heterologous) compared to untreated control (Fig 3-3 C). We next investigated if FMT from untreated *Muc2*^{-/-} and *Muc2*^{+/+} littermates was protective in antibiotics treated animals. Interestingly, *Muc2*^{+/+} receiving *Muc2*^{-/-} but not *Muc2*^{+/+} FMT exhibited significantly high rates of mortality and reduced recovery following DSS induced colitis (Fig 3-3 D). Notably, survival of antibiotic treated *Muc2*^{+/+} animals during DSS treatment was restored after receiving their own microbiota indicating a protective effect of a healthy gut microbiota (Fig 3-2 B). Surprisingly, *Muc2*^{+/+} FMT had no effect on *Muc2*^{-/-} survival and body weight loss upon DSS challenge (Fig 3-3 E). This was further confirmed by disease activity index (DAI; Fig 3-3 F) and intestinal permeability (Fig 3-3 G), where *Muc2*^{+/+} receiving *Muc2*^{-/-} FMT showed significantly higher DAI and intestinal permeability than the other groups.

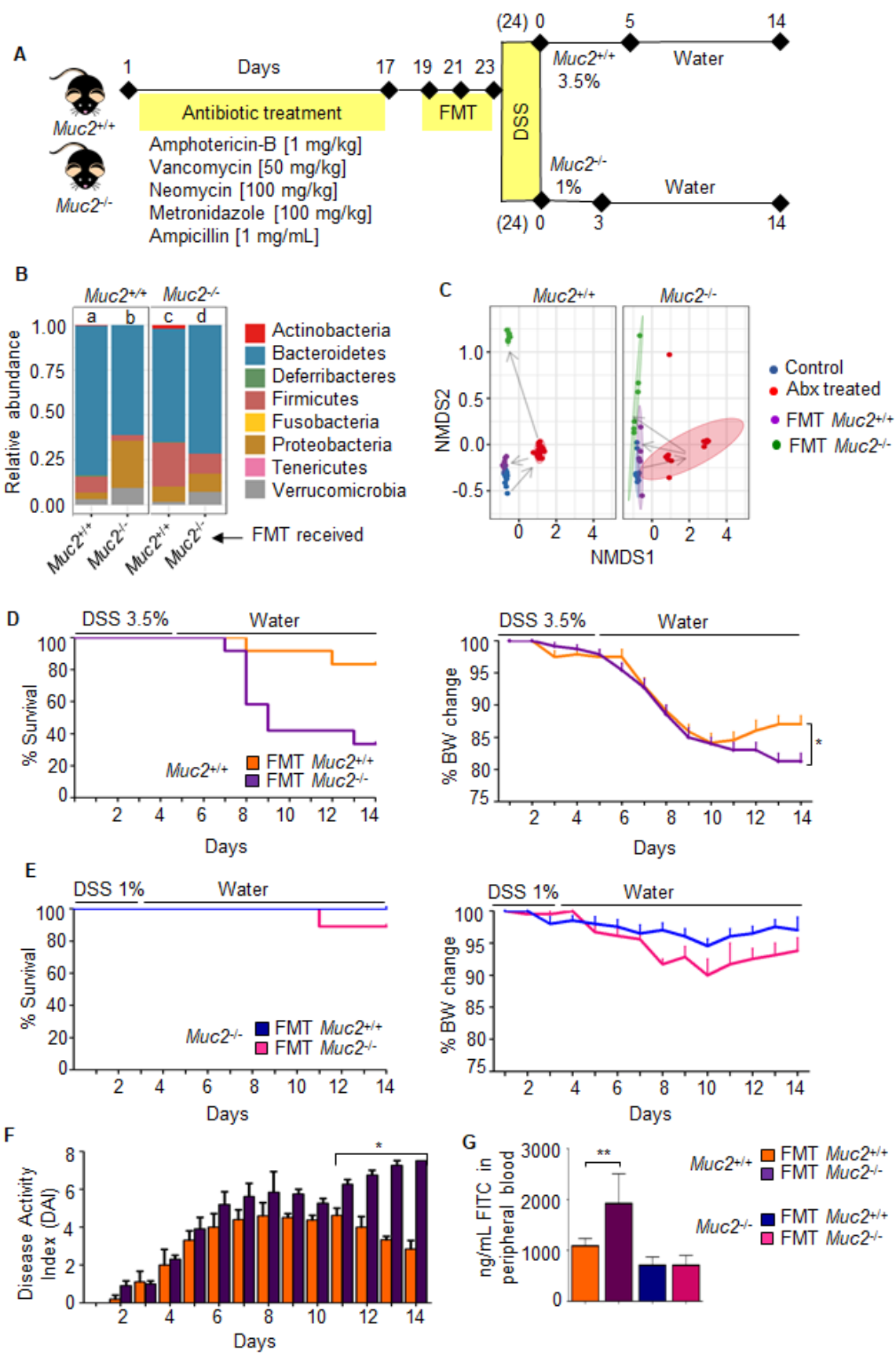


Figure 3- 3 *Muc2*^{-/-} microbiota increases susceptibility to DSS-induced colitis in *Muc2*^{+/+} littermates. **A.** Diagrammatic work plan, briefly, *Muc2*^{+/+} and *Muc2*^{-/-} antibiotic treated mice received fecal microbiota transplantation (FMT) every other day before giving DSS in drinking water. *Muc2*^{+/+} received 3.5% DSS for 5 days while *Muc2*^{-/-} received 1% DSS for 3 days, followed by *ad libitum* water. Mice were sacrificed on day 14 after following DSS treatment. **B.** 16s rRNA sequencing comparison of bacterial phyla abundance in *Muc2*^{+/+} and *Muc2*^{-/-} littermates receiving either homologous or opposite genotype FMT. **C.** Nonmetric multidimensional scaling (NMDS) analysis of samples was performed to plot population distribution between each sample, where a clear separation between groups is seen. **D.** Antibiotic treated *Muc2*^{+/+} mice receiving *Muc2*^{-/-} FMT showed increased mortality to DSS induced colitis and the surviving animals did not recover weight up to day 14. **E.** *Muc2*^{-/-} mice receiving *Muc2*^{+/+} FMT showed less severe illness and no mortality in response to DSS induced colitis. **F.** *Muc2*^{+/+} mice receiving *Muc2*^{-/-} FMT showed an increased in disease index activity (DAI) compared with those who received their own. **G.** On day 14, *Muc2*^{+/+} receiving *Muc2*^{-/-} FMT showed increased intestinal permeability as compared to *Muc2*^{+/+} receiving their own microbiota. n=4-6, *P< 0.05, **P< 0.01, FMT: fecal microbiota transplantation, DSS: dextran sulfate sodium, BW: body weight, FITC: fluorescein isothiocyanate.

Histopathology showed that colon samples of *Muc2*^{+/+} receiving *Muc2*^{-/-} microbiota (Fig 3-4 C, E and G) had significantly higher numbers of ulcerated lesions and damage to crypt architecture as compared with untreated *Muc2*^{+/+} (Fig 3-4 A) and *Muc2*^{+/+} mice receiving their own microbiota (Fig 3-4 D and F). At the height of disease on day 7 post DSS exposure, colonic tissues from *Muc2*^{+/+} receiving FMT (homologous or heterologous) showed a thicker muscularis and loss of crypts architecture (Fig 3-4 B and C). On examination of Swiss roll sections, *Muc2*^{+/+} receiving *Muc2*^{-/-} microbiota, showed marked damage throughout the colon length (Fig 3-4 E), while those that received their own microbiota, presented less damage in the proximal than the distal colon (Fig 3-4 D). On day 14, *Muc2*^{+/+} receiving *Muc2*^{-/-} FMT presented with worst histopathology (Fig 3-4 G), characterized by a thick muscularis mucosa and no signs of crypts regeneration whereas *Muc2*^{+/+} receiving their own FMT showed numerous crypt regenerating with epithelial cell proliferation (Fig 3-4 F). While day 7 histopathology was comparable in *Muc2*^{+/+} irrespective of the FMT source, at day 14 of disease restitution, *Muc2*^{+/+} that received *Muc2*^{-/-} FMT had significantly higher histological scores suggesting impaired recovery in the survivors (Fig 3-4 H). These results highlight that microbiota from *Muc2*^{-/-} when transplanted to healthy host (*Muc2*^{+/+}) increases disease susceptibility and impaired tissue healing and recovery following colonic injury.

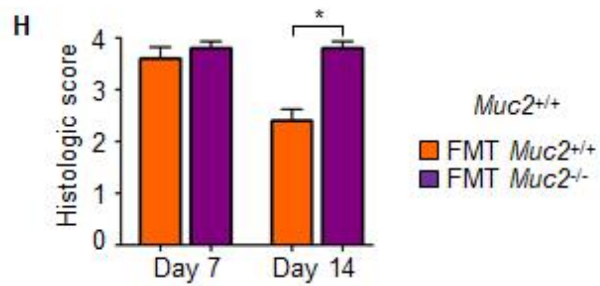
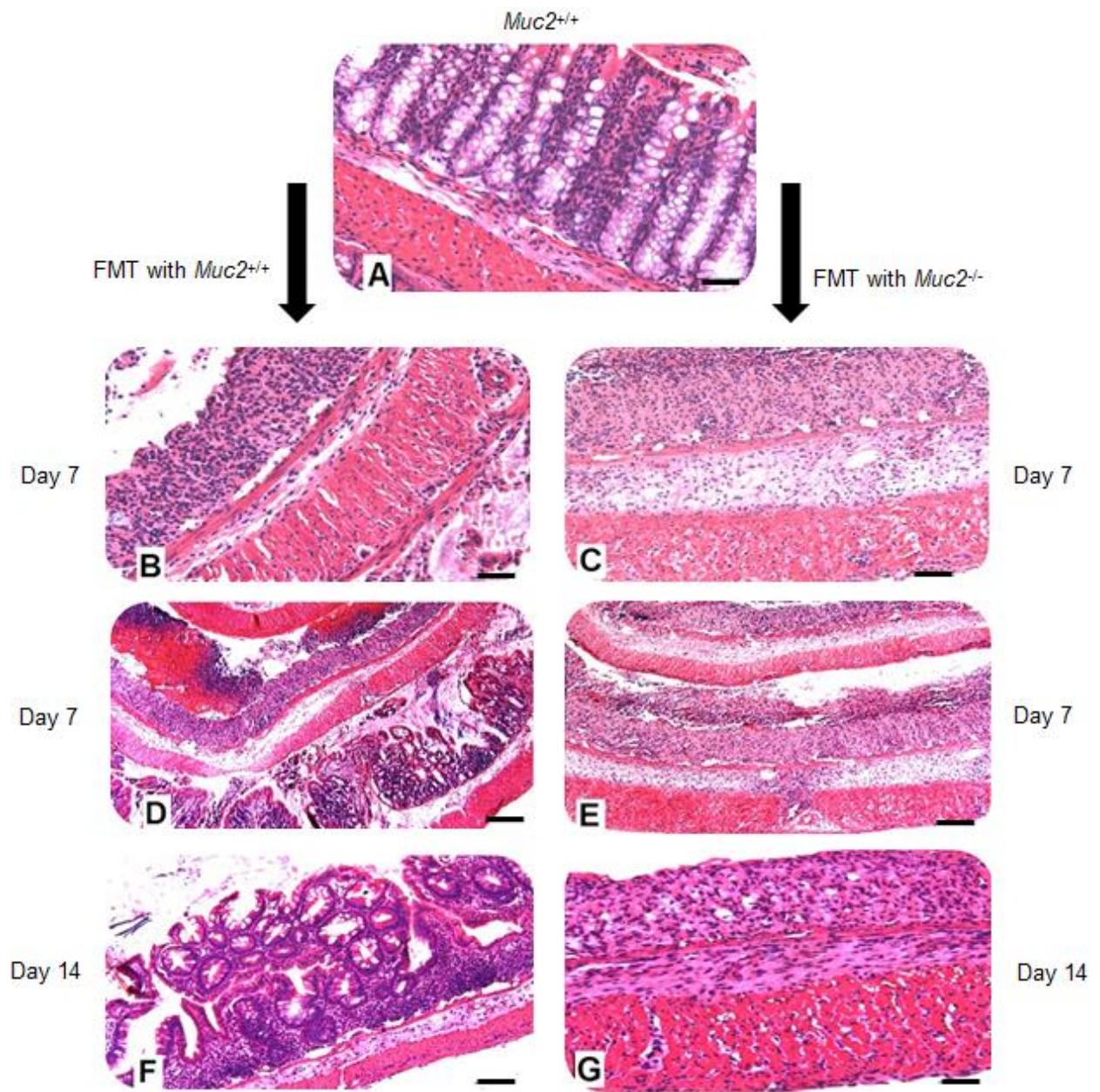


Figure 3- 4 *Muc2*^{+/+} receiving *Muc2*^{-/-} FMT impaired tissue healing and crypts regeneration following DSS-induced colitis. **A.** Hematoxylin and eosin stained Swiss-rolled colons from untreated control showing normal architecture with crypts filled with goblet cells. **(B, D & F)** *Muc2*^{+/+} receiving *Muc2*^{+/+} FMT and, **(C, E & G)** *Muc2*^{+/+} receiving *Muc2*^{-/-} FMT. Note loss of crypts architecture, ulcerated lesions and thickness of the muscularis in both *Muc2*^{+/+} receiving *Muc2*^{+/+} FMT **(B)** and *Muc2*^{+/+} receiving *Muc2*^{-/-} FMT **(C)** microbiota at day 7. Swiss roll sections showed that *Muc2*^{+/+} receiving *Muc2*^{+/+} FMT lesions were restricted to the mid and distal colon **(D)**. *Muc2*^{+/+} receiving *Muc2*^{-/-} FMT showed more intense tissue damage with complete loss of crypts and damage throughout the length of the colon **(E)**. In contrast, at disease resolution on day 14, *Muc2*^{+/+} receiving *Muc2*^{+/+} FMT show signs of crypts restitution and epithelial cell proliferation with a normal muscularis **(F)**, while no crypts regeneration and a thick muscularis were predominant in *Muc2*^{+/+} receiving *Muc2*^{-/-} FMT **(G)**. Scale bar: 50 μ m in A, B, C, F & G, 200 μ m in D & E. H. Blinded histologic score values at day 7 and 14 post DSS. At day 7, the histologic score was comparable between both groups, however, at disease recovery phase on day 14, *Muc2*^{+/+} receiving *Muc2*^{-/-} FMT had significantly higher histologic scores than the group that received homologous FMT. FMT: fecal microbiota transplantation. n=4-6, *P < 0.05.

3.4.4 Presence of tolerogenic immune cells in colonic lamina propria of *Muc2*^{-/-} littermates

We then aimed to explore the mechanisms through which *Muc2*^{-/-} tolerate their dysbiotic and/or colitogenic microbiota to cope with constitutive inflammation without developing raging colitis. A recent study⁵⁶ suggested that *Muc2*^{-/-} mice develop spontaneously colitis due to a reduction in CD103⁺CD11b⁻ and an increase in CD103⁻CD11b⁺ DCs population. Based on this, we characterized different populations of DCs to elucidate if the lack of a mucus barrier and altered microbial populations could induce this characteristic phenotype by changing immune cells

dynamics. Under basal conditions, *Muc2*^{-/-} littermates significantly higher levels of CD45⁺MHCII⁺ cell populations that are predominantly phagocytes (Fig 3-5 A), which could help in clearing incoming microbial penetrants at colonic surfaces. Expectedly, we also observed increased levels of colonic CD103⁺CD11c⁺ (R1) DCs population in *Muc2*^{-/-} (Fig 3-5 B). Furthermore, we quantified CD4⁺FoxP3⁺ cells in small intestine and colonic lamina propria lymphocytes (LPLs) and observed that *Muc2*^{-/-} exhibited higher populations of CD4⁺FoxP3⁺ Tregs population than *Muc2*^{+/+} littermates at basal level (Fig 3-5 C). These results clearly show that the intestinal microbiota in *Muc2*^{-/-} mice induced the recruitment of tolerogenic immune cells at colonic sites which allow them to mitigate the damage caused by higher levels of inflammatory cytokine at basal state.

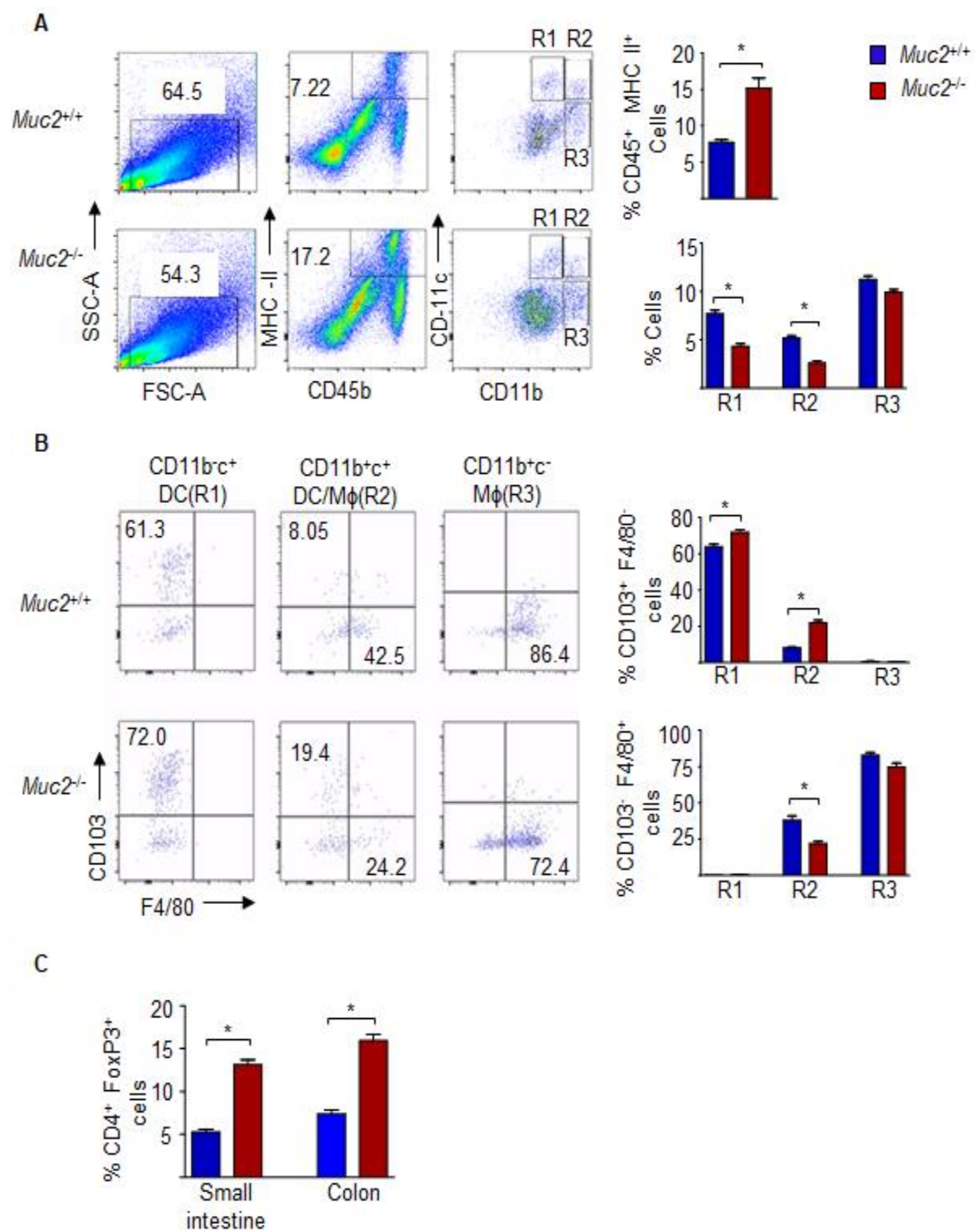


Figure 3- 5 Characterization of dendritic cells (DCs) and macrophages (Mφ) subsets in the colonic lamina propria of *Muc2*^{+/+} and *Muc2*^{-/-} littermates. Lamina propria cells were isolated and stained with antibodies for DCs/Mφ and a viability dye (7-AAD). **A.** FACS plot displaying live cells gated on MHCII and CD45 and then analyzed for CD11b⁺ and CD11c⁺ cells. **B.** Different regions of cells (R1, R2 and R3) expressing various levels of CD11b and CD11c were further gated for CD103 and F4/80. **C.** Increased population of CD4⁺FoxP3⁺ Tregs at basal level in small intestine and colonic lamina propria of *Muc2*^{-/-} mice compared to *Muc2*^{+/+} littermates. n= 3, *P < 0.05.

3.4.5 *Muc2*^{-/-} FMT into *Muc2*^{+/+} littermates increases the translocation of *C. rodentium* in the spleen and mesenteric lymph nodes

Having established that *Muc2*^{-/-} microbiota transplanted into *Muc2*^{+/+} littermates sensitizes the colonic mucosa to chemical-induced colitis, we next investigated whether a similar trend would occur with pathogen-induced colitis with the gram-negative bacteria *Citrobacter rodentium*. In *Muc2*^{+/+} receiving *Muc2*^{-/-} FMT, *C. rodentium* infection at 6 days post infection showed significant bacterial translocation with an increase in colony forming unit (CFU) in the spleen as compared to untreated controls, antibiotic treated and *Muc2*^{+/+} receiving their own FMT. The same trend was observed in *Muc2*^{-/-} mice, however the difference was not significant (Fig 3-6 A). Interestingly, only antibiotic treated *Muc2*^{+/+} showed *C. rodentium* translocation in the MLN. Strikingly, *Muc2*^{-/-} receiving *Muc2*^{+/+} FMT significantly inhibited *C. rodentium* translocation in the MLN whereas, in all the other treatment groups bacterial translocation occurred (Fig 3-6 B). As expected, increased bacterial translocation in mice receiving *Muc2*^{-/-} FMT correlated with

increased levels of serum lipopolysaccharide (Fig 3-6 C) and flagellin antibodies (Fig 3-6 D) in both genotypes.

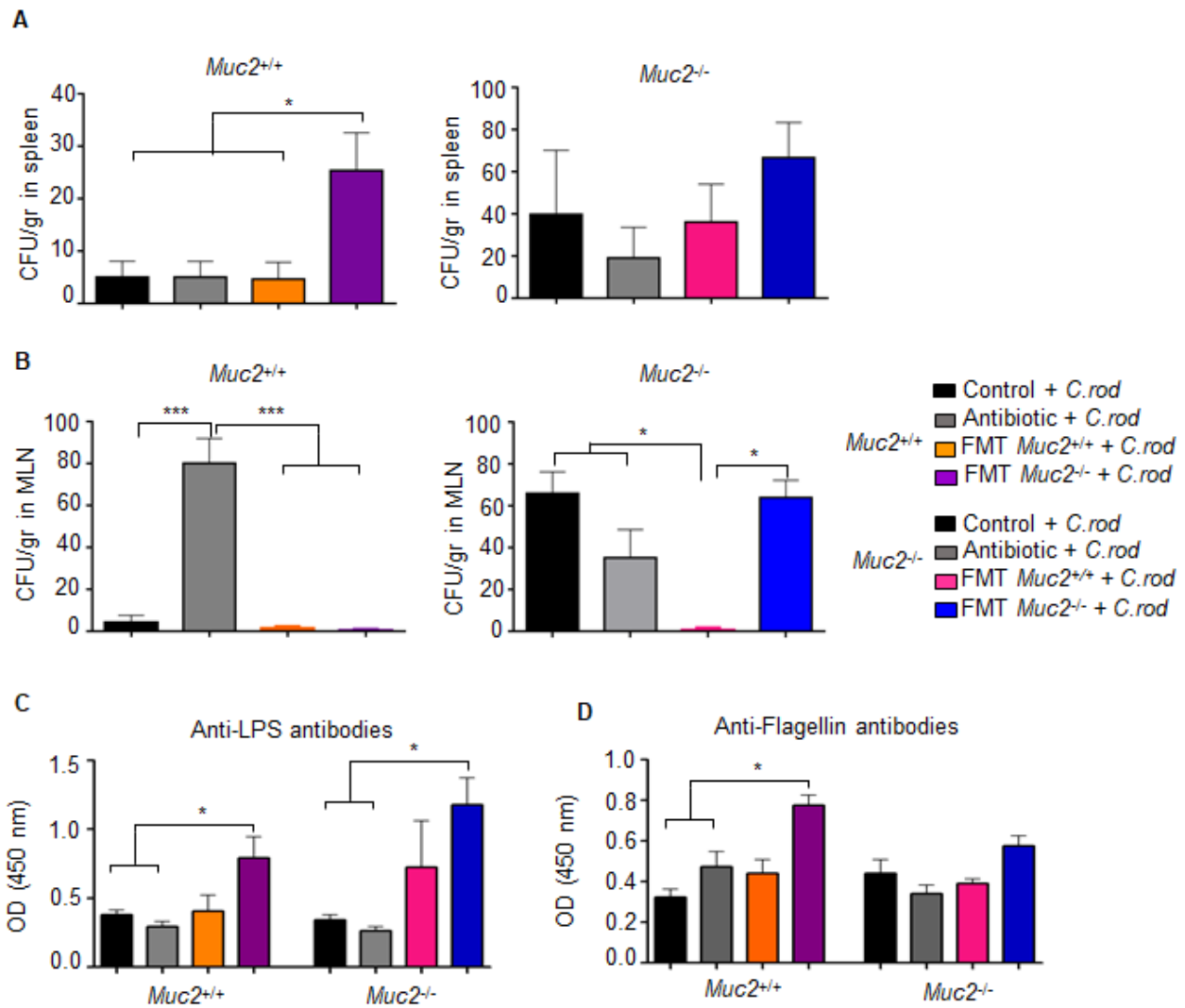


Figure 3- 6 *Muc2*^{+/+} receiving *Muc2*^{-/-} FMT increased *Citrobacter rodentium*-induced colitis and bacterial translocation. *Muc2*^{+/+} and *Muc2*^{-/-} littermates were treated with antibiotics and received FMT either from their own or opposite genotype and were infected with *C. rodentium* and sacrificed 6 days after infection. *C. rodentium* translocation to mesenteric lymph nodes (MLN) (A) and spleen (B) was measured by counts of colony forming unit per gram (CFU/gr) in LB agar with streptomycin plates. Antibodies for lipopolysaccharide (LPS) (C) and Flagellin (D) were detected by ELISA. FMT: fecal microbiota transplant. n=6, *P < 0.05, ***P < 0.001.

3.4.6 Muc2^{-/-} microbiota does not trigger pro-inflammatory responses in Muc2^{+/+} littermates

To investigate if *Muc2^{-/-}* microbiota was pro-inflammatory and/or colitogenic to *Muc2^{+/+}* littermates, mice were treated with antibiotics and received FMT from either *Muc2^{+/+}* or *Muc2^{-/-}* untreated animals. Animals were sacrificed on days 1, 5 and 10 after the last FMT and colon samples were analyzed for pro-inflammatory cytokine mRNA levels. Simultaneously, fecal samples were collected at the same time points and analyzed by 16s sequencing. Animals showed no significant change in body weight (Supplementary Fig 3-2 A) up to 10 days regardless of the FMT they received. No significant difference in pro-inflammatory cytokines IL-1 β , or IFN- γ mRNA levels was observed between *Muc2^{+/+}* receiving *Muc2^{-/-}* FMT or their own microbiota. TNF- α expression in *Muc2^{+/+}* was only significantly different at day 1. *Muc2^{-/-}* receiving *Muc2^{+/+}* FMT, showed a trend towards less pro-inflammatory cytokine expression but the difference was only significant at day 1. A trend in both genotypes receiving *Muc2^{-/-}* FMT to present with higher, but not significant, levels of pro-inflammatory markers was observed (Supplementary Fig 3-2B), however, this did not persist on days 5 and 10. These results clearly show that *Muc2^{-/-}* microbiota *per se* is not colitogenic when transplanted into *Muc2^{+/+}* littermates.

3.4.7 The impact of Muc2^{-/-} microbiota could be explained through OTUs from distinctive families that are transplantable to Muc2^{+/+} littermates

To determine if the biologic effects seen with *Muc2^{-/-}* microbiota over *Muc2^{+/+}* littermates was associated with a putative bacteria group, we performed 16s rDNA-sequencing analysis on day -17 (before antibiotics) and on days 1, 5 and 10 after receiving antibiotics and all three FMT.

On day 1 (Fig 3-7 A, blue dots), both *Muc2*^{+/+} and *Muc2*^{-/-} FMT with their own microbiota (Fig 3-7 A, panel b and c, respectively) displayed a bacterial composition somewhat similar to their pre-antibiotics state (Fig 3-7 A, red dots). However, at this same time point (day 1), a difference in composition was noticeable in *Muc2*^{-/-} receiving *Muc2*^{+/+} FMT (Fig 3-7 A, panel a) and specially contrasting in *Muc2*^{+/+} receiving *Muc2*^{-/-} FMT (Fig 3-7 A, panel d) that corresponded with a significant drop in α -diversity in *Muc2*^{+/+} at day 1 after receiving *Muc2*^{-/-} FMT (Supplementary Fig 3-3 B). When compared with their pre-antibiotics state, these differences are not maintained throughout time since the diversity returns to a more similar pre-antibiotic state as soon as 5 days after the last FMT and α -diversity was similar among treatments (Supplementary Fig 3-3 A). Based on these results, we next aimed to compare microbiota composition between both FMT in *Muc2*^{+/+} (FMT with *Muc2*^{-/-} vs FMT with their own microbiota) at day 1. Results revealed differences in bacterial composition with the ones receiving *Muc2*^{-/-} FMT having up to 6 times more Proteobacteria family Enterobacteriaceae as well as higher amounts of the Verrucomicrobiaceae family (Supplementary Fig 3-3B). In contrast, there was a decrease of the Actinobacteria family Coriobacteriaceae as well as some Proteobacteria families such as Pseudomonadaceae and Desulfovibrionaceae. The Tenericutes family Anaeroplasmataceae and the Deferribacteres family Deferribacteraceae numbers were also decreased, along with some Firmicutes families such as Ruminococcaceae, Lactobacillaceae, Lachnospiraceae, and Bacillaceae and some Bacteroidetes families like Prevotellaceae, Rikenellaceae and particularly S24-7 experienced a decrease in their populations when compared with the ones FMT with *Muc2*^{+/+} microbiota (Supplementary Fig 3-3 B).

OTUs among both FMT in *Muc2*^{+/+} were grouped by family and the five most abundant families were plotted to contrast their abundance per FMT treatment and time points (Figure 7B), these being Bacteroidaceae, Lactobacillaceae, S24-7, Enterobacteriaceae and Verrucomicrobiaceae families. At day 1 post-FMT, a dramatic difference among both FMT treatments was observed, where *Muc2*^{+/+} FMT with *Muc2*^{-/-} showed increase abundance in members of the Bacteroidaceae family (Fig 3-7 B and C), as well as members of the Enterobacteriaceae family (Fig 3-7 B and F) and Verrucomicrobiaceae (Fig 3-7 B and G), in contrast, members of the Lactobacillaceae (Fig 3-7 B and D) and the S24-7 family were reduced in these mice (Fig 3-7 B and E). *Muc2*^{-/-} FMT with their own microbiota also showed an increase in the Bacteroidaceae (Fig 3-7 C), Enterobacteriaceae (Fig 3-7 F) and Verrucomicrobiaceae (Fig 3-7 G) families. It is noteworthy that these changes in bacterial dynamics are not permanent since 10 days after the last FMT the differential abundance came back to their normal state. These results give strong evidence that bacteria belonging to the families previously mentioned played a primordial role in mucosal sensitization to DSS and infectious colitis when *Muc2*^{+/+} received *Muc2*^{-/-} FMT.

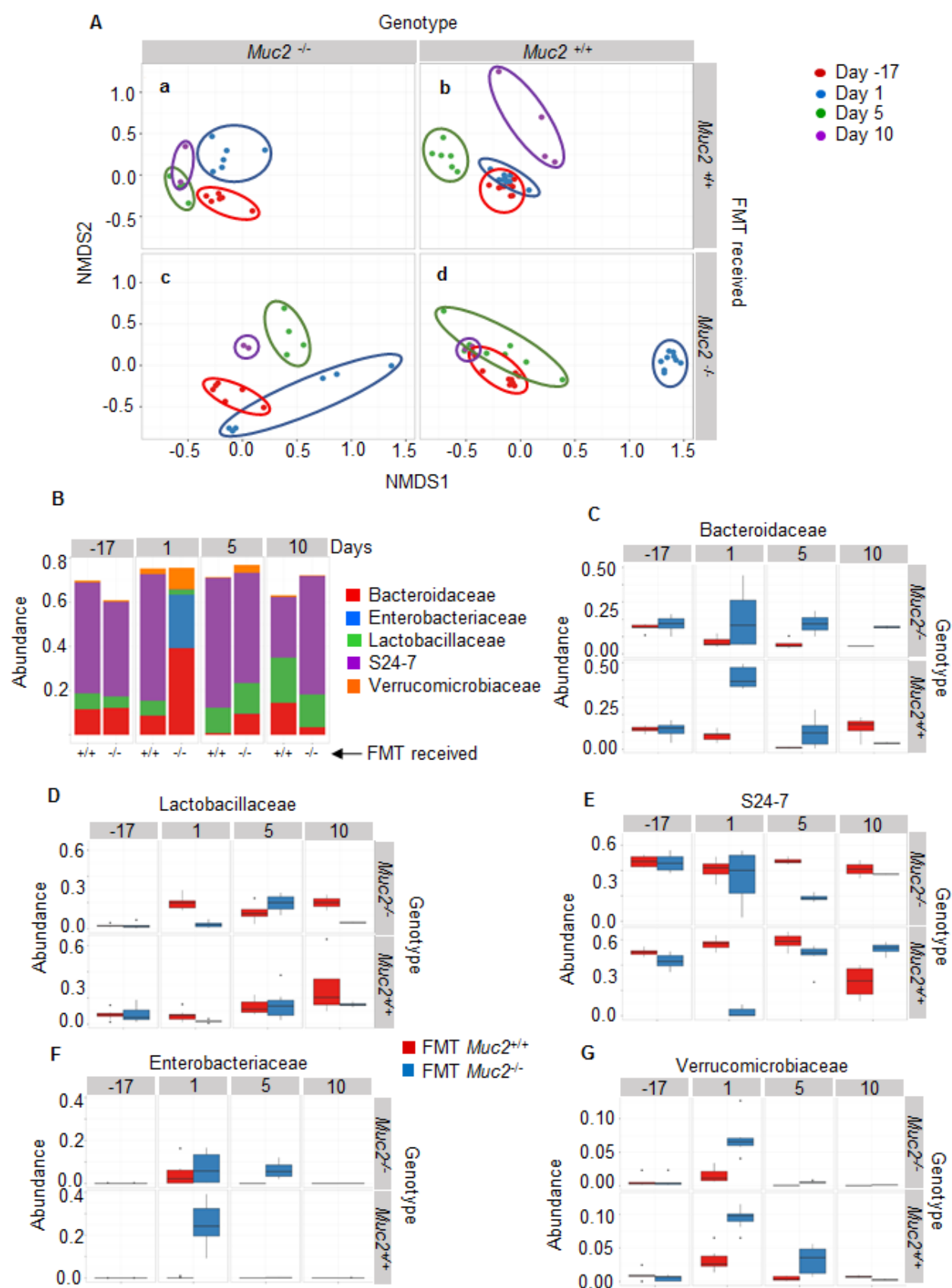


Figure 3- 7 The impact of *Muc2*^{-/-} microbiota could be explained through OTUs from distinctive families that are transplantable to *Muc2*^{+/+} littermates. A. Dissimilarity measured by non-metric multidimensional scaling (NMDS) illustrates the distance throughout samples that were antibiotic treated and FMT with *Muc2*^{-/-} and *Muc2*^{+/+} microbiota, time points are as follow: day -17: before antibiotic treatment, day 1, 5 and 10 correspond to days passed after the last FMT. (a) *Muc2*^{-/-} FMT with *Muc2*^{+/+}, (b) *Muc2*^{+/+} FMT with *Muc2*^{+/+}, (c) *Muc2*^{-/-} FMT with *Muc2*^{-/-}, (d) *Muc2*^{+/+} FMT with *Muc2*^{-/-}. **B.** Significantly different OTUs between *Muc2*^{+/+} FMT with *Muc2*^{-/-} and *Muc2*^{+/+} were grouped by Family and the top 5 were plotted in a bar graph to illustrate their difference in abundance. Plots **C, D, E, F** and **G** display the 5 families' dynamics at different time points. FMT: fecal microbiota transplant.

We also used phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt)⁵⁷ to predict metabolic differences and to identify their potentially distinctive functional features among the samples. Metagenomes were predicted from the 16S rRNA data and differences were focused in *Muc2*^{+/+} receiving either *Muc2*^{-/-} or their own FMT (Supplementary Fig 3-4) and Table 3-1. Among them, important pathways related with gram-negative pathogens are proposed to be upregulated in those receiving *Muc2*^{-/-} FMT, such as genes involved in biofilm of *Escherichia coli*, *Pseudomonas aeruginosa* and *Vibrio cholerae*, as well as genes related to lipopolysaccharide (LPS) biosynthesis, flagellar assembly and glutathione metabolism. Genes that were downregulated in *Muc2*^{+/+} receiving *Muc2*^{-/-} FMT included some involved in methane, carbon and butyrate metabolism. Therefore, we were interested in exploring the contributions of taxa to each of the predicted pathways across the sample groups. Several families within different phylum

were found to be related to those predicted changes in the metagenome. Bacteroidetes family S24-7 was represented in high proportion among all pathways, both up and downregulated and at different time points and FMT treatments. Likewise, the Bacteroidaceae family was also present among up and downregulated pathways throughout all the samples, but is less represented than S24-7. Interestingly the family Enterobacteriaceae was also present in both up and downregulated pathways, but with a noticeable increase to the pathway contribution at day 1 after FMT with *Muc2*^{-/-} microbiota (Supplemental Fig 3-4).

Table 3- 1 PICRUSt prediction of significantly different metabolic pathways between *Muc2*^{+/+} FMT with *Muc2*^{-/-} or homologous microbiota

Pathway	Kegg ID	p-value	Adjusted p-value	Normalized enrichment score
Biofilm formation - <i>Vibrio cholerae</i>	map05111	2.5e-05	0.0014	2.2
Biofilm formation - <i>Escherichia coli</i>	map02026	2.6e-05	0.0014	2.1
<i>Staphylococcus aureus</i> infection	map05150	7.1e-05	0.0017	-2.6
Aminoacyl-tRNA biosynthesis	map00970	7.9e-05	0.0017	-2.2
Lipopolysaccharide biosynthesis	map00540	8e-05	0.0017	1.9
Ribosome	map03010	9.9e-05	0.0018	-2.7
Methane metabolism	map00680	0.00012	0.0018	-2.7
Carbon metabolism	map01200	0.00029	0.0039	-1.8
Biosynthesis of siderophore group non-ribosomal peptides	map01053	0.00088	0.0099	1.8
Flagellar assembly	map02040	0.00092	0.0099	1.8
Carbon fixation pathways in prokaryotes	map00720	0.0035	0.032	-1.6
Cationic antimicrobial peptide (CAMP) resistance	map01503	0.0041	0.034	1.7

Glutathione metabolism	map00480	0.0052	0.038	1.7
Biofilm formation - <i>Pseudomonas aeruginosa</i>	map02025	0.0052	0.038	1.6
Butanoate metabolism	map00650	0.0061	0.041	-1.5

3.5 Discussion

Gut microbiota is essential to human health and the mucus layers provide them a niche and food source to thrive^{24,58}. Dysbiosis and depletion of the mucus layer have been implicated in colonic inflammation and largely associated with IBD^{59,60}. Therefore, understanding distinct roles of the microbiota in the presence or absence of the mucus layer is critical in understanding disease pathogenesis. Based on this premise, the primary aim of this study was to delineate their distinct and cooperative function in maintaining epithelial barrier function.

Muc2^{-/-} animals provide a unique opportunity to study shifts in specific taxa of gut bacteria and distinguish potentially harmful bacteria during intestinal dysbiosis that is common among IBD patients. *Muc2*^{-/-} littermates were able to harbor a stable microbiota in the absence of a mucus layer and fecal bacterial counts in both genotypes were comparable. Studies using *Muc2*^{-/-} mice in our laboratory and others have shown that they share several phenotypes typically seen in UC patients⁶¹. More recently, we have shown altered expression and secretion of the antimicrobial peptides β -defensins in *Muc2*^{-/-} littermates suggesting that Muc2 mucin may regulate the expression of antimicrobial peptides⁶². MUC2 mucin has also been shown to directly activate dendritic cells to secrete IL-8²⁷. Thus, we speculated that *Muc2*^{-/-} would have a different indigenous microbiota than *Muc2*^{+/+} littermates. Our results showed the presence of distinctly different microbiota

between Charles River obtained wild type and in-house bred *Muc2*^{-/-} animals. Thus, to investigate the role of the mucus layer, we normalized the microbiota between both genotypes by generating littermates. *Muc2*^{-/-} littermates also maintained low-grade colonic inflammation, which is likely because of the depleted mucus layer. The presence of the mucus layer prevents gut microbiota and luminal contents from activating the immune system ⁶³.

The role of mucus layer in development and regulation of the immune system is increasingly recognized as it separates gut bacteria from intestinal immune cells ⁶⁴. During IBD, commensal bacteria are the target of host immune aggression thus triggering an inflammatory phenomena and perpetuating mucosal destruction ⁶⁵. Our study clearly shows that close contact of bacteria with the intestinal epithelium creates a pro-inflammatory milieu evidenced by increased levels of TNF- α and IL-1 β that in turn potentiates intestinal permeability. Expectedly, antibiotics treatment of *Muc2*^{-/-} animals reduced the severity to DSS-induced mortality whereas *Muc2*^{+/+} littermates became more susceptible emphasizing the protective role of gut microbiota. This confirms the notion that gut microbiota is necessary for the proper functioning of the host and the immune system. *Muc2*^{-/-} littermates microbiota however, are distinctively different than *Muc2*^{+/+}, which could be linked to their characteristic phenotype and to their survival against low-grade colitis. IL-22 cytokine, mainly released by ILC3, has been found to play a key role in maintaining gut-microbial homeostasis, epithelial barrier function and stimulating tissue repair in the colon ^{66,67}. The ILC3-IL22 axis have also been found to be important in regulating immune response against extracellular pathogens (bacteria and fungus) as well as preventing their translocation, through the stimulation of antimicrobial peptides production (reviewed in ⁶⁸). A link between IL-22 and *Muc2*

has been suggested ^{69,70}, however the exact mechanism and how it is regulated it is not yet fully understood. Higher levels of IL-22 have been reported in the ileum of *Muc2*^{-/-} mice ⁷⁰. In this study, we found higher number of IL-22⁺ ILC3 population in *Muc2*^{-/-} colonic lamina propria as compared with *Muc2*^{+/+} littermates. We theorize that ILC3-IL-22 axis was inducing a tolerance state in *Muc2*^{-/-} littermates to cope with their basal inflammatory state without developing fulminant colitis. Accordingly, antibiotic treatment in *Muc2*^{-/-} animals increased IL-22⁺ ILC3 population and significantly reduced susceptibility to DSS-induced colitis.

Furthermore, we were concerned that prolonged antibiotic treatment (17 days) could promote inflammation as described previously ⁷¹. Nonetheless, we did not observe any evidence of the treatment affecting animal's health, weight loss, diarrhea or any indication of sickness that could compromise our study. Of interest was the finding that despite *Muc2*^{-/-} microbiota transplanted into *Muc2*^{+/+} littermates was not colitogenic, adding a trigger such as DSS rendered them highly susceptible to colitis associated with high mortality, increased intestinal permeability, rapid weight loss and failed crypts regeneration in the surviving mice. It is important to highlight that despite these bacteria not being colitogenic in wild types littermates, animals receiving *Muc2*^{-/-} FMT were tending towards increased weight loss and higher pro-inflammatory cytokines tissue levels. These results led us to investigate and propose that certain bacteria from *Muc2*^{-/-} microbiota was associated with the deleterious effects observed when transplanted into wild type littermates. We found that *Muc2*^{-/-} maintained a tolerogenic state against microbiota that allowed them to resist colonic injury. Mucosal tolerance is critical in maintaining intestinal homeostasis, and to provide an immune exclusion state against commensal microbiota ^{63,72}. This is mainly maintained by

lamina propria dendritic cells (DCs) as they sample the microbial content and stimulate T-regs to produce anti-inflammatory IL-10 and TGF- β ^{73,74}. We observed the presence of higher number of CD45⁺MHCII⁺ phagocytic cells in the lamina propria of *Muc2*^{-/-} than *Muc2*^{+/+} littermates. This no doubt will exert a protective effect as *Muc2*^{-/-} has higher gut microbial penetrants in the absence of a mucus barrier. Similarly, we also found increased populations of colonic CD103⁺CD11c⁺ DCs in *Muc2*^{-/-} critical for establishing tolerance and to withstand ongoing low-grade colonic inflammation ⁵⁶.

Since, differences in microbial dynamics present in untreated animals would not reflect the bacteria that are transplantable, we identified differences between both FMT groups (homologous and heterologous) in *Muc2*^{+/+} littermates. We found, via 16S sequencing, Bacteroidetes family Bacteroidaceae was significantly increased in both genotypes receiving *Muc2*^{-/-} microbiota, particularly in *Muc2*^{+/+} recipients (four-fold increase). Bacteroides is a clinically relevant group of bacteria and includes many known pathogens having unique secretion systems, endotoxins and virulence factor genes that provides them antimicrobial resistance ^{75,76}. A recent study using *Muc2*^{-/-} on 129S genetic background, linked the genus Bacteroides to higher histopathology scores at basal levels and increased susceptibility towards DSS induced colitis. There was also altered gene expressions associated with bacteria sensing and stress response ⁷⁷. Interestingly, we found that OTUs belonging to the Proteobacteria family Enterobacteriaceae were absent in *Muc2*^{+/+} that received their own FMT, however they were increased in *Muc2*^{-/-} FMT receiving groups. Another family that was significant increased within both genotypes receiving *Muc2*^{-/-} FMT was Verrucomicrobiaceae. This family is of special interest as it includes the mucin degrading bacteria

Akkermansia muciniphila, a commensal with known anti-inflammatory properties and associated with maintaining homeostasis. Their reduced levels have also been reported in patients with IBD and various metabolic disorders ^{78,79}. Furthermore, increase in Verrucomicrobiaceae abundance was only observed in *Muc2*^{+/+} after day 5 of FMT, which eventually came back to a normal on day 10. We also noticed a reduction in OTUs of Lactobacillaceae family, which are lactic acid producing bacteria and considered as important probiotics for their positive effects on hosts health ^{80,81}. Another dramatic change was the complete absence of OTU assigned to the Bacteroidetes S24-7 family in the *Muc2*^{+/+} after 1 day of FMT with *Muc2*^{-/-} that became comparable after day 10. To date, characteristics of the S24-7 family is unknown as they cannot be cultured under laboratory conditions. However, abundance of this family is high in murine fecal samples, demanding a deeper description and study ⁸². Our results also supports a recent report ⁸³ where it was established that within the first three weeks of colonization, the gut bacterial population goes through a series of changes until becoming stable, making evident the dynamics of microbiota population after being transferred to a new host.

PICRUSt is a valuable bioinformatics tool that allows to make predictions of genes and/or metabolic pathways that might be over or under-expressed ⁵⁷. We observed up regulated pathways that were related to gram-negative pathogens, such as genes involves in biofilm formation of *V. cholerae*, *E. coli* and *P. aeruginosa*. Biofilm formation plays an important role in bacterial infections, sheltering them from the host's immune defense and allowing them to attach, proliferate and spread inside of the host ⁸⁴. Similarly, we observed up regulation of genes involved in glutathione metabolism. Within bacteria glutathione is only present in Cyanobacteria and

Proteobacteria and is used as a protecting mechanism in different stress triggers such as low pH and oxidative stress. In addition, up regulation of genes involved in LPS biosynthesis were also predicted. The up regulation of these pathways correlates and could be explained due to a higher relative abundance of gram-negative Proteobacteria in *Muc2^{+/+}* receiving *Muc2^{-/-}* FMT.

Here, we have identified a distinctive gut microbiota in *Muc2^{-/-}* animals without a mucus layer that maintains a constitutive pro-inflammatory milieu in their colon. This particular microbiota did not trigger pro-inflammatory responses when transplanted orally into *Muc2^{+/+}* littermates but rather, sensitized the colonic mucosa for increased susceptibility towards chemical and infectious colitis. Unlike *Muc2^{-/-}*, *Muc2^{+/+}* microbiota played a protective role against chemical-induced colitis that was lost following antibiotic treatment. We conclude that both the colonic mucus layer and indigenous microbiota play key roles in conferring innate resistance against colonic injury and homeostasis.

3.6 Materials and Methods

3.6.1 Ethics statement and animals

8 to 12 weeks male and female C57BL/6 and *Muc2^{-/-}* mice were used. C57BL/6 mice were obtained from Charles River (Saint Constant, QC) while *Muc2^{-/-}* of the same genetic background were bred in house. Animals were backcrossed to generate *Muc2^{+/-}* breeding pairs to derive *Muc2^{+/+}* and *Muc2^{-/-}* littermates that were used for the experiments. All animals were housed under specific pathogen free conditions (SPF) in filter top cages and fed autoclaved food and water *ad*

libitum. Throughout the study animals were closely monitored to ensure healthy conditions and all experiments adhered to the University of Calgary Animal Care Committee standards.

3.6.2 Antibiotic treatment

Muc2^{+/+} and *Muc2*^{-/-} littermates were treated with antibiotics to decrease bacterial load as described previously⁴⁸. In short, mice were gavaged every 12h with an antibiotic cocktail as follows: for the first 3 days mice were gavaged with amphotericin-B (1 mg/kg) to suppress fungal growth. From day 4, ampicillin (1 mg/mL) was added to the drinking water; in addition, mice received orally vancomycin (50 mg/kg), neomycin (100 mg/kg), metronidazole (100 mg/kg) and amphotericin-B (1 mg/kg) for another 14 days. This combination ensures the safe and controlled delivery of antibiotics to each mouse while having a broad-spectrum effect.

3.6.3 Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) was achieved by collecting 0.1g of mice feces (about 4 fecal pellets), homogenizing them in 1 mL of sterile phosphate buffered saline (PBS) and centrifuged for 30 seconds at 1000 xg. Each mouse was gavaged three times with 200µl of the obtained supernatant every 48h.

3.6.4 DSS-induced colitis

Colitis was induced using dextran sulphate sodium (DSS; MP Biochemicals MW: 36,000-50,000) dissolved in tap water. *Muc2*^{-/-} animals received 1% for 3 days, while *Muc2*^{+/+} mice received 3.5%

for 5 days follow up by water *ad libitum* like previously described ²⁶. The cumulative disease activity index (DAI) was scored based of weight loss, stool consistency, blood loss and appearance (Table 3-2).

Table 3- 2 Disease activity index (DAI) in mice treated with dextran sodium sulphate (DSS)

	0	1	2	3
Weight loss	No loss	10-15%	15-20%	>25%
Stool consistency	Solid	Loose	Wet anal fur	Diarrhea
Blood loss	None	Little, dried on bum	Little, red on bum	Lots; fresh blood on bum & tail
Appearance	Lively/normal	Slower, not hunched	Hunched	Lethargic

3.6.5 OTU table construction

Raw reads were processed with cutadapt 1.8.3 ⁸⁵ to remove the primer sequences and any preceding adaptors. Subsequent processing was done using the UPARSE pipeline ⁸⁶ as implemented in usearch 8.1.1861. The forward and reverse reads were merged using the fastq_mergepairs option of usearch and subsequently filtered with usearch –fastq_filter and an expected error (EE) cut-off of 0.5 ⁸⁷. The filtered reads were de-replicated using usearch -derep_fulllength and then clustered with usearch -cluster_otus and the option ‘-minsize 2’ to remove singleton reads prior to clustering. Taxonomy was assigned to the representative sequences

using the RDP naïve Bayesian classifier ⁸⁸ with May 2013 version of the Greengenes database. The final OTU table was constructed with usearch -usearch_global and the options '-strand plus -id 0.97'. The entire procedure was run as a Snakemake pipeline ⁸⁹.

3.6.6 Diversity analysis

Downstream analysis was done in R 3.3.1 ⁹⁰ using phyloseq 1.16.2 ⁹¹ and vegan 2.4-1 ⁹². Mitochondrial and chloroplast sequences were removed along with OTUs that appeared only in one sample. Samples with less than 1000 sequences were also removed from the analysis. Alpha-diversity was measured using the Shannon index ⁹³. Differences in alpha-diversity between groups was tested using a Wilcoxon rank sum test, controlling the false discovery rate ⁹⁴ and using a cut-off of $p < 0.05$ for rejecting the null hypothesis of no difference between groups. Between-sample diversity was evaluated using the Bray-Curtis distance metric on proportionally normalized OTU counts and visualized with non-metric multidimensional scaling (NMDS). The generalized linear model framework as implemented in DESeq2 ⁹⁵ was used to identify OTUs in the Muc2^{+/+} group at that were differentially abundant between Muc2^{+/+} and Muc2^{+/+} FMT treated at Day1, controlling for differences at Day 0. This approach appropriately controls for over-dispersed data and variable library sizes ⁹⁶. A p-value cut-off of 0.05 was specified for rejecting the null hypothesis of no difference between groups.

3.6.7 DNA extraction and 16S rRNA gene sequencing

DNA extraction of fecal samples or tissues were carried out using a previously described protocol that enhanced DNA recovery from microbial communities with modifications ⁹⁷ to increase quantitative recovery of bacteria across different taxa (enzymatic pre-treatment with mutanolysin, lysozyme and proteinase K). Paired end reads of the V3 region of the 16S rRNA gene using bar coded Illumina sequencing as described previously ⁹⁸ with the modification that bar-codes are included in the forward primer. 250 nucleotides paired-end sequencing were carried out on a MiSeq Illumina sequencer providing complete overlapping sequence reads of the V3. These overlaps are used for correcting poor quality base calls and increasing sequencing accuracy. 30-60,000 16s rRNA reads were generated per sample.

3.6.8 Intestinal permeability

Fluorescein isothiocyanate (FITC) dextran was used to examine intestinal permeability. Briefly, mice were gavaged with 8mg FITC-dextran (3-5kDa, Sigma Aldrich), 3h later mice were anaesthetized by isoflurane (Pharmaceutical Partners of Canada, Richmond Hill, ON) and blood was collected by cardiac puncture, animals were then sacrificed by cervical dislocation. Whole blood was allowed to clot in the dark for 3h at room temperature (RT) and centrifuged at 10,000 x g for 10 min. Serum was transferred to a clean Eppendorf tube and diluted with an equal volume of PBS. An aliquot of 100µL of each sample was loaded onto a black bottom 96-well plate in duplicate, and fluorescence was determined with a plate reader (absorption 485nm, emission 535nm). This magnitude is expressed in relative fluorescence units.

3.6.9 Histology and staining

At the endpoint of the experiments, animals were anesthetized and sacrificed by cervical dislocation and the colon was excised. The colon was opened longitudinally along the mesenteric border and rolled distal to proximal end to form a Swiss roll. The Swiss roll was sectioned in half with one portion being used for histological analysis and the other one used for gene expression analysis. For histology, colonic tissues were fixed in Carnoy's solution, and embedded in paraffin blocks. 7µm tissue sections were rehydrated through an ethanol gradient to water and stained with hematoxylin and eosin (H&E; EMD Chemicals Gibbstown, New Jersey) to examine overall tissue morphology or Periodic acid Schiff's reagent (PAS, Sigma Aldrich Co.) to visualize neutral mucins.

3.6.10 Bacterial localization

Fluorescence *in situ* hybridization (FISH) was performed as described previously⁹⁹. Briefly 5 µm sliced Carnoy's fixed tissue was incubated with the total bacteria probe EUB338 5'-GCT GCC TCC CGT AGG AGT-3' [50ng/µL] coupled with Quasar 670 dye at 46°C overnight. FITC coupled-*Ulex europaeus* agglutinin (UEA) was used at [1:1000] to visualize the fucosylated residues in mucins and DAPI [1:1000] (Life Technologies) for nuclear counterstain. Tissue sections were visualized using an Olympus FV1000 scanning confocal inverted microscope.

3.6.11 Citrobacter rodentium infection and bacterial translocation measurement

A streptomycin-resistant derivative of *Citrobacter rodentium* strain DBS100 was utilized in our study. Mice were infected by oral gavage with 100 µl of an overnight culture of LB broth containing approximately 2.5×10^8 CFU of *C. rodentium*. To obtain colony forming units counts (CFU) numbers, mice were sacrificed at day 6 after infection and mesenteric lymph nodes and spleen was homogenized in sterile PBS and immediately streaked into LB agar plates with streptomycin [100 µg/mL], plates were incubated at 37°C for 24h and CFU counted.

3.6.12 Serum LPS/ flagellin antibodies detection assay

Levels of anti-flagellin and LPS antibodies in serum were detected by enzyme-linked immunosorbent assay (ELISA) using a previously described protocol ¹⁰⁰. All assays were performed using hemolysis free sera collected by centrifugation of blood in serum separator tubes (BD biosciences, USA).

3.6.13 Gene expression analysis

Total RNA was isolated from snap-frozen tissue using the Trizol reagent method (Invitrogen; Life Technologies, Burlington, ON) as per manufacturer's specifications, and the yield and purity determined by the ratio of absorbance at 260/280nm (NanoDrop, Thermo Scientific); only samples with a ratio of ~1.8 for DNA and ~2.0 for RNA were used. RNA from DSS-treated mice tissue was re-precipitated with lithium chloride precipitation solution (Ambion; Life Technologies, Burlington, ON). cDNA was prepared using a qScript cDNA synthesis kit (Quanta Biosciences)

and PerfeCTa SYBR Green FastMix (Quanta BioSciences). Real-time qPCR was performed using a Rotor Gene 3000 real-time PCR system (Corbett Research). Each reaction mixture contained 100 ng of cDNA, SYBR Green PCR Master Mix (Qiagen) and 1 μ M of primers. A complete list of the primer sequences and conditions used are listed in Table 3-3. The results were analyzed using the $2^{-\Delta\Delta CT}$ method and expressed as fold changes.

Table 3- 3 Primer sequences used for quantitative real-time PCR.

Name	Sequence		Annealing Temp.	Reference
	5`	3`		
16s V6	Fwd: AGGATTAGATACCCTGGTA Rev: CRRCACGAGCTGACGAC		55°	48
IL-1 β	Fwd: GCCTCGTGCTGTCTGGACCCA Rev: CTGCAGGGTGGGTGTGCCGT		65°	50
TNF- α	Fwd: ATGAGCACAGAAAGCATGATC Rev: TACAGGCTTGTCACCTCGAATT		56°	101
IFN- γ	Fwd: TCAAGTGGCATAGATGTGGAAGAA Rev: TGGCTCTGCAGGATTTTCATG		54°	101
Occludin	Fwd: AGAGGCTATGGGACAGGGCTCTTTGG Rev: CCAACAGGAAGCCTTTGGCTGCTCTTGG		60°	102
Claudin-2	Fwd: CCTCGCTGGCTTGTATTATCTCTG Rev: GAGTAGAAGTCCCGAAGGA		60°	102
Actin	Fwd: CTACAATGAGCTGCGTGTG Rev: TGGGGTGTTGAAGGTCTC		54°	50

3.6.14 Lamina propria lymphocytes (LPLs) isolation

Animals were euthanized at the end of the experiment and colon was surgically removed to isolate LPLs using an established protocol¹⁰³. In brief, colon was longitudinally cut open and cleaned for any fecal matter using HBSS. Then, it was cut into small 2-3 cm pieces to dissociate epithelial cells using HBSS containing EDTA and DTT in a 50 mL falcon tube and placed in an incubator shaker at 37°C for 20 min. Supernatant was discarded and remaining tissue was digested with collagenase to obtain LPLs.

3.6.15 Flow cytometry

LPLs were isolated and stimulated with PMA and ionomycin in the presence of brefeldin and monensin for 4h at 37° C in a humidified CO₂ incubator. Stimulated cells were stained for surface antigens with fluorescently labelled antibodies (Table 3-4) using previously described protocol. Cells were then stained with fixable viability dye FV510 for live/ dead discrimination followed by Intracellular staining using Fix/ Perm transcription factor kit (BD biosciences) and fluorescently labelled antibodies (Table 3-4). Data was acquired on BD FACSCanto flow cytometer and analysed using FlowJo software.

Table 3- 4 Antibodies for flow cytometry

Antibody- fluorochrome	Source; Cat #
IL4 - PE-Cy7	BD Pharmingen; 560699
KLRG1 – PerCP-Cy5.5	BD Pharmingen; 563595
IL22 - PE	BD Pharmingen; 516404
IFN γ - APC-Cy7	BD Pharmingen; 561479
Ly6G- FITC	BD Pharmingen; 561105
NK1.1 -FITC	BD Pharmingen; 561082
CD3- FITC	BD Pharmingen; 561798
CD19- FITC	BD Pharmingen; 561740
B220- FITC	BD Pharmingen; 561877
NKp46- AF700	BD Pharmingen; 561169
ROR γ t- AF647	BD Pharmingen; 562682
Viability dye FV510- BV510	BD Pharmingen; 564406
CD127- BV421	BD Pharmingen; 566377
CD45- BV786	BD Pharmingen; 565477

3.6.16 Statistical analysis

Data was analyzed using GraphPad Prism 6 (Graph-Pad Software, San Diego, CA) for all statistical analysis. Treatment groups were compared using analysis of variance (ANOVA) when more than two groups were compared. Student's t-test was used when only two groups were compared. Statistical significance was assumed at $P < 0.05$, n = total number of mice per group. Error bars in all the graphs represent mean \pm standard error of the mean (SEM).

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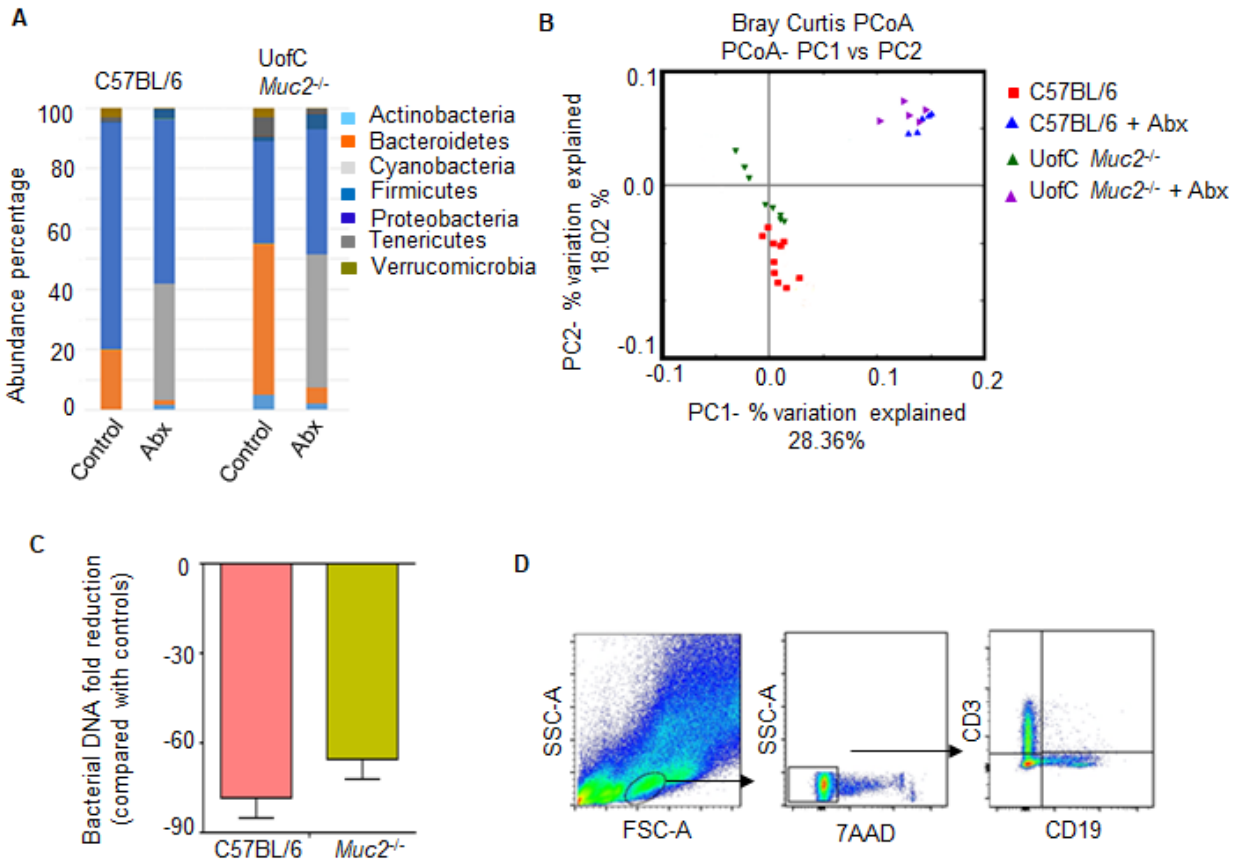
3.8 Conflict of interest

None

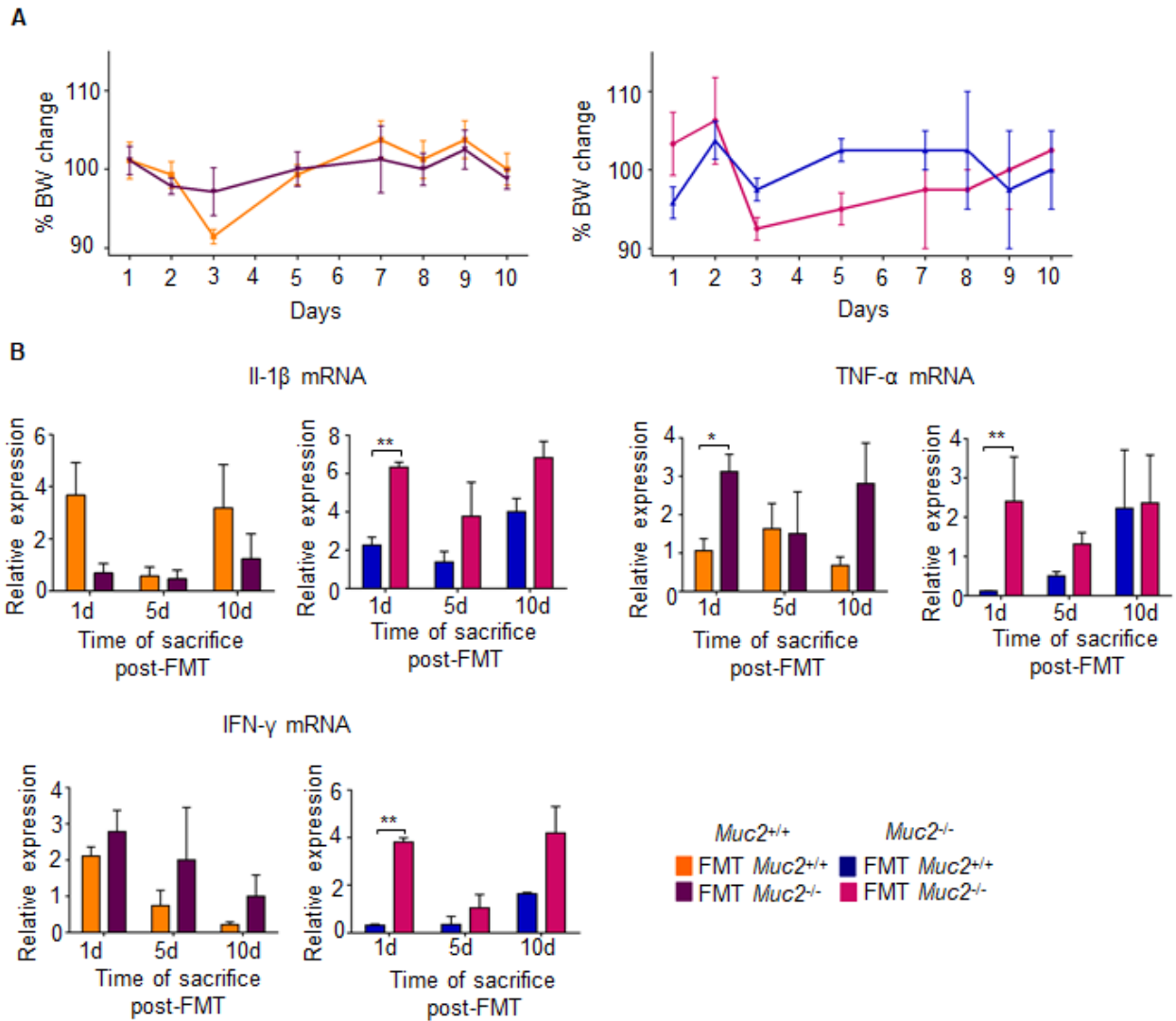
3.9 Authorships

A.L.C. and M.K conducted the experiments, analyzed data and wrote the manuscript. F.M. performed experiments. M.L.W. and M.S. executed bioinformatics analysis. K.C. secured funding, planned and supervised the experiments and edited the manuscript.

3.10 Supplemental

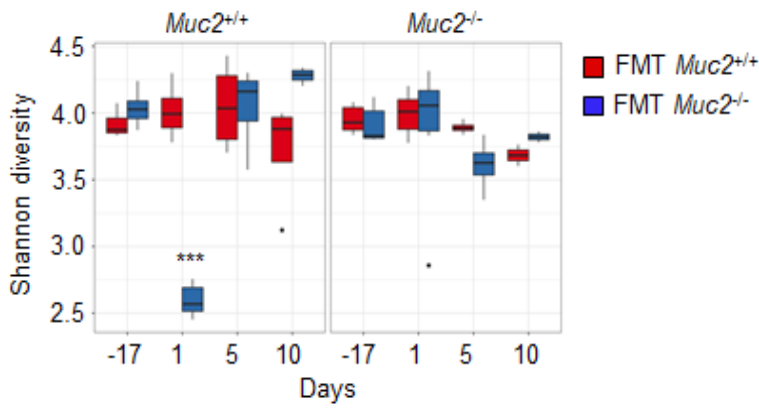


Supplemental Figure 3- 1 C57BL/6 and UofC *Muc2*^{-/-} have a different bacteria phyla distribution. **A.** C57BL/6 and UofC *Muc2*^{-/-} fecal samples were quantified and analyzed by MiSeq Illumina sequencing. Microbiota of inbred UofC *Muc2*^{-/-} is composed by lesser amounts of Firmicutes and higher amounts of Bacteroidetes compared with commercially bought wild-type C57BL/6 microbiota. Following antibiotics treatment there was a decrease in Bacteroidetes, a slight increase in Proteobacteria and an evident increase in Cyanobacteria is shown in both genotypes. **B.** Bray Curtis principal coordinates analysis (PCoA) was performed to plot population distribution between samples, a clear separation between groups and treatment is seen. **C.** Bacterial DNA load reduction in *Muc2*^{+/+} and *Muc2*^{-/-} following antibiotic treatment. Mice received a broad spectrum antibiotic cocktail for 17 days, fecal samples were taken at day 0 and day 18 of treatment and RNA extracted to analyze bacterial DNA load using primer V6 for bacterial 16S DNA. Results are expressed as bacterial DNA fold reduction of antibiotic treated mice compared with untreated controls. Abx: antibiotics. **D.** Lamina propria lymphocytes from *Muc2*^{+/+} and *Muc2*^{-/-} littermates were isolated and gated through FSC-A vs SSC-A and further interrogated by the ratios of height and width in forward and side scatter to select only single cells population. Thereafter, only live cells were selected using 7-AAD. Live CD19⁺ B cells and CD3⁺ T cells were selected for further analysis. n=6.

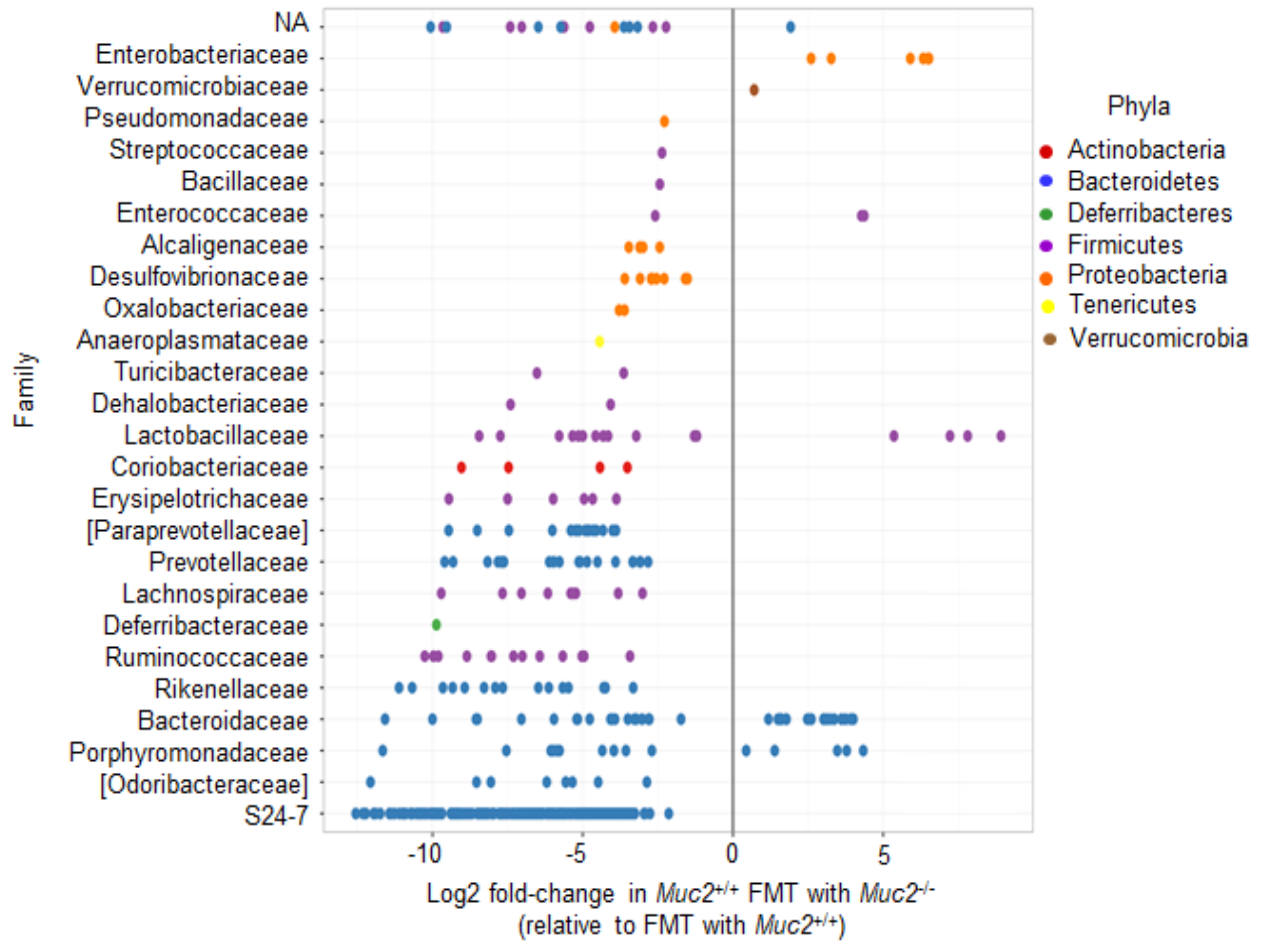


Supplemental Figure 3- 2 Pro-inflammatory cytokine levels in *Muc2*^{+/+} and *Muc2*^{-/-} following FMT up to 10 days. *Muc2*^{-/-} and *Muc2*^{+/+} mice were treated for 17 days with an antibiotic cocktail and received FMT with either *Muc2*^{-/-} or *Muc2*^{+/+}. Animals were sacrificed 1, 5 and 10 days after FMT. **A.** No significant differences in weight change were observed between the four groups. **B.** *Muc2*^{+/+} receiving *Muc2*^{-/-} FMT showed no significant increase in pro-inflammatory cytokines compared with controls receiving their own FMT. Relative expression as compared to its own untreated control. BW: body weight, FMT: fecal microbiota transplant, d: days n= 3-4 *P < 0.05, **P < 0.01.

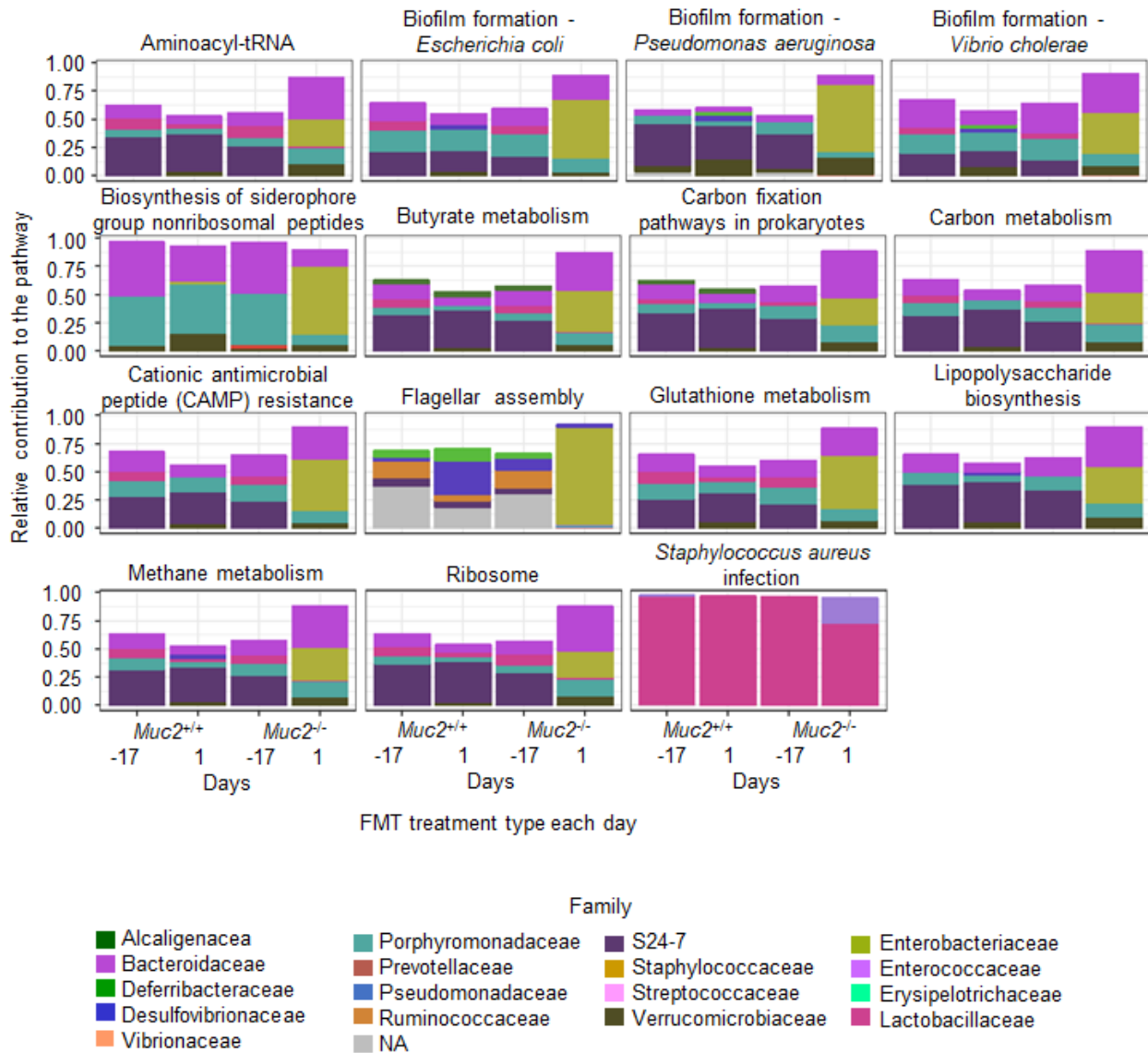
A



B



Supplemental Figure 3- 3 *Muc2*^{+/+} receiving *Muc2*^{-/-} FMT changed bacteria families' abundance and decreased alpha diversity. **A.** Alpha diversity of *Muc2*^{+/+} and *Muc2*^{-/-} was characterized by Shannon's index in *Muc2*^{+/+} and *Muc2*^{-/-} littermates at different time-points; before antibiotic treatment (day -17), and 1, 5 and 10 days after receiving the last FMT (Day 1, 5 and 10, respectively). Notice the drop in species diversity in the *Muc2*^{+/+} group at day 1 when they received *Muc2*^{-/-} FMT. Wilcoxon rank sum test for differences in Shannon diversity between *Muc2*^{+/+} treated and *Muc2*^{-/-} treated P-values are adjusted for multiple testing using the Benjamini and Hochberg method¹⁰⁴. **B.** Differential abundance, at the family level, between *Muc2*^{+/+} receiving either *Muc2*^{-/-} or own FMT. Results are shown as fold-change in *Muc2*^{+/+} receiving *Muc2*^{-/-} FMT relative to *Muc2*^{+/+} receiving *Muc2*^{+/+} FMT. Log2 fold-changes are plotted for significantly different ($p < 0.05$) family for each of the comparisons. FMT= fecal microbiota transplant, *** $P < 0.001$.



Supplemental Figure 3- 4 Relative contribution of bacterial families to predicted metagenome. Bacteria families' contribution to PICRUSt predicted pathways significantly different between *Muc2*^{+/+} receiving *Muc2*^{-/-} and *Muc2*^{+/+} FMT.

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Chapter Four: **Manuscript II**

Cooperative roles of colonic microbiota and Muc2 mucin in mediating innate host responses
against *Entamoeba histolytica*

Aralia Leon-Coria, Manish Kumar, France Moreau, Kris Chadee

4.1 Abstract

Amebiasis is the disease caused by the protozoan parasite *Entamoeba histolytica* (*Eh*), a health problem in developing countries and one of the leading causes of parasite-induced death. The reason why most infections remain asymptomatic is not known, suggesting that microbiota and/or host immune responses play major roles in innate host defense. *Eh* shares an ecological niche with microbiota that resides, feeds and interacts with the MUC2 mucus layer. Here we explored the interconnected relationship between colonic microbiota and the mucus layer in *Eh*-induced secretory and pro-inflammatory responses in *Muc2*^{-/-} and *Muc2*^{+/+} littermates and germ-free mice. In *Muc2* genotypes with normalized microbiota, *Eh*-induced a modest host response with water/mucus secretions and pro-inflammatory cytokine release in closed colonic loops. However, following microbial disruption with a cocktail of antibiotics, *Eh* elicited aggressive robust water and mucus secretions and enhanced release of the pro-inflammatory cytokines IFN- γ and TNF- α , and chemokines MCP-1, KC and MIP-2. Remarkably, water and mucus secretions were restored to basal levels following homologous microbial fecal transplants in antibiotic-treated *Muc2*^{+/+} littermates. Surprisingly, the transcription factor for secretory goblet cells, *Math1* was suppressed in the presence of *Eh* in the proximal colonic loops with a corresponding increase in *Math1* activity in the non-looped distal colon that was microbiota dependent. This was confirmed using germ-free mice that showed significant decrease in pro-inflammatory and mucus secretagogue responses towards *Eh*, with reduced *Math1* and *Muc2* mRNA expression and number of filled goblet cells throughout the colon. These results signify that intestinal commensal microbiota plays an important role in shaping innate host defenses against *Eh* and that dysbiosis can render the colonic epithelium susceptible to *Eh*-induced pro-inflammatory responses and tissue injury.

4.2 Author summary

Entamoeba histolytica (*Eh*) is a human protozoan parasite and the causative agent of amebiasis. This disease can cause severe ulcerations and tissue destruction in the colon that can disseminate to the liver through blood, causing amoebic liver abscess, or to other organs like the brain or lungs. When *Eh* colonizes the colon, it shares its habitat with resident intestinal microbiota that lives, feeds and multiplies on the outer mucus layer. Thus, both microbiota and the mucus layer play critical roles in a layered host defense against potential invasive pathogens. At present, it is not known how *Eh* thrive or changes its behavior in the ecological niche within the microbiota-mucus interface. Here we show that a dysbiotic state worsens the outcome of *Eh* infection by increasing pro-inflammatory responses independently of the mucus layer, as well as boosting mucus release from colonic goblet cells. However, the absence of microbiota in germ free mice prevented the development of a proper immune response towards the parasite allowing *Eh* to gain close contact with the epithelium resulting in epithelial disruptions. This study shed new light on the role of indigenous microbiota colonizing the mucus layer and forming a living shield as the first line of innate host defense against *E. histolytica*-induced pro-inflammatory responses and tissue injury.

4.3 Introduction

Entamoeba histolytica (*Eh*) is a human protozoa parasite that causes the disease amebiasis and in 2013, *Eh* was responsible for approximately 11,300 deaths ¹. Interestingly, of those individuals infected with the parasite, only 10% develops symptoms of the disease while the rest remains asymptomatic. To date, the variation in disease outcome has not been fully explained, but several studies have suggested that this difference could be due to host's health conditions as well as immune fitness. More recently, with molecular tools for studying the microbiome, the idea that specific bacteria within the microbiota could modulate *Eh* pathogenesis and predispose to intestinal amebiasis ² and amebic liver abscess ³ (ALA) has been proposed.

MUC2 is the major secretory mucin in the gastrointestinal tract that forms a barrier between the single layer of epithelial cells and luminal contents that not only contains nutrients but is also loaded with potential pathogenic microorganisms. When *Eh* is ingested through contaminated food or water, it colonizes the colonic outer mucus layer, which is rich in a diverse community of bacteria. The human intestine hosts approximately 100 trillion microorganisms ⁴, mainly bacteria, that form the microbiota that regulates host homeostasis by promoting digestive health as well as stimulating a balanced immune system ^{4,5}. It is known that *Eh* stimulates goblet cell mucus secretion ⁶ as an innate host defense mechanism to counter *Eh* adherence to intestinal epithelial surfaces ⁷. In disease pathogenesis, *Eh* cysteine proteases cleave MUC2 in the non-glycosylated domains, weakening its structure and facilitating *Eh* contact with epithelial cells ⁸. Germ-free and gnotobiotic experiments with guinea pigs have established that the presence of gut microbiota is required for *Eh* pathogenicity ⁹.

In recent studies, the link between microbiota and *Eh* infection in humans has been suggested with varying results depending on location and subjects of the study. A study in India have shown a decrease in mostly butyrate-producing bacteria (*Clostridium coccoides*, *C. leptum*, *Eubacterium*, *Lactobacillus*, *Bacteroides*) and an increase in *Bifidobacterium* in stool samples from *Eh* positive patients ¹⁰. More recently, a study in the Cameroon found higher α -diversity and a decrease in β -diversity in individuals positive for *Eh* infection with increase members of the Clostridiales Ruminococcaceae family and a decrease in *Prevotella copri* ¹¹. The decrease in *P. copri* was confirmed in a longitudinal study done in children living in a slum in Bangladesh and the presence of this bacteria was correlated with higher incidence of diarrhea when compared with asymptomatic *Eh* positive children ¹². Analysis of bacterial diversity in ALA patients was not able to significantly relate its incidence with any specific bacteria, however, co-infection with bacteria was present in most of the ALA samples, with a notable higher abundance of *Klebsiella* ³. Various studies ^{2,13-15} have demonstrated that microbiota can significantly alter the outcomes of different protozoan infections, however mechanisms underlying these relationship remain poorly understood.

In the present study, we explored the distinct contributions of microbiota and the Muc2 mucus barrier in *Eh*-induced innate and pro-inflammatory responses critical in disease pathogenesis. Our findings show that indigenous commensal microbiota that colonizes the outer Muc2 mucus layer plays an important role in fortifying innate host defense against *Eh* and that dysbiosis (antibiotic and/or alterations in the mucus layer) renders the colonic epithelium susceptible to *Eh*-induced pro-inflammatory responses and tissue injury.

4.4 Results

4.4.1 Muc2 deficient and sufficient antibiotic-treated mice elicits an enhanced pro-inflammatory cytokine and chemokine response when inoculated with Entamoeba histolytica (Eh)

To quantify the distinct roles of indigenous microbiota and/or the presence of an intact Muc2 mucus layer in *Eh*-induced innate host responses in closed colonic loops¹⁶, *Muc2*^{+/+} and *Muc2*^{-/-} littermates were pre-treated or not with a broad spectrum antibiotic (Abx) cocktail and compared with germ-free mice (GF). *Eh* inoculated in colonic loops in *Muc2*^{+/+} presented with watery and mucoid secretions under intense pressure and bloating (gas bubble formation). Abx-treated animals showed similar responses but also presented with bloody secretions (Fig 4-1 A) as compared to PBS-inoculated controls. *Muc2*^{-/-} littermates in absence of a Muc2 mucus layer showed similar ballooning effects with abundant watery secretions (Fig 4-1 B). Similarly, colonic loops in GF mice presented with ballooning mucoid secretions under intense pressure (Fig 4-1 C). Overall, no significant differences were observed in gross pathology scores between the Muc2 genotypes and GF mice inoculated with *Eh*. Similarly, Abx treatments did not modify gross pathology scores (Fig 4-1 D).

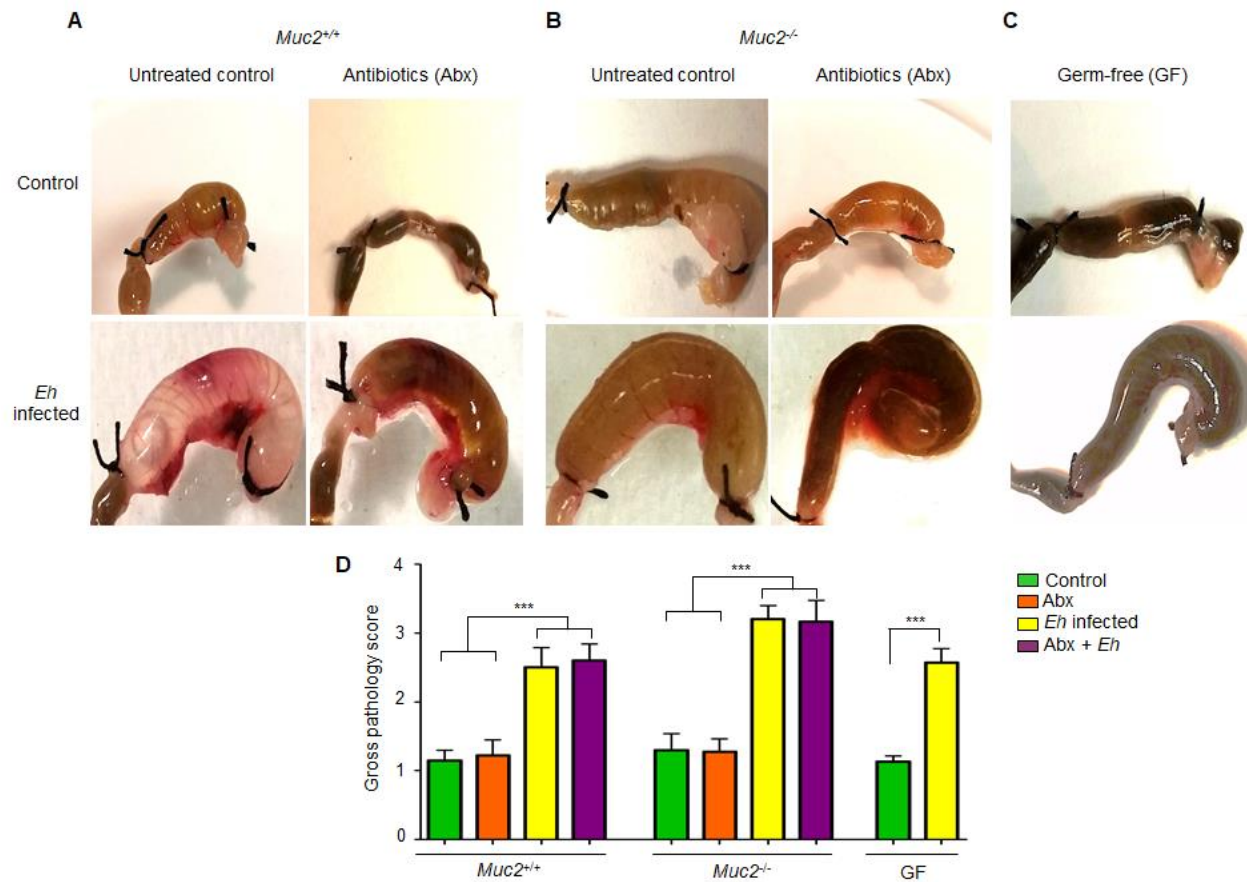


Figure 4- 1 Gross pathology of colonic loops in *Muc2*^{+/+}, *Muc2*^{-/-} and germ-free (GF) mice inoculated with *Entamoeba histolytica* (*Eh*). *Eh*-colonic loops were performed in antibiotic treated (Abx) and untreated *Muc2*^{+/+} and *Muc2*^{-/-} littermates and in GF mice. Gross pathology was measured and scored among treatments as indicated in Material and Methods. **A.** *Eh* inoculated in *Muc2*^{+/+} colons elicited extensive mucoid secretions with bloating. **B.** *Eh* in *Muc2*^{-/-} mice showed a similar ballooning effect with abundant watery secretion under intense pressure. **C.** Colonic loops in GF mice evoked a less robust watery/mucoid secretions with less ballooning as *Muc2* genotypes. **D.** Gross pathology score of colons. Note no differences in gross pathology were observed among *Eh*-inoculated groups compared to homologous controls. n= 6, *** P < 0.001.

To determine whether Abx treatment altered *Eh*-induced host inflammatory responses in *Muc2* genotypes, colonic tissues and luminal contents were analyzed for pro-inflammatory cytokines and chemokines. Interestingly, following Abx treatment, basal IFN- γ and TNF- α pro-inflammatory cytokine expression decreased in colonic tissues in both *Muc2*^{+/+} and *Muc2*^{-/-} littermates as compared to untreated controls (line; Fig 4-2 A-C). However, as predicted, *Eh*-inoculated colonic loops showed increased pro-inflammatory cytokines expression regardless of the presence or absence of a mucus layer. Surprisingly however, Abx-treated mice inoculated with *Eh* showed significant increase in the pro-inflammatory cytokines IFN- γ and TNF- α mRNA expression as compared with non Abx-treated *Eh* inoculated littermates (Fig 4-2 A-C). A similar increase in IFN- γ and TNF- α protein levels were noted in the luminal contents of Abx-treated *Muc2*^{+/+} and *Muc2*^{-/-} littermates inoculated with *Eh* compared as compared to none Abx-treated controls (Fig 4-2 D-F). Likewise, chemokine levels (MCP-1, KC and MIP-2) in the luminal contents of Abx-treated animals inoculated with *Eh* were significantly increased compared with non Abx-treated controls (Fig 4-2 G-I). Several other cytokines and chemokines were assessed by multiplex, however their differences were not significant. These results clearly show that a dysbiotic state induced by Abx-treatment predisposes the host for robust pro-inflammatory responses toward *Eh* regardless of the presence or absence of a functional mucus barrier.

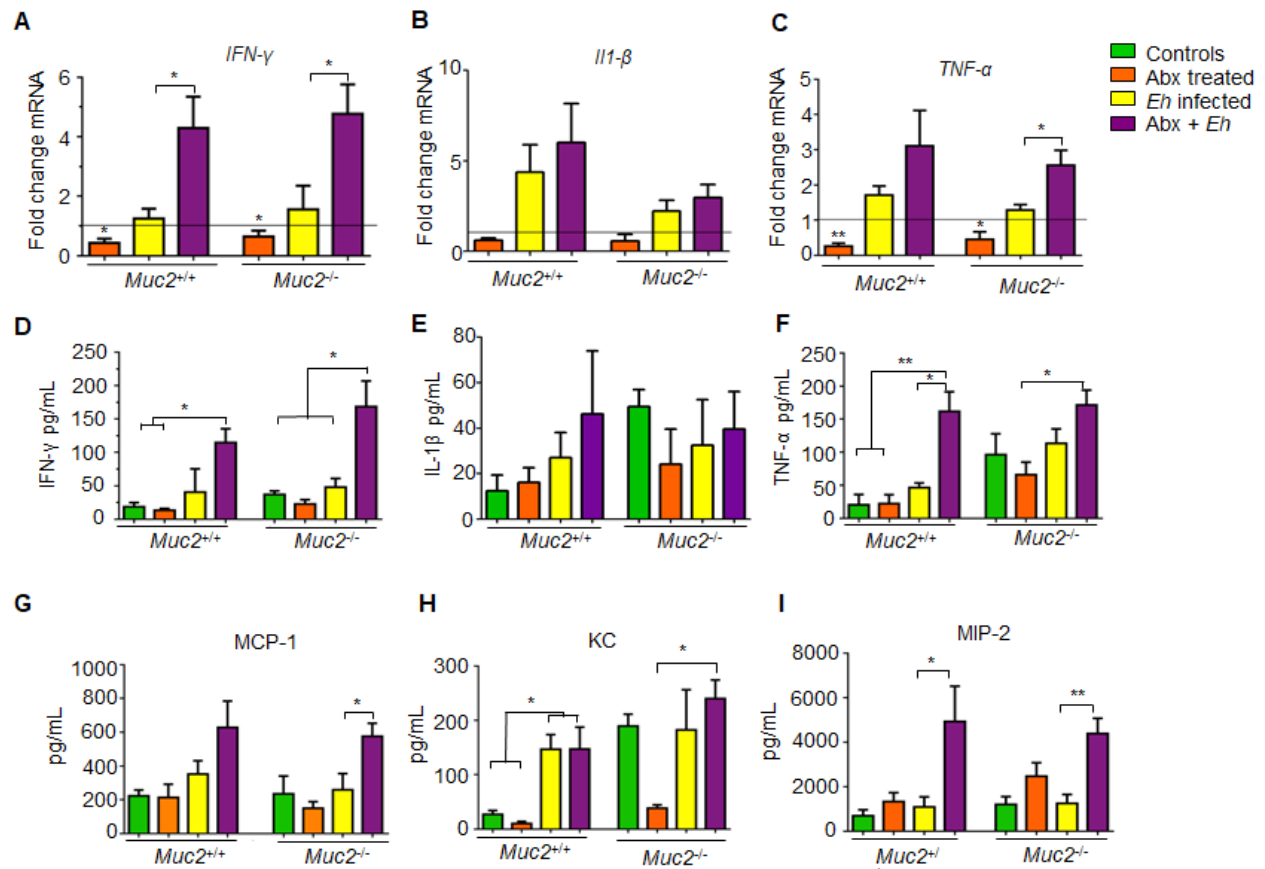


Figure 4- 2 *Entamoeba histolytica* (Eh) infection in antibiotic treated mice exacerbates pro-inflammatory responses in $Muc2^{+/+}$ and $Muc2^{-/-}$ littermates. A-C. $Muc2^{+/+}$ and $Muc2^{-/-}$ littermates pro-inflammatory cytokines mRNA expression in colonic tissue were measured by qPCR and compared with their corresponding untreated control (line). D-F. Colonic luminal pro-inflammatory cytokines and chemokines (G-I) protein levels were measured using a mouse cytokine/chemokine array. n= 6. *P < 0.05, **P < 0.01.

4.4.2 Eh in antibiotic-treated mice evokes a robust mucus secretagogue response

As an intact Muc2 mucus layer and hyper secretion of mucus are critical determinants of innate host defense against *Eh*¹⁷, we determine if Abx treatment in *Muc2*^{+/+} littermates affected mucin biosynthesis and secretion. This was monitored in colonic tissues stained with periodic acid Schiff (PAS) reagent to visualize the mucus layer and filled goblet cells, transcription factors for goblet cell lineage and immunostaining for Muc2 mucin within goblet cells. *Muc2*^{+/+} controls and Abx-treated animals showed normal colonic architecture with numerous goblet cells (arrow) filled with PAS⁺ material (Fig 4-3 A-top panel). However, when inoculated with *Eh*, *Muc2*^{+/+} showed prompt robust mucus secretory response (Fig 4-3 A-bottom left) with coalescence of mucin granules and mucus streaming out in the lumen. In Abx-treated animals, *Eh* elicited enhanced mucus secretion (Fig 4-3 A-bottom right) forming a thick mucus plug over the mucosal surface (Fig 4-3 A-bottom right inset). Mucus secretagogue effects evoked by *Eh* correlated well with the number of filled goblet cells per crypt. In particular, there was a significant decrease in the number of filled goblet cells in control and Abx-treated mice inoculated with *Eh* as compared with controls that received PBS (Fig 4-3 B). Interestingly, Abx treatment alone did not affect basal *Muc2* gene expression, however, in response to *Eh*, both controls and Abx treated animals showed significant up regulation of *Muc2* mRNA expression (Fig 4-3 C). In addition, we also quantified the transcription factor for secretory lineage *Math1*. While Abx treatment had no effect on *Math1* expression, exposure to *Eh* significantly decreased *Math1* gene expression in controls but had no effect on Abx-treated animals (Fig 4-3 D). As *Math1* affects all secretory lineage we analyzed *Spdef* expression [SAM pointed domain containing ETS transcription Factor (SPDEF)], the transcription factor that is critical for terminal goblet cell differentiation¹⁸ and observed the same trend

(Fig 4-3 E). Based on the decrease numbers of filled goblet cells in *Eh*-inoculated animals with a corresponding increase in *Muc2* gene expression, immunostaining of colonic loops was done to visualizing *Muc2* mature mucin granules within goblet cells. Even though a similar pattern to the PAS staining was observed with the *Muc2* antibody, immunostaining revealed mucin granule-granule coalescence and mucus streaming from goblet cells in the deep crypts in response to *Eh* (Fig 4-3 F arrows). There was a paucity of filled goblet cells with mucin in Abx + *Eh* inoculated animals (Fig 4-3 F arrows) demonstrating intense mucus secretion with cavitation and/or mucus depleted goblet cells.

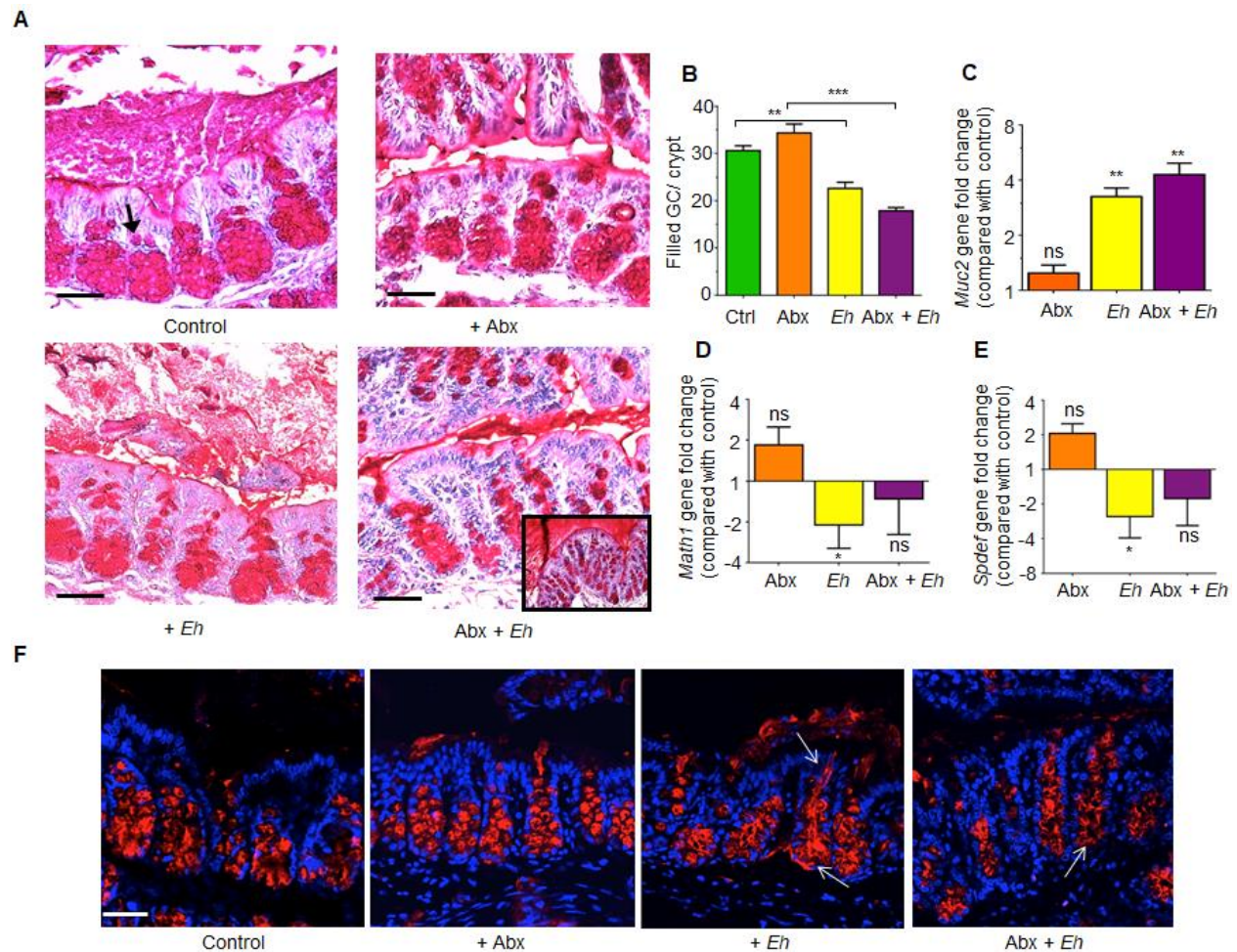


Figure 4- 3 *Entamoeba histolytica* (*Eh*) in antibiotic-treated mice triggers robust mucus and *Muc2* mRNA expression. **A.** Colonic loops from *Muc2*^{+/+} mice were fixed in Carnoy's solution to preserve the mucus layers and stained with Periodic acid Schiff (PAS) reagent to visualize secreted and goblet cell mucus. Top left untreated control proximal colon with densely packed pear-shaped collection of goblet cells in the crypts filled with PAS⁺ material (arrow). Top right is the Abx- treated group showing similar morphology to the controls. Bottom left is *Eh*-inoculated colonic loops with secreted mucus is a watery exudate and less PAS⁺ cells in the crypts. Bottom right is the Abx treated mice inoculated with *Eh* showing fewer filled PAS⁺ cells in the crypts and with a dense mucus plugs on the surface of the mucosa (inset). **B.** Quantification on the number of filled goblet cells per crypt. **C.** Gene expression for *Muc2*, **(D)** *Math1* and **(E)** *Spdef*. **F.** Confocal microscopy using *Muc2* specific antibody (red) and counterstained with DAPI (blue) to visualize *Muc2* filled goblet cells in sections for A above. Scale bar = 50 μ m. n= 6. *P < 0.05, **P < 0.01, ***P < 0.001.

To quantify mucin and non-mucin glycoprotein secretions, mucus in *Muc2*^{+/+} littermates were metabolically labeled with ³H-glucosamine that incorporates into galactose, N-acetylgalactosamine and N-acetylglucosamine glycans in newly synthesized mucin. The ³H-labeled glycoproteins secreted in response to *Eh* were then fractionated into high molecular weight V₀ mucin and non-mucin components by Sepharose 4B column chromatography (Fig 4-4A)⁶. Abx treatment had no effect on constitutive mucin or non-mucin glycoproteins secreted in the colon as compared with untreated control (Fig 4-4 A, B and C orange panels). Expectedly¹⁶, *Eh* significantly stimulated not only V₀ mucin but also non-mucin glycoprotein secretions (Fig 4-4 A, B and C yellow panels). Surprisingly, Abx-treated mice inoculated with *Eh* (Abx + *Eh*) showed enhanced secretions of both mucin and non-mucin glycoproteins (Fig 4-4 C, D and E purple panels) as compared to animals that were not treated with Abx but inoculated with *Eh* (*Eh* infected group). To exclude the possibility that the enhanced mucus secretory effect was not due to the Abx but rather, dysbiotic microbiota, animals were transplanted with their own microbiota (FMT) following Abx treatment and then inoculated with *Eh*. Remarkably, FMT normalized both ³H-mucin and non-mucin glycoproteins secretions equivalent of animals inoculated with *Eh* that did not receive Abx (Fig 4-4 C, E and F gray). Taken together, these results indicate that dysbiotic microbiota provokes enhanced mucin and non-mucin secretions in response to *Eh*.

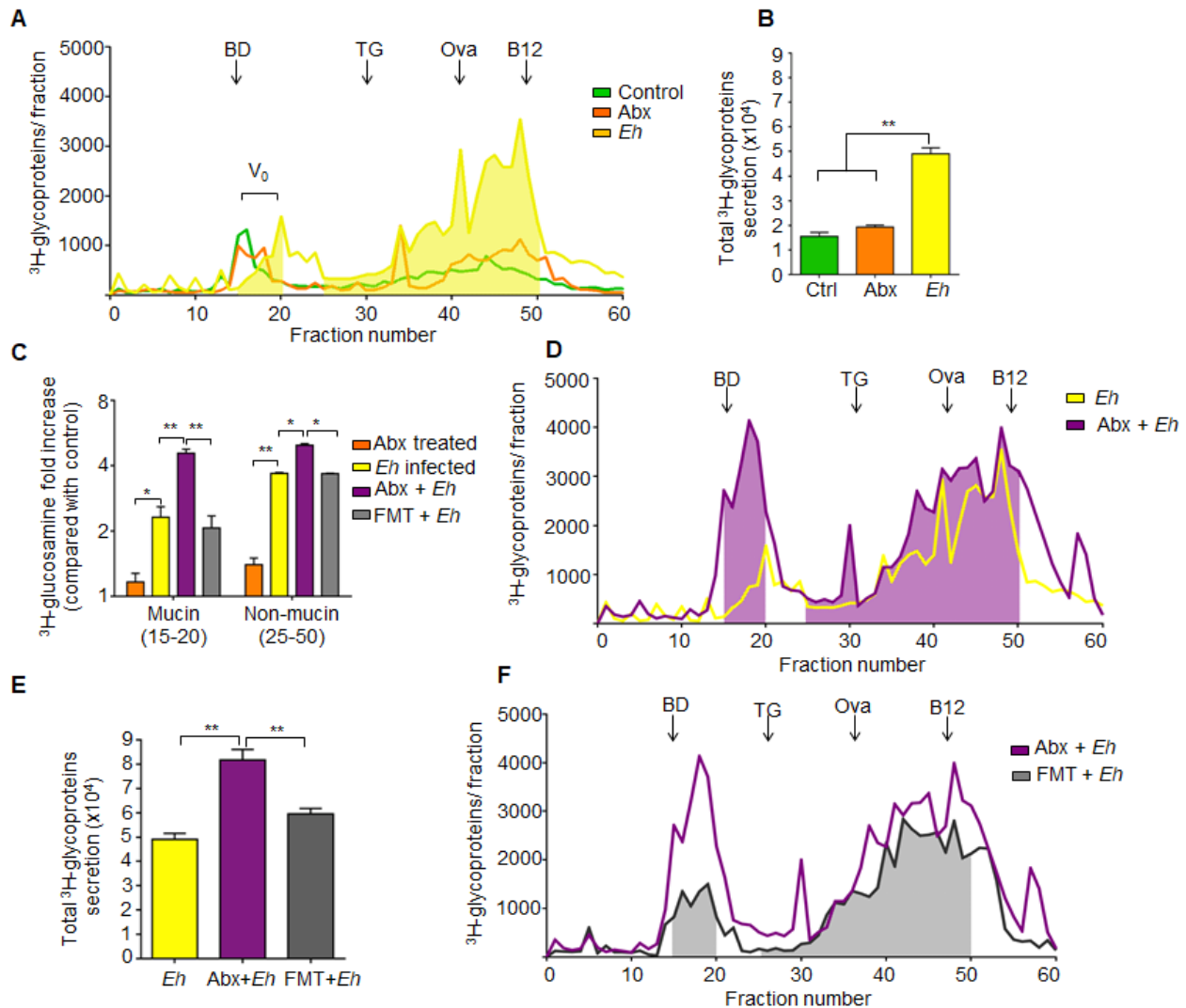


Figure 4- 4 Reduction in bacterial load with antibiotics increases *Eh*-induced colonic mucus and non-mucus secretions. To quantify the release of newly synthesized mucus, mucin was metabolically labeled with ^3H -glucosamine that incorporates into mucin galactose and N-acetylgalactosamine glycan residues. (A, D, F) In response to *Eh* in closed colonic loops, secreted ^3H -labeled glycoproteins were fractionated on a Sepharose 4B column to separate high molecular weight mucin (V_0 ; fractions 15-20) from non-mucin glycoproteins (fractions 25-50). (B, E) Total ^3H -counts (CPM) released constitutively in control, Abx-treated and fecal microbial transplant (FMT) animals in response to *Eh*. C. Fold change in ^3H -mucin V_0 mucin and non-mucin release among the treated groups as shown in the legend. The column was equilibrated with BD: blue dextran (2,000 kilodaltons), TG: thyroglobulin (660 kilodaltons), Ova: chicken ovalbumin (42.7 kilodaltons), B12: Vitamin B12 (1.4 kilodaltons). $n = 8$, $*P < 0.05$, $**P < 0.01$.

4.4.3 Eh suppresses Math1 transcriptional activity in colonic cells

Based on the differential *Math1* gene expression (Fig 4-3 D), we next investigated the fate of the secretory goblet cells in the colon following Abx treatment and in response to *Eh*. To interrogate this, we used *Math1*^{GFP} mice containing the green fluorescent protein (GFP) reporter for *Math1*-expressing goblet cells. In the colon, *Math1* is expressed in epithelial cells to differentiate into Muc2-producing goblet cells lineage. We have recently used *Math1*^{GFP} mice to quantify goblet cells by flow cytometry and by microscopy to follow the fate of goblet cells in DSS-induced colitis¹⁹. Basally, *Math1*^{GFP} activity was higher in the proximal than the distal colon in control animal. However, following Abx treatment, while fluorescence activity in the proximal colon remained unchanged there was a significantly decrease in *Math1* activity in the distal colon (Fig 4-5 A, B). Surprisingly, control mice inoculated with *Eh* showed a significant decrease in *Math1*^{GFP} activity in the proximal colon with a corresponding increase in activity in the distal colon. In Abx treated mice while *Math1*^{GFP} activity in the proximal colon remained the same as control animals, in response to *Eh* *Math1* activity in the distal colon was completely silenced (Fig 4-5 A, B). These results suggest that *Eh* suppresses *Math1* activity in the proximal colon where *Eh* are contained within loops but enhances *Math1* activity distally through a mechanism that is microbiota-dependent.

To determine if the down regulation in *Math1* activity was a direct effect from *Eh* or initiated by *Eh*-induced inflammation, LS174T human colonic goblet were inoculated with *Eh* and *MATH1* mRNA expression quantified. Within 30 min, *Eh* significantly decreased *MATH1* mRNA expression that remained low up to 1h (Fig 4-5 C). As predicted, glutaraldehyde-fixed equivalent

numbers of *Eh* had no effect on *MATH1* mRNA expression, suggesting a requirement for only live parasites (Fig 4-5 C). In acute intestinal amebiasis, IL-1 β is one of the most important pro-inflammatory cytokines elicited by the cysteine protease 5 (*EhCP5*) RGD motif ligating host cells integrins²⁰ and this cytokine had no effect on *MATH1* transcription (Fig 4-5 C). These data suggest that *Eh* had a direct effect on *MATH1* transcription that was dependent on microbiota.

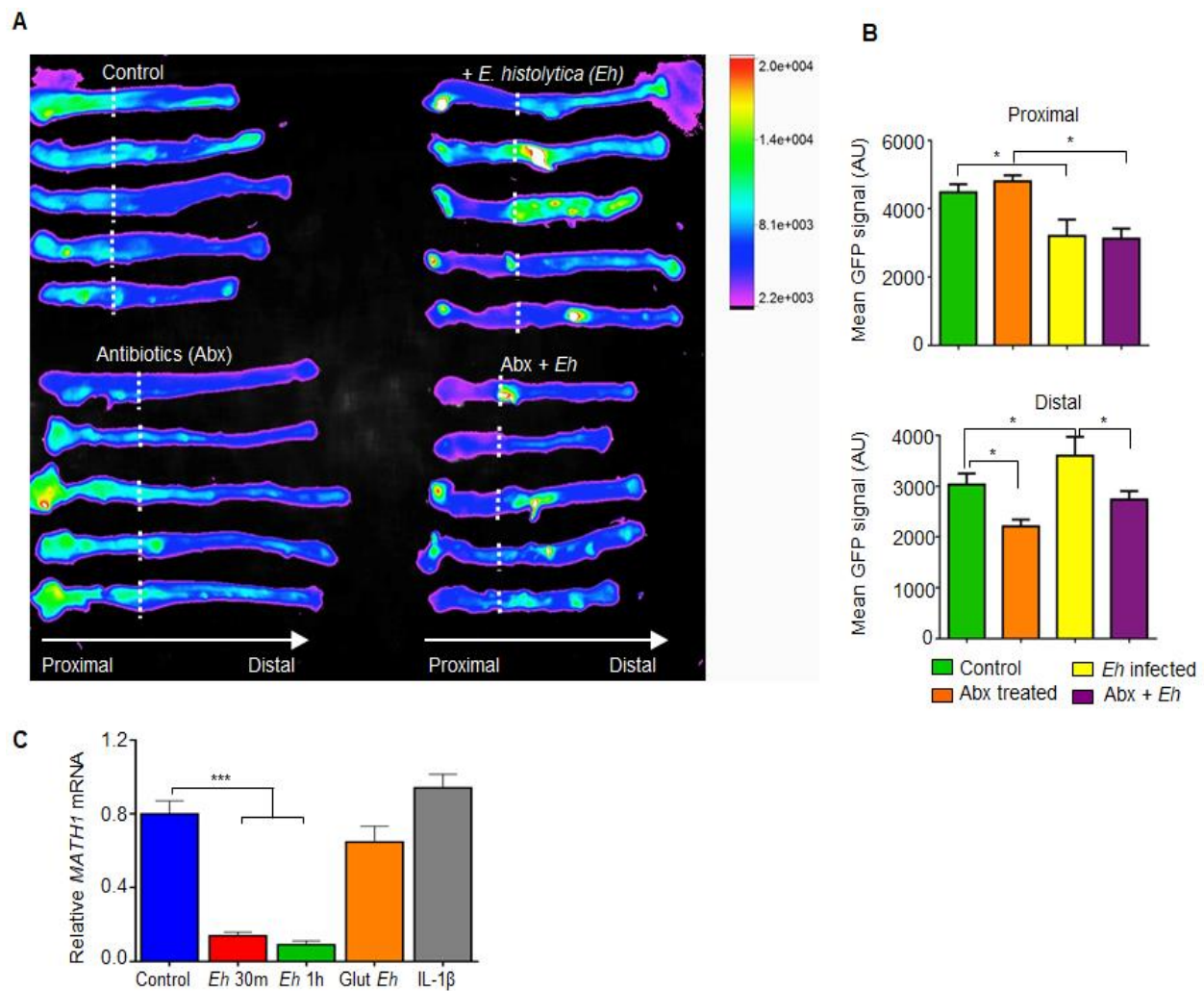


Figure 4- 5 *Entamoeba histolytica* (*Eh*) suppresses Math1 mRNA expression in colonic goblet cells *ex vivo* and *in vitro*. **A.** Math1 expression heatmap in Math1^{GFP} mice colons, the dotted line indicates the colonic loop ligation. **B.** Each colon was divided into three sections (proximal, middle and distal) and GFP signal was quantified. **C.** Monolayers of LS 174T human goblet cells were either exposed to *Eh* for 30 min (m), 1 h, fixed *Eh* with glutaraldehyde 2.5% (Glut), or pre-treated with IL-1β. mRNA expression levels of transcription factor *MATH1* were measured by qPCR and normalized with *GAPDH* levels. GFP: green fluorescent protein, AU: arbitrary units. n=6, *P < 0.05. ***P < 0.001.

4.4.4 Colonic microbiota is required for development of innate host defense against Eh

The role microbiota plays in shaping the development of innate host defenses against *Eh* is not known. Based on the results above, microbial dysbiosis induced with Abx in *Muc2*^{+/+} and *Muc2*^{-/-} specific pathogen-free (SPF) littermates markedly enhanced pro-inflammatory cytokine and mucin secretory responses toward *Eh*. To interrogate the distinct role of microbiota in the development of innate host defenses we quantified *Eh*-induced host responses in colonic loops of germ free (GF) mice. As expected, in response to *Eh*, SPF mice elicited a prompt increase in the expression of the pro-inflammatory cytokines TNF- α and IL-1 β mRNA whereas no response was observed in GF mice (Fig 4-6 A). This is interesting as enhanced watery secretions were observed in *Eh*-inoculated colonic loops of GF mice (Fig 4-1 D). Surprisingly, basal *Math1* and *Muc2* mRNA expressions were very low in GF mice and *Eh* infection did not cause a further decrease as compared to *Eh* inoculated SPF animals (Fig 4-6 B and C). A similar decrease in myeloperoxidase (MPO) activity, a marker for neutrophils influx into the colon, was also noted in GF mice. This is in contrast to *Eh* in SPF mice that induced 4- and 3-fold increase in MPO activity in the proximal and distal colon, respectively (Fig 4-6 D). A dependency for microbiota in *Eh*-induced inflammation was shown by treating SPF mice with Abx that reduced MPO activity to those seen in GF mice in both the proximal and distal colon (Fig 4-6 D). As *Muc2* and pro-inflammatory cytokine gene expression was associated with increased mucin secretion (Fig 4-6 A and C), we quantified the number of filled goblet cells (GC) in GF mice. In SPF, there was a significant reduction in filled GC in response to *Eh* as GC are actively releasing mucus in response to *Eh* (see below) whereas in GF mice we did not observe a decrease in filled GC. In fact, GF mice had less numbers of filled GC in the colon (Fig 4-6 E).

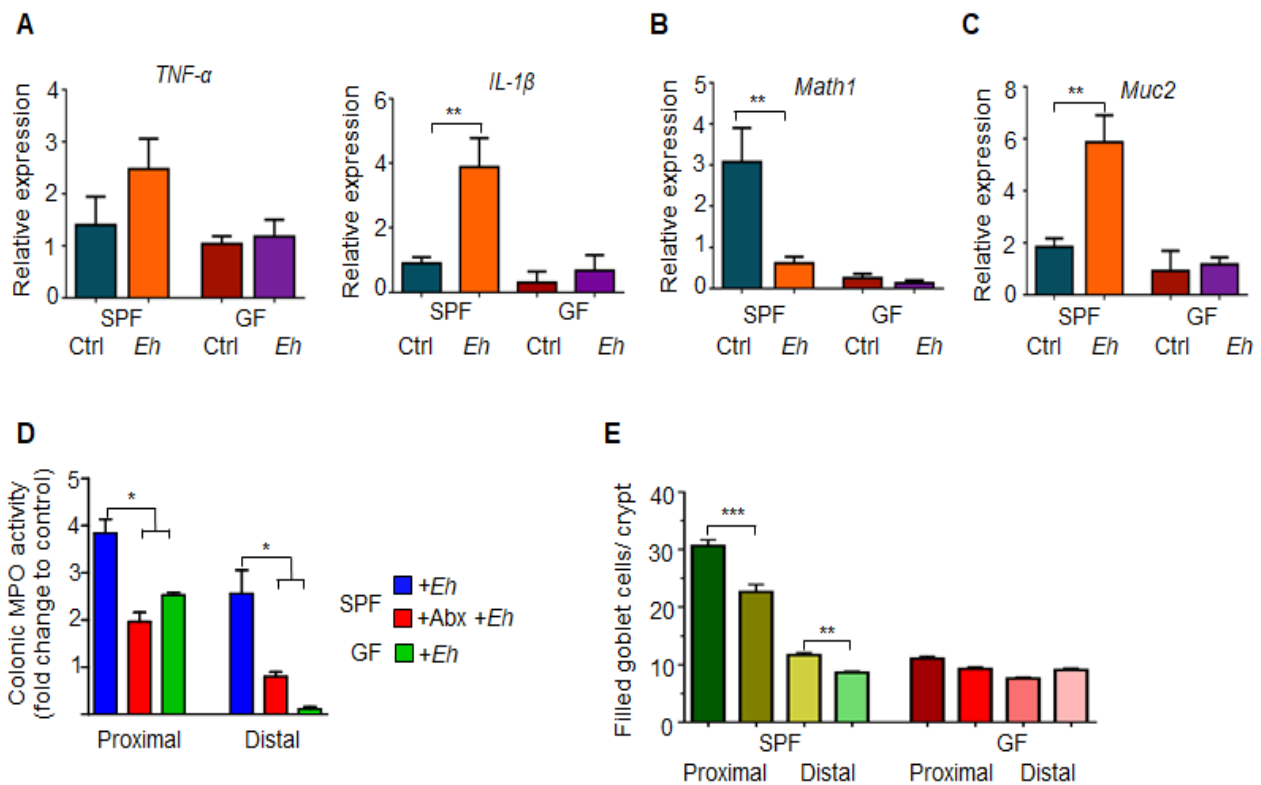


Figure 4- 6 Germ-free mice have a deficient immune response towards *E. histolytica*. Amebic colonic loops were performed in germ-free (GF) mice and pro-inflammatory response towards *Eh* was measured and compared with specific pathogen free (SPF) animals. **A.** Relative expression of pro-inflammatory cytokines *TNF-α* and *IL-1β* in the proximal portion of the colon. **B.** Relative expression of the transcription factor *Math1*, and **(C)** *Muc2* in the proximal colon. **D.** Myeloperoxidase activity in the proximal and distal colons of SPF and GF mice inoculated with *Eh*, fold change was compared with untreated SPF mice. **(E)** Number of filled goblet cells/crypt was blindly counted on distal and proximal colons in GF and SPF mice. Abx: antibiotics, MPO: myeloperoxidase. n=6, *P < 0.05, **P < 0.01.

Colonic tissues were fixed in Carnoy's to preserve the mucus layers and stained with periodic acid-Schiff reagent to visualize mucus, goblet cells and *Eh*. In response to *Eh*, there was hyper secretion of mucus in SPF mice with cavitated (empty shown by the arrow) GC (Fig 4-7 A), thick adherent dense inner mucus (IM) and a loose outer mucus layer (OM) with *Eh* (Fig 4-7 A). In GF mice the adherent mucus layer in the proximal colon was patchy with low numbers of GC. Nonetheless, *Eh*-induced intense mucus secretions from GC in the shallow crypts (Fig 4-7 B, arrows). Most striking however, unlike SPF were we rarely observe *Eh* in contact with the epithelium, in GF mice *Eh* were occasionally found bound to surface and adjacent epithelial cells (Fig 4-7 C; arrows and inset) and at places, showed signs of epithelium erosion in direct contact with *Eh* (Fig 4-7 D arrows). Taken together, these results underscore a critical role for microbiota in the development of an effective mucus barrier and host pro-inflammatory cytokine responses in innate host defense against *Eh*.

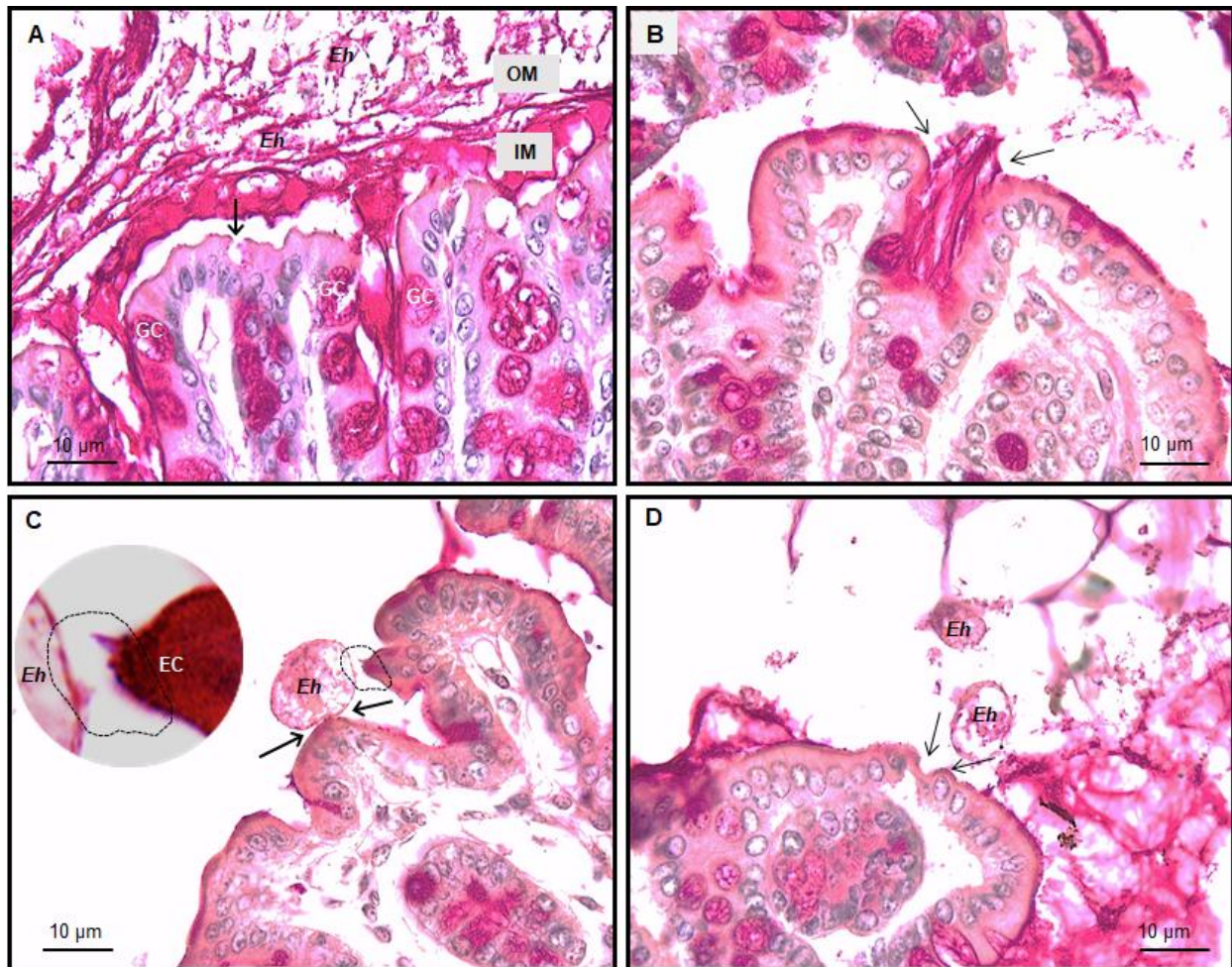


Figure 4- 7 *E. histolytica* disrupts the colonic epithelium in germ-free mice. Colonic loops inoculated with *Eh* in specific pathogen-free (SPF) and germ-free (GF) mice were fixed in Carnoy's solution and stained with periodic acid-Schiff reagent. **A.** Representative images of SPF animals inoculated with *Eh* showing thick adherent inner mucus (IM) layer and a loose outer mucus (OM) layer, with cavitated goblet cell (GC; arrow) due to mucus depletion shown. **B.** Intense mucus secretion in response to *Eh* in GF (arrows) mice; note the lower number of goblet cells in the surface epithelium and crypts. **C.** *Eh* attaching to GF colonic epithelial cells (EC) (arrows), the epithelial cell on the right appear to be plucked out (inset) by *Eh*. **D.** The arrows point at disruption of the single layer of epithelial cells by *Eh* in close proximity to the erosion.

4.5 Discussion

A major deficiency in our knowledge gap is the relationship between *Eh* and colonic microbiota in parasite colonization, disease pathogenesis and innate host defenses. As microbiota colonizes and utilizes MUC2 mucin substrates as a food source to maintain homeostasis, it stands to reason a delicate balance must exist to sustain asymptomatic *Eh* infections. *Eh* colonizes in/on the MUC2 mucin layers and here it interacts freely with colonic microbiota without adverse effects on the host. At present, we do not know the distinct contribution of the microbiota and/or the Muc2 barrier in fortifying innate host defenses against *Eh*. This was the impetus for this study where we interrogated the distinct roles of both colonic microbiota and the mucus barrier in early responses towards *Eh* using colonic loops as a short-term infection model. The major findings of our study revealed that microbial dysbiosis played a critical role in *Eh*-induced water and mucus secretion and pro-inflammatory cytokine responses that was restored following fecal microbial transplants. Moreover, studies in germ free mice revealed that microbiota was critical for shaping the intestinal landscape for the development of goblet cells and formation of an effective mucus barrier and in educating the host pro-inflammatory cytokine responses to limit *Eh* binding and erosion of the surface epithelium.

We have previously shown that *Muc2*^{-/-} are highly susceptible to *Eh*-induced secretory and pro-inflammatory responses compared to commercially bought WT animals on the same genetic background¹⁶. In this study, we used *Muc2*^{+/+} and *Muc2*^{-/-} littermates to normalize the microbiota and surprisingly showed no differences in gross pathology scores among the genotypes. This highlights that the use of littermates are essential for microbiota studies as it greatly reduces

the variability caused when using animal models in research ²¹. Here, the absence of a mucus barrier did not leave *Muc2*^{-/-} mice with a noticeable disadvantage to *Eh* as compared to mucus sufficient littermates, thus demonstrating that the protective role of the mucus barrier is intimately related to the host microbiota. It is well known that an Abx regime provokes an alteration in microbial abundance ²². This particular dysbiotic state is characterized with an increase in facultative anaerobic bacteria within the Enterobacteriaceae family, and has proven to be an indication of a non-homeostatic state in both animal models as well as in important human gastrointestinal diseases ²³. This switch to a more oxygenated luminal environment, could explain the exacerbated reaction towards *Eh*, characterized by an increase in pro-inflammatory cytokines IFN- γ and TNF- α , as well as chemokines MCP-1, KC and MIP-2. *Eh*, despite being a microaerophilic organism, possesses an arsenal of virulence factors to live in the colon ²⁴, but also has various mechanisms that not only protects it, but also allows *Eh* to invade into highly oxygenated environments such as in the case of extra-intestinal amebiasis ²⁵.

In addition to mucin staining, Periodic-acid Schiff (PAS) reagent allowed us to visualize *Eh* and we consistently fail to observe *Eh* in close contact with the epithelium. This is interesting as the host is able to sense *Eh* secreted components and/or the altered environment to mount water and mucus secretions as well as pro-inflammatory cytokine and chemokine responses. Abx treatment alone did not affect basal mucus production or the numbers of filled goblet cells, *Muc2* gene expression or total ³H-glycoprotein secretion. Curiously, previous studies have shown that macrolides Abx have an inhibitory effect on mucus production in airways ²⁶ and is used therapeutically in the treatment for chronic obstructive pulmonary disease (COPD), reducing airway goblet cells

production of MUC5ac mucin and alleviating COPD symptoms ²⁷. A similar effect has been described with Muc2 mucin in the gastrointestinal tract, utilising different Abx treatment regimes that reduced the number of goblet cells and *Muc2* gene expression in mice ²⁸, as well as mucus layer thickness ²⁹. At present, no other studies have reported Abx-induced mucus increase. In our study, we did not observe a reduction but rather, a slight increase in mucus production in Abx-treated mice and could be explained by the Abx regime used in our study.

The mucus layer, secreted by goblet cells, is a key player in maintaining intestinal homeostasis, mainly acting as a barrier and limiting contact between the epithelial cells and any potential hazard contained in the lumen ^{30,31}. Math1 is a transcription factor that differentiates intestinal stem cells into a secretory lineage, which includes Paneth cells, enteroendocrine cells and goblet cells ³². Paneth cells are absent in the colon, likewise, enteroendocrine cells are more abundant in the small intestine, but some of them, like L, D and Enterochromaffin cells (EC), can still be found in colon and rectum ³³, although, they form only about 1% of the cells in the colon. In our study, we conclude that Math1 activity visualized using Math1^{GFP} mice had a greater effect on goblet cells than on any other secretory colonic cell lineage. An interesting finding in our study is that *Eh* decreased Math1 activity in the proximal colon where parasites were contained within colonic loops with a corresponding increase in Math1 activity in the distal colon. The effects of pro-inflammatory cytokines and/or neuronal signalling downstream to the distal colon could explain this remote effect. To date, there is a paucity of information on how intestinal pathogens affect the transcription factor Math1. Studies with the nematode parasite *Trichinella spiralis* showed an increase in *Math1* mRNA expression in the small intestine as well as induction of goblet cell

metaplasia when the parasite was present ³⁴, suggesting that Math1 has a protective role in the intestine. DSS-induced colitis in rats had no effect on Math1 activity ³⁵. Unfortunately, the exact mechanism by which this transcription factor exerts its protective activity is not yet fully described. Although the effect of the Abx cocktail we used was generic, this regime reduced Math1 activity basally only in the distal colon. This regional effect could be due to the greater reduction in bacterial load and dysbiosis affecting the distal colon but this is only speculation.

Studies done in germ free (GF) guinea pigs showed that the presence of microbiota is necessary for *Eh* to express its pathogenicity ⁹. Similarly, infection of GF mice with the parasite *Giardia duodenalis* failed to induce the characteristic pathology ³⁶. Likewise, GF mice infected with the protozoa *Leishmania amazonensis* presented with an innocuous infection and absent immune response towards the parasite ³⁷. The exact mechanism that explains this phenomenon is not clearly understood, but clearly suggests that microbiota plays a fundamental role in establishing the pathogenicity of these protozoa. Our results are in concordance with previous observations, as *Eh*-inoculated in colonic loops in GF mice failed to induce the characteristic pro-inflammatory response in spite of modest water and mucus secretions in the colon. This phenomenon could be due to an undeveloped immune response in GF animals that rendered them with a limited ability to produce cytokines in response to a colonic pathogen. A requirement for cytokines was shown with *Schistosoma mansoni* infection in GF mice, where it is known that TNF- α was required for optimal proliferation of the parasite in the host ³⁸. The only parasite that showed a worst outcome in GF mice is infection with the protozoa *Trypanosoma cruzi* ³⁹. The exact mechanisms to explain this altered susceptibility are not known.

Our finding on reduced number of goblet cells correlates with previous reports where a characterization of the colonic epithelium in GF mice showed decreased numbers of goblet cells⁴⁰. Reduction in goblet cells appear to be systemic as it was also observed in paranasal sinuses⁴¹ and in the conjunctiva⁴² in these mice. GF mice have a thinner and penetrable intestinal mucus layer⁴³ and Muc2 monomers with shorter O-glycans⁴⁴ compared to conventional SPF mice. Based on this, we hypothesise that the reduction in thickness and changes in the biochemical structure of Muc2 rendered the mucus barrier more susceptible to *Eh* cleavage. It is possible that *Eh* glycosidases and proteases could degrade GF mucus with higher efficiency as they have shorter glycans and absence of commensal microbiota. As *Eh* utilizes microbiota and cleaved Muc2 substrates as its primary source of food, it is tempting to speculate that *Eh* in the GF colon need to find alternatives nutrient sources forcing the parasite to move closer to the epithelium. This could explain why we consistently see *Eh* in direct contact with the epithelium with epithelial erosions in the GF colon, a condition we never see in SPF mice.

These studies clearly show a requirement for colonic microbiota in forming the first line of innate host defense against *Eh* independent of the Muc2 mucus layer. Disruption of microbiota with Abx sensitized animals for exacerbated pro-inflammatory responses and high output water and mucus secretion toward *Eh* that was normalized with fecal microbial transplants. In the absence of microbiota, *Eh* failed to induce pro-inflammatory responses that together with a dysfunctional mucus layer allowed *Eh* to contact and disrupt the epithelium.

4.6 Materials and methods

4.6.1 Animals

8 to 10 weeks old *Muc2*^{+/+} and *Muc2*^{-/-} littermate mice on a C57BL/6 background were used in the experiments. *Math1*^{GFP} mice (also known as *Atoh1*^{tm4.1Hzo})⁴⁵ were purchased from Jackson laboratory and bred in-house. Germ-free (GF) mice on a C57B/6 background were purchased from the Microbiome Centre at the University of Calgary. All animals were housed under specific pathogen-free conditions (SPF) in filter top cages and fed autoclaved food and water *ad libitum*. Throughout the study, animals were closely monitored to ensure healthy conditions, in addition, all experiments adhered to the University of Calgary Animal Care Committee standards.

4.6.2 Cultivation and harvesting of E. histolytica

E. histolytica (HM1:IMSS) trophozoites were cultured in TYI-S-33 medium containing 100 U/ml penicillin/streptomycin at 37°C under axenic conditions. After 72 h, logarithmic-growth-phase *Eh* cultures were harvested by chilling on ice for 9 min, pelleted at 200 × g, and washed twice with PBS. Trophozoites were subjected to routine passage through liver of gerbils to maintain high virulence.

4.6.3 Antibiotic (Abx) treatment

Muc2^{+/+} and *Muc2*^{-/-} littermates were treated with Abx to decrease bacterial load as described previously⁴⁶. Briefly, mice were gavaged every 12 h with an Abx cocktail as follow: for the first

3 days mice were gavaged with amphotericin-B (1 mg/kg) to suppress fungal growth. From day 4, ampicillin (1 mg/mL) was added to the drinking water; in addition, mice received orally vancomycin (50 mg/kg), neomycin (100 mg/kg), metronidazole (100 mg/kg) and amphotericin-B (1 mg/kg) for another 14 days. This combination ensures the safe and controlled delivery of Abx to each mouse while having a broad-spectrum effect.

4.6.4 Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) was achieved by collecting 0.1g of mice feces (about 4 fecal pellets), homogenized in 1 mL of sterile phosphate buffered saline (PBS) and centrifuged for 30 seconds at 1000 x g. Each mouse was gavaged with 200µl of the obtained supernatant every 48 h for a total of three times.

4.6.5 Colonic loops an in vivo mucin secretion

To quantify mucin secretion *in vivo*, mice were fasted overnight and injected intraperitoneally with 20 µCi of ³H-glucosamine (PerkinElmer, Waltham, MA) in PBS for 3 h to metabolically label newly synthesized mucin, as described previously^{16,47}. In this process ³H-glucosamine is converted into galactose, *N*-acetyl-glucosamine and *N*-acetyl-*D*-galactosamine in the mucin monomer. Colonic loops were used as a model for short-term infection studies (3 h after infection), as described previously⁴⁸. Briefly, *Muc2*^{+/+} and *Muc2*^{-/-} mice were anesthetized with isoflurane inhalant anesthesia (Pharmaceutical Partners of Canada, Richmond Hill, ON). A laparotomy was performed, and the colon was exteriorized and ligated with 3-0 black silk sutures (Ethicon,

Somerville, NJ; Peterborough, ON, Canada) at the proximal end and ~2 cm below. Care was taken to keep the mesenteries, blood vessels, and nerves intact. Virulent log-phase *Eh* trophozoites (1×10^6) in 100 μ L PBS (pH 7.3) were inoculated into the loop. To quantify the secretion of high molecular weight (V_o) mucin and non-mucin components, secreted ^3H -labeled glycoproteins were fractionated using a Sepharose 4B columns as described previously^{16,47}.

4.6.6 Gross pathology scoring

Gross pathology of colonic loops was assessed on a scale of 1 to 4, as follows: 1, normal colon (uniform thickness, no colon dilation or distension, no blood in luminal contents); 2, minimal damage (visible mucosal thickening and colonic distension, visible mucosal exudates, and expanded loop occupying <50% of the abdominal cavity); 3, extensive damage (thickening of the colonic mucosa, visible dilation of surface blood vessels, colon distension with visible luminal contents, mucosal exudates, and expanded loop occupying 50% of the abdominal cavity); 4, inflamed colon (extensive colon thickening, colon surface with extensive inflamed dilated blood vessels with or without haemorrhage, extensive colon distension with or without visible brown or bloody luminal contents, mucosal exudates under extreme pressure leading to ballooning of the colon, and expanded loop occupying >50% of the abdominal cavity).

4.6.7 Histology and staining

At the endpoint of the experiments, animals were anesthetized and sacrificed by cervical dislocation and the colon was excised. For histology, colonic tissues were fixed in Carnoy's

solution, and embedded in paraffin blocks. 7µm tissue sections were rehydrated through an ethanol gradient to water and stained with Periodic acid Schiff's reagent (PAS, Sigma Aldrich Co.) to visualize neutral mucins.

4.6.8 Quantification of pro-inflammatory cytokines and chemokines

Total RNA was isolated from snap-frozen tissue using the Trizol reagent method (Invitrogen; Life Technologies, Burlington, ON) as per manufacturer's specifications, and the yield and purity determined by the ratio of absorbance at 260/280nm (NanoDrop, Thermo Scientific). Only samples with a ratio of ~1.8 for DNA and ~2.0 for RNA were considered. cDNA was prepared using a qScript cDNA synthesis kit (Quanta Biosciences). Real-time qPCR was performed using a Rotor Gene 3000 real-time PCR system (Corbett Research). Each reaction mixture contained 100 ng of cDNA, SYBR Green PCR Master Mix (Qiagen) and 1µM of primers. A complete list of the primer sequences and conditions used are listed in Table 4-1. Results were analyzed using the $2^{-\Delta\Delta CT}$ method and expressed as fold changes. Luminal pro-inflammatory cytokines was analyzed using a mouse 31-plex cytokine–chemokine panel (Eve Technologies, Calgary, AB, Canada).

Table 4- 1 Primer sequences used for quantitative real-time PCR. Murine primers as previously described¹⁶

	Name	Sequence 5` 3`	Annealing Temp
Murine	IL-1 β	Fwd: GCCTCGTGCTGTCGGACCCA Rev: CTGCAGGGTGGGTGTGCCGT	60°
	TNF- α	Fwd: ATGAGCACAGAAAGCATGATC Rev: TACAGGCTTGTCACCTCGAATT	56°
	IFN- γ	Fwd: TCAAGTGGCATAGATGTGGAAGAA Rev: TGGCTCTGCAGGATTTTCATG	54°
	Muc2	Fwd: CCCAGAAGGGACTGTGTATG Rev: TGCAGACACACTGCTCACA	56°
	Actin	Fwd: CTACAATGAGCTGCGTGTG Rev: TGGGGTGTGAAGGTCTC	54°
	Math1	Fwd: AAAGGAGGCTGGCAGCAA Rev: TGGTTCAGCCCGTGCAT	58°
	Spdef	Fwd: GACTCACACTCAAGGGGCAA Rev: TCAGAAGAGTCGTCCGTCCT	58°
Human	MATH1	Fwd: TGCATTCTCGACTTTCGAGGACA Rev: AACTTGCCTCATCCGAGTCACTGT	56°
	GAPDH	Fwd: GGATTTGGTCGTATTGGG Rev: GGAAGATGGTGATGGGATT	56°

4.6.9 Muc2 fluorescence staining

For visualizing Muc2, 7 μ m sections of Carnoy's fixed tissue was incubated with H-300 antibody [1 μ g/ml] at 4°C overnight and secondary anti-rabbit antibody coupled with Alexa 594 and DAPI (Life Technologies) was used for nuclear counterstain. Tissue sections were visualized using an Olympus FV1000 scanning confocal inverted microscope.

4.6.10 Math1 expression via non-invasive whole-body imaging ex vivo

To detect Math1 associated GFP expression, colons of differently treated Atoh1^{GFP} mice were surgically removed and imaged *ex vivo* using an In-Vivo Xtreme 4MP-imaging platform (Bruker, Billerica, MA, USA). Colons were positioned horizontally from the proximal to the distal side and imaged with the luminal side facing the camera. The imaging protocol contained two steps: reflectance imaging (2 sec exposure time) and fluorescent imaging with excitation at 470 nm and emission at 535 nm (5 sec exposure time). Binning was kept constant at 2 x 2. Images from the In-Vivo Xtreme were acquired and analyzed using Bruker molecular imaging software MI SE (version 7.1.3.20550). Math1 associated GFP expression in the colon under different treatment conditions was quantified by measuring the mean fluorescence (after background subtraction) in a constant region of interest (ROI). ROI were either defined as whole colon area (proximal to distal part) or split into proximal, median and distal part for quantification.

4.6.11 LS174T cell culture and Entamoeba histolytica in vitro assay

Human adenocarcinoma colonic goblet cells (LS174T) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 20 mM HEPES, and 100 U/ml penicillin/streptomycin. Cells were passaged with 0.25% trypsin-EDTA (Thermo) once 90% confluence was reached. For experiments, LS174T cells were seeded in 24-well plates in triplicate at a density of 2.5×10^4 and cultured until a confluent monolayer was formed. To determine if *Eh* directly modulated *MATH1* expression in the absence of microbiota, LS174T cells were exposed to 2.5×10^5 trophozoites/ml at 37°C for 30 and 60 min. Glutaraldehyde-fixed

(2.5% for 15 min and washed twice in PBS) *Eh* was used to determine a requirement for live parasites. LS174T cells were pre-treated with human IL-1 β (Peprotech, Cedarlane, Burlington, ON, Canada) at a concentration of 20 ng/mL for 16h to determine if pro-inflammatory cytokines could modulate *MATH-1* expression.

4.6.12 Myeloperoxidase activity assay (MPO)

MPO activity in mouse colon samples (50 mg of fresh-frozen tissues) was assessed as a marker for neutrophil influx as previously described ⁴⁹. Briefly, tissue was homogenized in 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer (pH 6.0). Homogenized tissue was freeze-thawed three times, sonicated, and centrifuged (10,000 g for 10 min at 4°C) for collection of clear supernatant. The reaction was initiated by addition of 1 mg/ml dianisidine dihydrochloride (Sigma, St. Louis, MO) and 1% H₂O₂, and change in optical density was measured at 450 nm.

4.6.13 Statistical analysis

Data was analyzed using Graphpad Prism 6 (Graph-Pad Software, San Diego, CA) for all statistical analysis. Treatment groups were compared using analysis of variance (ANOVA) when more than two groups were compared. Student's t-test was used when only two groups were compared. Statistical significance was assumed at $P < 0.05$, n = total number of mice per group from two independent experiments. Error bars in all the graphs represent mean \pm standard error of the mean (SEM).

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4.8 Author Contributions

ALC and KC conceived and designed the experiments. ALC, MK and FM performed the experiments. ALC analyzed the data. ALC and KC wrote the paper.

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Chapter Five: **Discussion, conclusions and future directions**

The colonic intestinal tract is multilayered and includes the luminal microbiota, the bi-layered Muc2 mucus barrier and a monolayer of epithelial cells. Luminal microbiota plays a key role in promoting digestive health, in limiting important pathogens, as well as in the development of a balanced immune system. The bi-layered mucus acts as a protective barrier and limits the contact between the luminal content and the epithelial cells. The outer mucus layer is constantly exposed to commensals and pathogenic organisms, whilst the inner layer is sterile. Epithelial cells form a physical barrier between the mucosal immune system and the luminal content and spread out among them are goblet cells, the specialized secretory cells that produce Muc2. Alteration in microbial composition, a condition known as dysbiosis, as well as the disruption of the mucus barrier function have been associated with gastrointestinal diseases, such as inflammatory bowel disease (IBD), colorectal cancer and irritable bowel syndrome. Despite the wealth of knowledge on the role of microbiota to health and Muc2 mucin in epithelial barrier function, little is known about their synergistic role in colonic health. Therefore, it was of interest in this study to delineate distinct and cooperative function of commensal microbiota and the Muc2 mucus barrier in maintaining epithelial barrier function critical in intestinal homeostasis.

5.1 Muc2 deficient mice have a distinctive microbiota

Muc2^{-/-} mice were examined to determine if the lack of a protective Muc2 barrier provided them with a distinctive microbiota than their mucus sufficient littermates. At the beginning of this study,

cecum, distal and proximal colon, colonic luminal samples and fecal pellets were analyzed for bacterial phyla distribution. The same trend in microbial composition was observed throughout the samples, reason for which it was decided to do all the 16s sequencing studies based on fecal pellet samples only. Interestingly, *Muc2* deficient and sufficient littermates had similar species richness (alpha diversity), but the species diversity (beta diversity) differs. This finding is of particular interest since it shows that even in the absence of a mucus barrier, which provides shelter and food, bacterial communities are able to settle, live and occupy niches. *Muc2* deficient and sufficient littermates shared the same phyla distribution. However, they had differences in some bacterial families. *Muc2*^{-/-} mice presented higher abundance of Proteobacteria families Desulfovibrionaceae and Pseudomonadaceae, and an increase in the Bacteroidetes family Bacteroidaceae and the Firmicutes family Lachnospiraceae. *Muc2*^{+/+} mice showed increased abundance of the Firmicutes Lactobacillaceae and the Bacteroidetes S24-7 family. These differences in abundance make evident that the mucus barrier takes part in shaping bacterial composition. Furthermore, these changes represent the basal differences between both genotypes. However, it was of much interest to study differences in bacterial population between both fecal microbiota transplantation (FMT) in *Muc2*^{+/+}, since both groups show dissimilarities in susceptibility, and tissue resolution. The results of 16s sequencing showed that the effect of *Muc2*^{-/-} microbiota, when transplanted to mucus sufficient littermates, could be explained due to an increase in members of the Bacteroidaceae, Enterobacteriaceae and Verrucomicrobiaceae families, and a corresponding decrease in members of the S24-7 and Lactobacillaceae families. Hence, these results reinforce the belief of the importance a functional mucus barrier has in shaping a healthy intestinal microbiota. Likewise, the changes in bacterial distribution associated with an increase in intestinal injury susceptibility and

impaired tissue healing overall translates into an increase in Gram-negative pathobionts (mainly from Proteobacteria phylum) and a decrease in fermenter and short chain fatty acids producing bacteria. These findings provide important insights for the development of therapeutic treatment or into prevention of different intestinal pathologies.

5.2 The distinctive *Muc2*^{+/+} and *Muc2*^{-/-} microbiota and its role in chemical and pathogen-induced colitis models

Intestinal injury is associated with increased inflammatory markers and increased permeability¹. Here, I showed that *Muc2*^{-/-} mice have a constitutive inflammatory and permeable phenotype that is microbiota driven, since the administration of broad-spectrum antibiotics to these mice reduced their inflammatory cytokines and permeability levels to those equivalent to mucus sufficient mice. The antibiotic regime utilized in this thesis was based on a published study² that reported a reduction in bacterial load without compromising the mice's health. Antibiotics were administered orally, twice a day for 17 days. Animals were monitored daily, and no signs of weight loss, diarrhea or sickness was observed regarding the genotype of the mice.

Dextran sulfate sodium (DSS)- induced colitis model was chosen for studying the effect of both microbiota and mucus in intestinal injury. This chemical-induced colitis model allows the assessment of gut integrity and host response to microbiota mediated by epithelial cells and immune response. Also, it allows the investigation of the disease progress at the onset, progression and recovery phases. Susceptibility to DSS under antibiotic-induced dysbiosis was only affected in mucus sufficient mice, making evident that a healthy microbiota is required for protection

against colonic injury. Interestingly, *Muc2*^{-/-} mice susceptibility to DSS after antibiotics was not dramatically altered like in mucus sufficient mice. It is possible that this phenomenon could be explained due to their immune cells population. Here, I showed that *Muc2*^{-/-} have increased numbers of CD103⁺CD11C⁺ dendritic cells, which promotes a tolerogenic environment. Moreover, these mice presented increased numbers of IL-22 producing innate lymphoid cells-3 (ILC3), which has been shown that have a protective role in maintaining intestinal homeostasis when the mucus barrier is compromised³. This increase in tolerogenic and in homeostasis-related cells could be explained as a mechanism to counteract the microbiota being in contact with the epithelium, which, as previously discussed, causes a basal inflammatory condition and increased permeability in these mice. Since it has been observed that ulcerative colitis patients have a reduced and compromised mucus barrier⁴, these findings contribute to the knowledge of physiological effects in response to a disrupted epithelial barrier that would advance the field to a better understanding of inflammatory bowel disease (IBD). A schematic diagram of the herein outlined findings is shown in Fig 5.1.

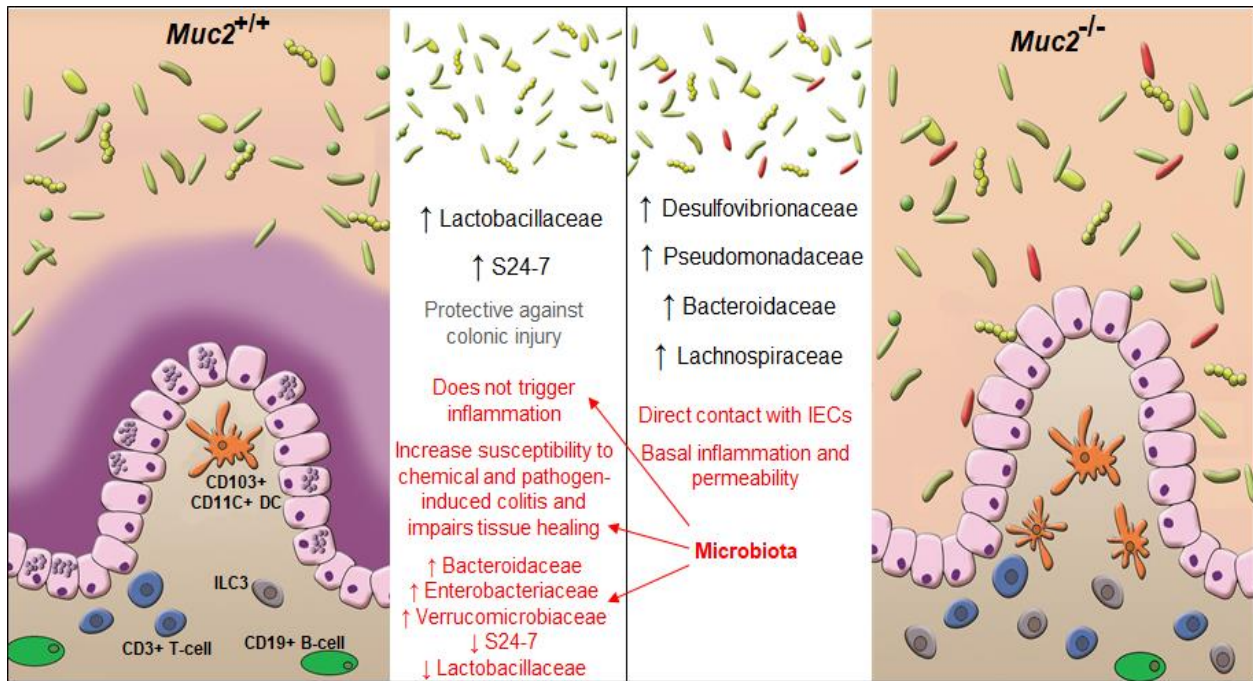


Figure 5- 1 Conceptual diagram of findings in the role of microbiota and an intact mucus layer in epithelial barrier function and colonic injury. Compared with their *Muc2^{+/+}* littermates, *Muc2^{-/-}* mice have higher abundance of members of the families Desulfovibrionaceae, Pseudomonadaceae, Bacteroidaceae and Lachnospiraceae and lower abundance of those within the Lactobacillaceae and S24-7 families. *Muc2^{+/+}* microbiota has a protective role against chemical and pathogen-induced colitis, whereas *Muc2^{-/-}* microbiota's presence was irrelevant to confer protection against colonic injury. The reason for this is a higher number of CD3⁺ T cells, IL-22 producing innate lymphoid cells-3 (ILC3) cells and CD103⁺CD11C⁺ dendritic cells (DC) in *Muc2^{-/-}* mice. The lack of a protective Muc2 barrier allows bacteria to contact epithelial cells, which promotes a basal state of inflammation and intestinal permeability in *Muc2^{-/-}* mice. When transplanted to *Muc2^{+/+}*, *Muc2^{-/-}* microbiota *per se* does not induce inflammation in mucus sufficient littermates, although it does increase their susceptibility to both DSS- and *Citrobacter rodentium*- induced colitis. It also impairs the tissue healing process. This effect could be explained due to an increase in the number of particular members of the families Bacteroidaceae, Enterobacteroidaceae (both containing important pathobionts), an increase in Verrucomicrobiaceae family (bacteria with mucolytic activity), and a corresponding decrease in the S24-7 family, and Lactobacillaceae (important fermenters).

5.3 Microbiota is required for proper immune response against *Entamoeba histolytica*

It is well-known that the presence of commensal microbiota is required for *Eh* to express its pathogenicity⁵. However, an explanation of the mechanisms underlying this phenomenon and the effect of the absence of commensal microbiota in the immune system in the context of ameba infections warranted further investigation. Amebic colonic loops was used as a short-term infection model⁶ and have been very useful to study the innate host response to *Eh*. By using this model, I characterized the host response towards *Eh* in the presence or absence of a mucus barrier, using *Muc2*^{-/-} and *Muc2*^{+/+} littermates, and under different microbiota conditions, using germ-free mice and antibiotic cocktail to induce dysbiosis. A dysbiotic state in SPF made them hyper responsive for increased pro-inflammatory cytokine, chemokine and secretory responses to *Eh*. Interestingly, *Eh* decreased *Math1* transcription, both *in vivo* and *in vitro*. Since *MATH1* expression is required for goblet cells specification, this finding requires further investigation to unravel the mechanism by which *Eh* targets secretory lineage maturation. This study is the first to characterize the host response to *Eh* in germ-free (GF) mice. The fact that *Eh* contacted epithelial cells only in GF mice could be explained by a reduction in goblet cells number⁷, which in turn produced a thinner mucus layer⁸ with different composition of O-glycans⁹. Signs of epithelial disruption were also observed in GF mice. Of particular interest was the fact that despite the closeness of *Eh* to epithelial cells, the typical pro-inflammatory and secretory response to *Eh* was absent. These results reinforce the importance of microbiota in the development of a proper host response towards pathogens. Further investigation will need to be done to better understand the connection between commensal microbiota and immune response to this parasite. A schematic illustration on how *Eh*-microbiota-mucus is interconnected is shown in Fig 5.2.

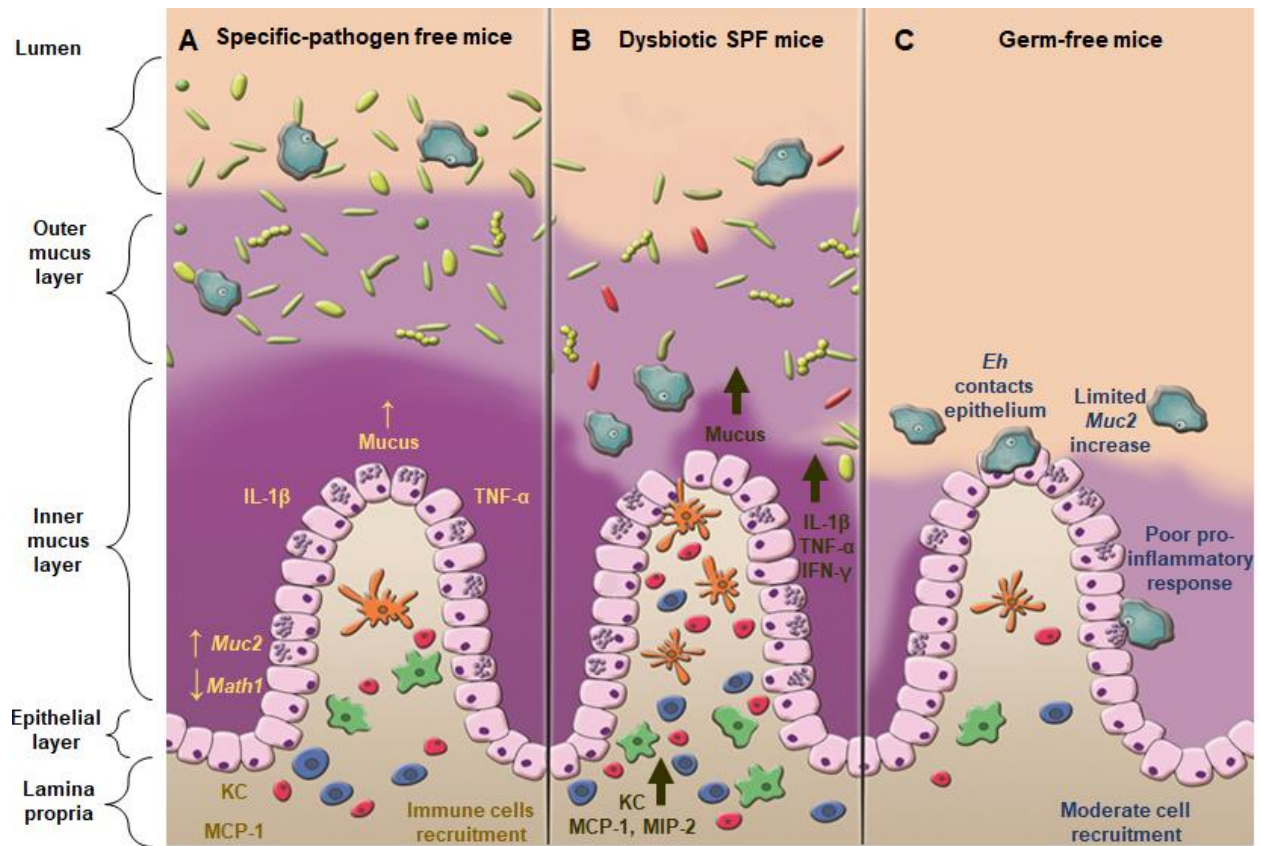


Figure 5- 2 Host response to *E. histolytica* varies under intestinal barrier disruption in the colonic loop model. **A.** In specific-pathogen free mice (SPF), with a functional intestinal barrier, the presence of *Eh* evokes an inflammatory response characterized by increased water and mucus secretion, secretion of pro-inflammatory cytokines IL-1 β and TNF- α , and chemokines KC and MCP-1 with corresponding immune cells recruitment. There is also a reduction of *Math1* transcription factor and an increase in *Muc2* expression. **B.** Antibiotic-induced dysbiosis in SPF mice evoked robust mucus secretagogue response and increased pro-inflammatory cytokines and chemokines release. **C.** In germ-free mice, *Math1* and *Muc2* transcript, as well as pro-inflammatory cytokine expression remain unaltered. Due to a thinner and biochemical different mucus layer, *Eh* readily contacted epithelial cells and disrupted the epithelium.

5.4 Future directions

Since their generation, in 2002¹⁰, *Muc2*^{-/-} mice have become extremely useful to study and characterize the role of Muc2 mucus in intestinal health. Indeed, their usages in different studies have pushed the mucus barrier field forward as well as studies aimed at characterizing distinctive bacterial population¹¹, antimicrobial peptides^{12,13} and immune cells¹⁴. Despite mucus and microbiota being at center stage for their roles in intestinal health and disease, it is still unclear how microbiota and the mucus layers act cooperatively to maintain epithelial barrier function. One of the major functions of gut microbiota is to break down non-digestible carbohydrates into short-chain fatty acids (SCFA) that serves as an important energy source for intestinal epithelial cells¹⁵. Additionally, SCFA have many immunomodulatory effects, attenuating colonic inflammation by modulating immune cell functions¹⁶⁻¹⁸. The results of this study suggest that the increased susceptibility of *Muc2*^{+/+} mice when transplanted with *Muc2*^{-/-} microbiota was due to a decrease in fermenters and SCFA-producer bacteria. In the microbiota field there are still many answers to solve, and an interesting study to follow-up would be the quantitative analysis of SCFA profiles under the different conditions used in this study. Based on the microbial composition data, I predict a reduction of these bacterial metabolites, particularly butyrate, in *Muc2*^{+/+} fecal transplanted with *Muc2*^{-/-} microbiota. Similarly, the increase production of SCFA stimulated either by FMT or by probiotics have shown promising results for the development of future therapies for IBD^{19,20}.

Tissue regeneration in the intestinal epithelium has IL-22 in a leading role²¹. This study suggests that the increase of IL-22-producing innate lymphoid cells 3 (ILC3) observed in *Muc2*^{-/-} mice following antibiotic treatment could explain why these mice did not show an increased

susceptibility when challenged with DSS. The specific blockage of IL-22 in these mice previously challenged with DSS could help to elucidate if this cytokine and/or ILC3 cells play a key role in maintaining intestinal homeostasis when the mucus barrier is compromised. Such studies are valuable for understanding diseases such as IBD, particularly UC where a reduction in mucus layer thickness is observed.

One of the novelties of this thesis was the study of the host response to *Eh* infection in germ-free (GF) mice. Results showed that these mice lack the characteristic pro-inflammatory response to *Eh* infection but maintained watery secretions. The trend in parasite infections is that GF mice do not show signs of disease or fail to show a proper immune response to the parasite²²⁻²⁴. However, there are cases, like the one with the parasite *Trypanosoma cruzi*, in which infection in GF mice had a worse outcome than conventional mice²⁵. For this reason, it would be prudent to study if the observed host response to the parasite was not caused by a delayed immune response in GF mice. This could be easily assessed by increasing the colonic loop time to up to 6 h. Similarly, quantification of mucus secretion, using metabolic labeling with ³H-glucosamine could discern if mucus secretagogues activity is impaired and whether glycosylation of Gal and GalNAc glycans are altered. These studies will underline critical developmental defect in Muc2 mucin biosynthesis, secretion, and glycoform alterations in GF mice demonstrating a complex interconnection between microbiota and mucus.

5.5 Conclusions

This study has unraveled that during a dysbiotic state, intestinal barrier functions are compromised and becomes vulnerable to epithelial damage. Understanding the role of the microbiota in colonization to an intact mucus layer, in epithelial barrier function and disease is of paramount importance. The studies comprised in this thesis cover several interesting findings about the interconnected relationship between microbiota and mucus in promoting colonic homeostasis. Firstly, I have shown that the absence of a mucus layer fosters a dysbiotic microbiota, which in turn promotes a constitutive inflammatory milieu. Secondly, switching to a more tolerogenic immune cell phenotype potentially counteracts basal inflammation caused by the absence of a mucus layer. Thirdly, dysbiotic microbiota increases susceptibility to intestinal injury when transplanted to mucus sufficient animals. Finally, this study shows the importance of commensal microbiota in the development of an appropriate innate immune response against the human parasite *Eh* independent of the presence of a Muc2 mucus layer. Taken together, the research presented in this thesis has contributed significantly to the field of intestinal barrier function and highlight the critical link between the mucus barrier and commensal microbiota in maintaining intestinal homeostasis.

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