MICROBIAL CONTRIBUTION TO PROTEOLYTIC HOMEOSTASIS IN THE GUT

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ABSTRACT

Proteases mediate a vast array of physiological functions, and their enzymatic activity must be tightly regulated by protease inhibitors. Proteolytic homeostasis is crucial in the gastrointestinal tract, and disturbances are associated with several gastrointestinal disorders including irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), colorectal cancer (CRC), and celiac disease (CeD). Perturbed proteolytic balance is hypothesized to contribute to disease pathophysiology by inducing structural and functional changes in the intestinal barrier and promoting inflammation, and modulation of this proteolytic imbalance through delivery of protease inhibitors represents a therapeutic target. Studies addressing proteolytic homeostasis in the gut have focused on proteases and protease inhibitors produced by the host, while largely ignoring the potential contribution of the microbiota. This is a major oversight given the wide repertoire of proteases and protease inhibitors produced by the intestinal microbiota, as well as the strong association between alterations in microbiota and several chronic intestinal disorders. We hypothesized that increased microbial proteolytic activity can elicit deleterious effects on the host, while microbial protease inhibitors such as those produced by probiotic strains may have a therapeutic role in gut inflammatory disorders. The aims of this thesis were: (1) to investigate the contribution of microbial proteolytic activity to the processes underlying disease pathogenesis, and, (2) to test the therapeutic potential of bacterial probiotic strains naturally expressing protease inhibitor genes. We first employed a reductionist model wherein gnotobiotic mice were colonized with microbial communities expressing a specific proteolytic functional phenotype *in vitro*. Colonization of germ-free mice with microbiota with high proteolytic activity resulted in increased systemic translocation of live bacteria and low-grade inflammation. Some of these effects were mitigated after treatment with the probiotic *B. longum* strain expressing its serine protease inhibitor (Srp). Moreover, treatment with this Srp-expressing *B. longum* strain prevented immunopathology in a mouse model of gluten sensitivity. Together, these results demonstrate the relevance of microbiota-derived proteases and protease inhibitors in the modulation of host responses and gut homeostasis.

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LIST OF ABBREVIATIONS

AJ	zonula adherens junction
AMP	anti-microbial peptide
CCL2	chemokine ligand 2
CD	Crohn's disease
CD4	cluster of differentiation 4
CeD	celiac disease
CRC	colorectal cancer
EU	endotoxin unit
FMT	fecal microbiota transplantation
F4/80	epidermal growth factor-like module-containing mucin-like hormone receptor-like 1
GALT	gut-associated lymphoid tissue
GC	goblet cell
GFD	gluten-free diet
GI	gastrointestinal
GMO	genetically modified organism
HBP	heptose-1,7-bisphosphate
HLA	human leukocyte antigen
HNE	human neutrophil elastase
H&E	hematoxylin & eosin
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IEL	intraepithelial lymphocyte
IL	interleukin
ILF	isolated lymphoid follicle
ISC	short-circuit current

LAL	limulus amebocyte lysate
LP	lamina propria
MC	mast cell
MICA	major histocompatibility class I polypeptide-related sequence A
MMP	matrix metalloprotease
MPO	myeloperoxidase
MYD88	myeloid differentiation primary response gene 88
NLS	Natren life start
NOD	nucleotide oligomerization domain
NOD	non-obese diabetic
NLR	nucleotide oligomerization domain-like receptors
PAR	protease-activated receptor
PAS	Periodic acid-Schiff's reagent
PBS	phosphate buffered saline
pIgR	polymeric immunoglobulin receptor
PMN	polymorphonuclear
PRR	pattern recognition receptor
PSA	polysaccharide A
RFU	relative fluorescence unit
SCFA	short chain fatty acid
serpin/Srp	serine protease inhibitor
SFB	segmented filamentous bacteria
sIgA	secretory immunoglobulin A
SLPI	secretory leukocyte peptidase inhibitor
TCA	trichloroacetic acid
TCR	T cell receptor

TGFβ	transforming growth factor beta
TG2	tissue transglutaminase 2
TJ	tight junction
TIMP	tissue inhibitor of matrix metalloprotease
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
Treg	regulatory T cell
UC	ulcerative colitis
V:C	villus to crypt
5-HT	5-hydroxyltryptamine

DECLARATION OF ACADEMIC ACHIEVEMENT

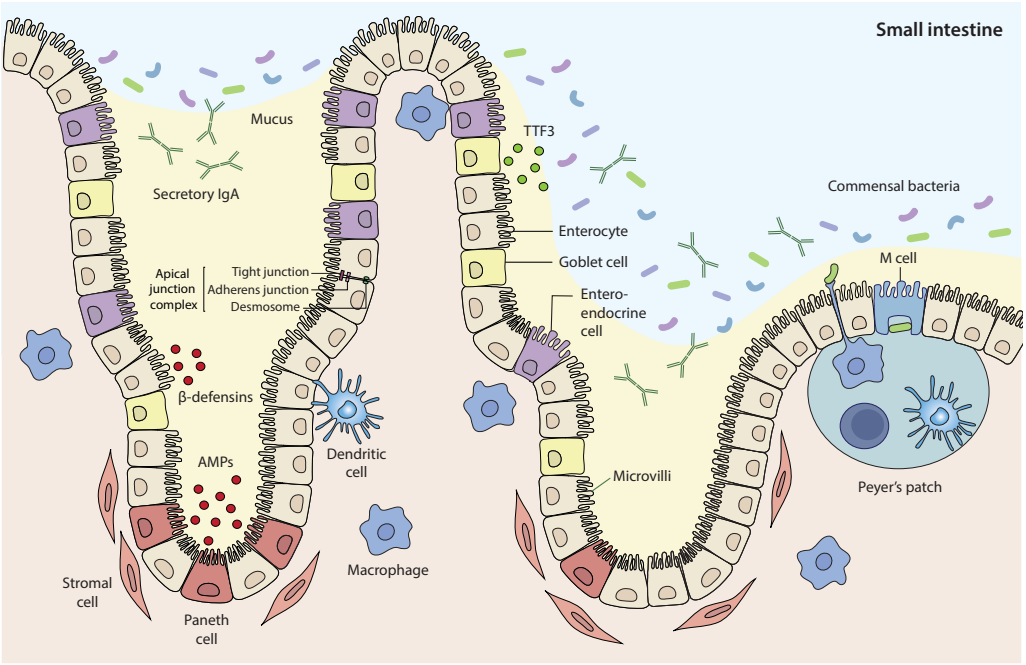
The project in Chapter 3 was initially conceived by Dr. Elena F. Verdu. Experiments were designed by the combined efforts of Jasmine Dong, Dr. Verdu, and Dr. Alberto Caminero. All experiments within Chapter 3 were performed by Jasmine Dong and Dr. Caminero. Data analysis and interpretation of results were conducted by Jasmine Dong under the guidance of Drs. Verdu and Caminero. Insightful direction was provided by Dr. Stephen Collins and Dr. Premysl Bercik, who were members of Jasmine Dong's advisory committee. Experiments in Chapter 4 were conducted and manuscript was written by Jasmine Dong and Justin McCarville with contributions from collaborators at Nestle Research Centre. Jasmine Dong wrote this dissertation with contributions from Dr. Verdu.

CHAPTER 1
INTRODUCTION

INTRODUCTION

1.1 The intestinal epithelial barrier

The gastrointestinal (GI) tract is highly specialized in terms of its anatomy and function. In the small intestine, long, thin villi and absorptive epithelial cells with apical microvilli maximize surface area for digestion and nutrient absorption. The large intestine lacks villi, and specializes in the reabsorption of water, metabolism of undigested dietary residue and expulsion of waste. In addition to dietary and environmental antigens, the GI tract is the largest reservoir of commensal bacteria. The intestinal epithelial barrier must therefore limit exposure of luminal antigens to the underlying immune system, while permitting absorption of ions, water and nutrients[1]. Physical, functional, and immune components, such as epithelial cells, tight junctions, mucus, anti-microbial peptides, and secretory IgA, collectively form a multilayered intestinal barrier (Figure 1.1).



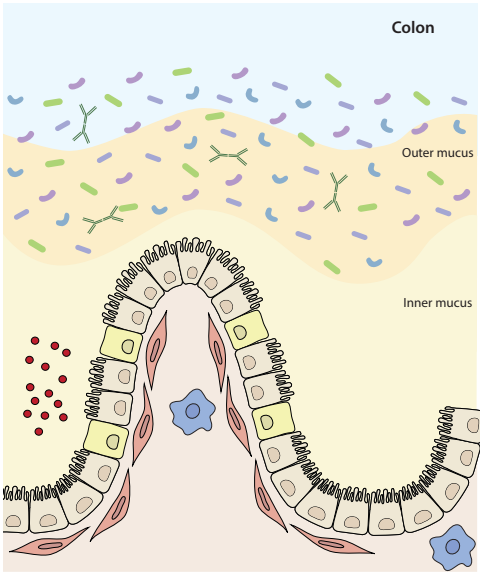


Figure 1.1. Intestinal epithelial barrier. Epithelial cells form a single layer lining the intestinal tract, which limits exposure of luminal antigens to the underlying mucosal immune system. The commensal microbiota, mucus, antimicrobial peptides, sIgA, and immune compartments are all critical in maintaining barrier function in the small intestine (top) and colon (bottom).

The single layer of epithelial cells lining the intestine mostly consist of enterocytes, as well as more specialized goblet cells, Paneth cells, and neuroendocrine cells[2]. Enterocytes regulate the paracellular and transcellular passage of small molecules, ions, and solutes. Epithelial cells are held together by apical junctional complexes which comprise the tight junctions (TJ), zonula adherens junction (AJ), and desmosomes. Paracellular permeability is controlled by TJs in particular, which form integral membrane proteins (claudin, occludin, junctional adhesion molecules) and functional complex proteins (zonula occludens), which directly interact with the cytoskeleton to strengthen the intestinal barrier[3]. Transcellular passage across the epithelium can occur through aqueous pores or actively via endocytosis, and is the primary route for transport of nutrients and electrolytes. Tight junctions are dynamic structures, as they undergo rearrangement and redistribution in response to cytokines, xenobiotics, pathogens, and stress[4], and can therefore modify intestinal permeability in both physiological and pathophysiological states.

Beyond forming an anatomical barrier, intestinal epithelial cells are actively involved in immune defense of the barrier through the production of anti-microbial peptides (AMPs) and mucus. Secretory epithelial cells known as Paneth cells are exclusive to the small intestine and are specialized in the production of AMPs, although AMPs are produced by nearly all epithelial cell types throughout the GI tract. Defensins are the predominant family of AMPs in mammals and have broad antimicrobial activity against bacteria, fungi and some enveloped viruses. In particular, β-defensins are produced throughout the GI tract[5] by numerous epithelial[6, 7] and non-epithelial cells[8]. Beta-defensins participate in the recruitment of adaptive immune cells, as well as trigger the activation and degranulation of mast cells, releasing histamine and prostaglandin D_2[9]. Thus, besides protecting the intestinal barrier from bacterial infiltration through their direct bactericidal activity, AMPs can act as a bridge between innate and adaptive immunity.

The mucus layer of the GI tract is essential for separating luminal antigens from direct contact with the epithelium. Goblet cells (GCs) secrete secretory mucin glycoproteins (Muc2), bioactive molecules such as epithelial membrane-bound mucins, and trefoil peptides such as TTF3 along the length of the GI tract. GCs are found in increasing frequency towards the distal colon, where they comprise roughly 25% of all epithelial cells compared to 10% in the proximal small intestine[10]. Furthermore, penetrability of the small intestinal mucus layer is relatively high compared to the colon, where the mucus is thickest[11]. Continual replenishment of mucus by GCs forces bacteria towards the lumen to prevent their adhesion and penetration of the intestinal epithelium. Furthermore, the mucus layer concentrates AMPs produced by crypt Paneth cells and enterocytes near the epithelium, creating an outwards antibacterial gradient into the lumen[12-14]. Host factors such as cytokines induce GC hyperplasia and mucus hypersecretion in response to parasitic infections via Th2 cytokines[15-17], or under immunoregulatory conditions via IL-10[18] or IL-22[19]. Thus, immune signals can regulate GC function, mucus secretion, and affect the environment of the intestinal microbiota.

Secretory IgA (sIgA) is the most abundant class of antibodies in the intestinal lumen. The majority of IgA is secreted at the mucosa where it serves as another defensive barrier against luminal microbes. IgA is initially secreted into the lumen by undifferentiated crypt enterocytes via the polymeric immunoglobulin receptor (pIgR)[20]. In the lumen, this secretory form of IgA can bind to the mucus layer, inhibit the adherence and translocation of microbes, and neutralize their toxins[21, 22] in a process known as immune exclusion. Additionally, sIgA can induce anti-inflammatory responses in the intestinal mucosa. In the small intestine, sIgA can facilitate uptake of pathogens into Peyer's patches and isolated lymphoid follicles (ILFs)[23], where recognition of sIgA by dendritic cells can lead to the induction of regulatory T cell (T_{reg}) responses[24, 25]. Therefore, sIgA can modulate antigen sampling, shape host immunity, and contribute to intestinal barrier integrity.

Collectively, epithelial cells and their many intracellular and extracellular factors coordinate to protect the host from incoming pathogenic insults and regulate the commensal microbiota. As such, defects in the multilayered intestinal epithelial barrier may cause dysregulated immune activation in the gut and is central to the pathogenesis of GI diseases[26-28].

1.2 The gut mucosal immune system (GALT)

The GI tract is also equipped with a highly sophisticated mucosal immune system known as the gut-associated lymphoid tissue (GALT). To maintain homeostasis, the gut mucosal immune system must balance protective immunogenic responses against pathogens while preventing peripheral immune responses towards innocuous antigens such as food antigens in a process known as oral tolerance. Perturbations in these mechanisms can precipitate chronic diseases such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), celiac disease (CeD), and food allergy. Due to the complexity of the mechanisms involved, the pathogenesis of many GI disorders remains largely unknown. Antigen-specific responses occur at immunological induction sites

within the GALT, which include the mesenteric lymph nodes (MLNs), Peyer's patches of the small intestine, and ILFs and cryptopatches found throughout the intestinal tract are induced. Intestinal effector effector sites such as the lamina propria (LP) and surface epithelial layer are sites of action for diverse populations of innate and adaptive immune cells[29].

1.2.1 Innate mucosal immunity

Innate immune cells are poised for rapid effector functions in the intestinal mucosa. Monocytes/macrophages and dendritic cells are key sentinel cells found throughout the connective tissue and LP in the gut, developing from specific, bone-marrow derived precursors. These mononuclear cells are responsible for the uptake and presentation of antigens, and therefore play complementary roles in induction and maintenance of tolerance. Challenging to define phenotypically, mouse and human macrophages can be identified by their cell surface markers F4/80 (epidermal-like module-containing mucin-like hormone receptor-like 1) and CD68. In contrast, there are numerous DC subsets that exhibit great plasticity. DCs are specialized antigen-presenting cells that can migrate to secondary lymphoid organs such as PP or MLN to prime naïve T cells and induce their differentiation. Through signals received from co-stimulatory molecules and cytokines, DCs can actively shape the functional polarization and overall magnitude of T cell responses, for example through the activation of the cytokine transforming growth factor-β (TGF-β). In the intestine, $CD103^+$ DCs are of particular interest due to their role in immune regulation and development of tolerance[30]. Macrophages, however, are the most abundant mononuclear cell population in the gut. In particular, LP CX_3CR1^+ macrophages derive from $Ly6C^+$ blood monocytes and can sample bacterial antigens from the gut lumen to prime naïve T cells[31]. Macrophages are also highly phagocytic cells which perform important homeostatic functions such as tissue turnover, wound healing, epithelial cell renewal, and clearance of dead cells and microbes. Among the array of cytokines elaborated by macrophages, they are an important source of IL-10, a key immunoregulatory cytokine[32-34].

Mast cells (MCs) are present throughout the GI tract, but are mostly localized to the LP and submucosa. Cross-linking of their IgE receptors, Fc epsilon receptors, induces degranulation and release of histamine and the effector protease chymase into the intestinal mucosa. Activation of MCs are essential for physiological processes of tissue turnover and repair, and their products participate in diverse pathways such as epithelial barrier function, motility, vascular permeability, microbial detection, and neural axes. However, aberrant MC activation can lead to inflammation, epithelial barrier dysfunction, and disturbances in gut motility.

Eosinophils are concentrated in the GI tract, particularly within the small intestinal LP. Eosinophils derive from bone marrow progenitor cells under the influence of IL-3, IL-5, and granulocyte-macrophage colony stimulating factor, and migrate to the GI tract via CCR3/eotaxin-1. Eosinophils have traditionally been considered for their effector function in parasitic infections and allergic disease through the release of their toxic granule contents[35]. More recently, an immunomodulatory role for eosinophils has been elucidated, as their cytokine profile has the propensity to activate DCs and induce IgA class-switching. Thus, eosinophils may be important regulators of the microbiota through production of antibacterial granule proteins and modulation of innate mucosal immunity[36].

Neutrophils are the first immune cells recruited to sites of inflammation responding to signals produced largely by resident immune cells. These polymorphonuclear cells (PMNs) primarily control invasion of microbes through massive release of granule contents such as AMPs, myeloperoxidase (MPO), hydrolytic enzymes and lactoferrin[37]. Mice deficient in PMNs experience significantly increased bacterial translocation following induction of colitis[38], highlighting the importance of neutrophils in controlling the microbiota. During an acute inflammatory response, neutrophils can facilitate recruitment of monocytes to inflamed sites and stimulate adaptive immunity. As well, they aid in the resolution of inflammation and the wound healing response[37]. Although neutrophils are a critical innate component in the intestine, their excessive

recruitment and activation under pathogenic circumstances amplify chronic inflammation and tissue damage.

1.2.2 Adaptive mucosal immunity

Lymphocytes are located in the epithelium and lamina propria, and are widely distributed within both inductive sites and effector sites of the intestine. Mucosal T cells are activated through the T cell receptor (TCR)-CD3 complex, and can be broadly classified into type 'a' conventional T cells expressing TCRαβ and either the CD4 or CD8αβ co-receptor, and type 'b' non-conventional T cells expressing either TCRαβ or TCRγδ and CD8αα. Type 'a' mucosal T cells mainly populate the lamina propria, while type b T cells are more common in the epithelium. Of the type 'a' T cells, $CD4^+$ T cells also represent the majority of the T cells in the lamina propria and mostly display an antigen-specific effector memory phenotype[39].

Most intraepithelial lymphocytes (IELs) in the gut are divided into "natural" (or previously known as type 'b') and "induced" (or previously known as type 'a') based on their mechanisms of activation. "Natural" IELs develop in the thymus and are activated in response to self-antigens, while "induced" IELs arise from type 'a' conventional T cells that become activated in response to peripheral antigens in secondary lymph nodes[40]. These conventional IELs express the CDαβ heterodimer (CD4) and an αβTCR. In the mouse intestinal tract, significantly more IELs can be isolated from the small intestine which are mostly non-conventional IELs expressing CDαα homodimer and either αβTCR or γδTCR. In the colon, conventional IELs are the dominant population. IELs perform both regulatory and effector functions, ranging from immune surveillance, immune tolerance, wound repair, maintenance of gut barrier function, and protection from infectious agents[40]. Uniquely poised at the front line of mucosal immunity in direct contact with epithelial cells, IELs must thwart incoming pathogens while avoiding excessive inflammatory responses that damage the epithelial barrier.

The GALT is the main induction site for mucosal IgA-producing B cells, and are relatively abundant in the GI mucosa compared to other host tissues. IgA-secreting

plasma cells also increase in number distally with the highest proportion found in the colon. The canonical pathway of B cell activation involves the uptake of luminal antigens via microfold cells or epithelial cell endocytosis as conduits to DCs, which go on to mediate T cell-dependent B cell activation. T cell-independent intestinal IgA responses are typically in response to highly conserved microbial antigens, and a large proportion of intestinal IgA responses arise from antigen-specific B cell responses[41].

1.3 Intestinal microbiota

The commensal microbiota is an incredibly diverse ecosystem that has co-evolved with its host[42, 43]. In the GI tract, the commensal microbiota actively shapes physiology, metabolism, epithelial barrier function, and innate and adaptive immunity[44]. The intestinal microbiota can be characterized based on its diversity or richness of the ecosystem, resistance to perturbation, and its ability to revert following a perturbation[45]. Perturbations could be induced by the diet, the immune system, xenobiotic exposures, or early colonization events[45]. The gut microbiota associated with a situation of health is predominantly constituted by the phyla Bacteroidetes and Firmicutes, followed by the Actinobacteria and Verrucomicrobia phyla. Abundances of Proteobacteria are relatively low in healthy individuals[46], while higher proportions of Proteobacteria have been described in patients with chronic GI diseases[47]. Opportunistic pathogens, such as *Bacteroides thetaiotaomicron*, *Bacteroides fragilis* and *Escherichia coli* [43] are also part of the GI microbiome.

Following birth, the early colonizing *Bifidobacteria* spp. are specialized in metabolizing milk oligosaccharides, allowing them to outcompete other species such as *Escherichia coli* and *Clostridium perfringens*[48, 49]. Species richness and complexity is thought to increase during the first year of life, settling at approximately 3 years of age as the microbiome more closely resembles that of a healthy adult[50]. Spatial heterogeneity is also a key feature of the microbiome and considerable variation in microbial composition is found along the GI tract. The number of bacteria increases progressing distally in the

GI tract, ranging from 10^5 in the upper small intestine, to 10^{12} per gram of contents in the colon[51].

Aerobic species are predominant in the upper small intestine, while anaerobic species are found in the colon. The dominant bacterial families of the small intestine and colon reflect physiological differences along the length of the GI tract. The high pH, oxygen, and antimicrobials in the small intestine reduces bacterial density and allows growth of facultative anaerobes that can tolerate bile acids and antimicrobials while competing for simple carbohydrates[52]. Despite the lower bacterial load of the SI, there is still an important presence of mucosal and luminal bacteria, such as those belonging to Lactobacillaceae and Enterobacteriaceae, which are strategically located to interact with nutrients and dietary antigens. The surface of the small intestine is colonized by adherent species such as segmented filamentous bacteria (SFB), Lactobacillaceae, and *Helicobacter* spp. Compared to the colon, there is lower bacterial diversity and greater proportions of *Clostridium* spp. and certain members of Proteobacteria in the small intestine[52].

The caecum and colon represent the largest reservoir for bacteria in the gut. The lack of simple carbon sources favors the growth of fermentative anaerobes such as the Clostridia and Bacteroidaceae families, which degrade polysaccharides that would normally be resistant to small intestinal metabolism[53]. Bacterial density is highest in the distal colon where Gram-negative anaerobes are abundant, and Firmicutes and Bacteroidetes are the primary phyla[51, 54]. Microbial communities also differ along the cross-sectional axis of the gut, forming discrete niches in the lumen, mucus layers, and colonic crypts[55, 56]. While *Bacteroides, Bifidobacterium, Streptococcus,* Enterobacteriacae*, Enterococcus, Clostridium, Lactobacillus* and *Ruminococcus* are the predominant luminal microbial genera cultured from stool, *Clostridium, Lactobacillus, Enterococcus and Akkermansia* are the predominant mucosa and mucus-associated genera detected in the mucus layer and epithelial crypts of the small intestine[54]. Gnotobiotic studies demonstrate that a microbial presence is necessary for immune development and homeostasis[57,58]. Abnormal intestinal morphology, immune defects, and

structureless secondary lymphoid tissues [59] are normalized after microbial colonization of germ-free mice [60]. Germ-free mice have reduced number of IgA-secreting B cells while levels of total B cells is similar to conventionally raised mice, and colonization with commensals greatly stimulates IgA responses[61,62]. Thus, the intestinal microbiota is deeply complex in its developmental dynamics and structural organization.

1.3.1 Host-microbiome interactions

Active cross-talk between the intestinal microbiota and host immunity is important in achieving and maintaining homeostasis[63]. The innate immune system must monitor the immense array of bacterial antigens and respond appropriately to changes in the microbial landscape. Germline-encoded pattern recognition receptors (PRRs) such as the extracellular Toll-like receptors (TLRs) and the intracellular nucleotide-binding oligomerisation domain 2 (NOD)-like receptors (NLRs) are expressed by epithelial and immune cells of the host to recognize microbial components and metabolites[64]. Activation of PRRs by bacterial ligands trigger signaling cascades that regulate expression of inflammatory and antimicrobial mediators, including interleukin (IL)-6, tumor necrosis factor alpha (TNF-α) and IL-1β[65]. Mice deficient in the TLR signalling adaptor myeloid differentiation primary response protein 88 (MYD88) have an altered microbiota profile[66, 67]. Similarly, modification of microbial composition is associated with deficiency in NOD2, a PRR which recognizes bacterial peptidoglycan. NOD2-deficient mice have an increased proportion of mucosa-associated bacteria, which is associated with increased susceptibility to intestinal inflammation[68-70]. Additionally, human NOD2 polymorphisms are associated with dysbiosis in intestinal inflammatory disorders and is a genetic risk factor for disease[70-72]. The adaptive immune system can shape microbial communities as well, primarily through B cell production of sIgA which preferentially targets mucosa-associated microbial species and species with colitogenic potential[73].

The microbiota produces an extremely diverse repertoire of metabolites and products which influence immune development and fine tuning of the immune response. For example, short-chain fatty acids (SCFAs) are generated from microbial fermentation

of undigested complex carbohydrates and can act as histone deacetylase inhibitors to promote a tolerogenic, anti-inflammatory immune phenotype[74-76]. Bacterial metabolism of tryptophan generates ligands that bind aryl hydrocarbon receptors present on host immune cells and epithelial cells, leading to protection against intestinal inflammation[77]. Immune modulation by microbial products such as formyl peptide, D-glycero-β-D-manno-heptose-1,7-biphosphate
(HBP), and polysaccharide A (PSA)[78] are also able to interact with host cells or host products, to influence immune responses and disease risk. Microbial components such as LPS show species-dependent variations in immune activation status of the host, which may potentially affect the development of immune-mediated diseases[79]. Certain commensal species can degrade sIgA, and transfer of microbiota with low fecal sIgA can shift the host status from high to low fecal sIgA and alter colitis susceptibility[80]. Despite the number of studies investigating host-microbe interactions, the multitude of mechanisms remain largely unknown. Gnotobiotic mouse models using murine gut bacteria or bacteria from the human GI tract (humanized models) have been instrumental in our understanding of this bidirectional dialogue, in the context of disease pathogenesis and therapies targeting the microbiota.

1.4 Contribution of microbes to gut inflammatory disorders

The propensity for commensal microbiota to overcome host responses and colonize the intestine underscores its pathogenic potential. Indeed, a large number of immune-mediated diseases have been associated with intestinal dysbiosis[81-83], which is defined as a compositionally and functionally altered microbial profile that is linked to a pathological state[84]. The widespread inter-individual variability in microbiota composition of healthy individuals precludes the definition of a single “healthy” versus “dysbiotic” microbiota. Despite the number of investigations, there is still no consensus to ascribe a compositional or functional microbial signature to IBD, CeD, or any other gut inflammatory disorder. Nonetheless, the burden of data supports a role of intestinal

microbiota in the pathogenesis of gut inflammatory disorders such as IBD and CeD, and there is some consistency among the reported compositional dysbiosis in intestinal disease, including a reduction in overall species richness or alpha diversity, an expansion of pathobionts, and a loss of commensal species in which their reconstitution can reverse the dysbiosis-associated phenotypes.

It is unclear whether dysbiosis plays a causal or consequential role in gut inflammatory disorders, and animal models offer evidence for both cases. Transfer of dysbiotic microbiota can result in transfer of the dysbiosis-associated disease phenotype, implicating a causal role for dysbiosis in some intestinal pathologies[85-87]. Furthermore, replenishing the microbiota with certain commensal species or their metabolites can reverse dysbiosis-associated phenotypes and protect from intestinal inflammation[88, 89]. In chronic colitis mouse models, administration of *F. prausnitzii* significantly decreased colitis severity, colonic MPO activity, and proinflammatory markers[90]. Numerous clinical and animal studies also suggest the influence of the microbiota over susceptibility to chronic inflammatory diseases especially in early life[91, 92]. *In vivo* studies have also shown differences in disease susceptibility depending on the microbiota composition in mouse models of DSS-induced colitis, metabolic disorders, type 1 diabetes, and gluten sensitivity[93-95]. These studies have generated interest in the potential of expanding the use microbiota-targeted therapies to restore homeostasis in other human disorders.

1.4.1 Inflammatory bowel disease

IBD is comprised of two main subtypes of chronic inflammatory disorders: Crohn's disease (CD) and ulcerative colitis (UC). The pathogenesis of IBD stems from an interplay between genetic and environmental components leading to a chronic intestinal inflammation[72] either throughout the GI tract in CD or limited to the colon as in UC[96]. Genome-wide association studies have identified several overlapping genetic risk alleles shared between CD and UC, but due to the concordance rate in monozygotic twins of 30-35% in CD compared to 10-15% in UC, this suggests an especially important role for UC. Currently, the initiating events and causes of IBD progression are still unclear. Multiple

UC susceptibility genes include those related to innate and adaptive host immunity, intestinal epithelial barrier integrity, and microbial defense[96]. Defects in the intestinal epithelial barrier have been proposed as a major mechanism for IBD pathogenesis in both CD and UC[97,98]. A compromised barrier facilitates the exposure of resident microbiota to the host immune system, leading to proliferation of commensal-specific and pathogen-specific T cells[99-101] and further epithelial injury[102, 103].

Infiltration of monocytes, neutrophils, and lymphocytes to the mucosa are key drivers of the inflammatory milieu in IBD[104-106]. For instance, anti-inflammatory IL-10-producing macrophages of the lamina propria shift towards an inflammatory phenotype in IBD, producing IL-23, TNF, and IL-6[107]. Significant polymorphonuclear leukocyte migration across the epithelium has been shown to correlate with epithelial damage, disease activity, and symptoms in active IBD[108]. Tissue-infiltrating neutrophils have been identified as the main source of IL-23 in the colon of IBD patients, the key cytokine during the early initiation and progression of IBD[109]. The degree of neutrophil infiltration is used as a surrogate marker for disease activity and severity in UC[110, 111], and neutrophils are necessary for development of colitis in animal models using chemically-induced colitis[112, 113], further supporting their central role in IBD pathogenesis.

Dysregulated interactions between the host immune system and microbiota are central in the pathogenesis of both CD and UC. Several genetic risk alleles for CD such as NOD2 are involved in host-microbe interactions[58,114,115]. Lower bacterial diversity and alterations in the ratio of Firmicutes to Bacteroidetes have also been associated with CD[116-119]. Decreased total fecal bacteria, reduced bacterial diversity of Ruminococcaceae, and lower numbers of Bifidobacteria and Lachnospiraceae have all been observed in fecal microbiota from IBD patients compared to healthy controls[118, 120-124]. Other studies have also reported increases in bacterial species belonging to the Actinobacteria and Proteobacteria phyla in IBD patients[125]. Pathogenesis of UC involves an abnormal inflammatory response to commensal microbiota[126], and alterations in microbiota has also been described in UC patients[127-129] and animal models of colitis[120]. Beyond these associative studies, there is little mechanistic insight behind the observed IBD-associated

alterations in microbiota. We have previously found that colonization of germ-free mice with microbiota derived from UC patients low in Firmicutes induced a proinflammatory Th17 phenotype in the host and increased sensitivity to colitis[130]. Mechanisms underlying these effects are still unknown, and further studies should investigate functional aspects of the microbiota that could be drivers of inflammation and disease. Importantly, there is a lack of investigation into the enormous repertoire of factors such as proteases and protease inhibitors elaborated by the microbiota and their ability to modulate intestinal inflammation.

1.4.2 Celiac disease

CeD is a chronic autoimmune enteropathy triggered by a complex group of dietary proteins, called collectively "gluten", in genetically susceptible individuals. Currently, the only treatment for CeD is life-long adherence to a gluten-free diet (GFD) which has many limitations[131-134,135]. The human leukocyte antigen (HLA)-DQ2/DQ8 is necessary for disease onset, but only confers between 30-40% of the risk[136,137], suggesting that environmental factors are a key contributor to disease. Gluten is composed of gliadin and glutenin proteins with immunogenic peptides which, once deamidated by tissue transglutaminase 2 (TG2), have high avidity for HLA-DQ2/8 molecules[138,139]. HLA-DQ2/8$^+$ antigen-presenting cells are then able to activate a host of lamina propria CD4$^+$ T cells specific against gluten, leading to inflammatory T cell, generation of autoantibodies, and atrophic enteropathy, characterized by villus blunting and lymphocytic infiltrates[140,141]. While the CD4$^+$ T cell response has been thoroughly described in the pathogenesis of CeD, increased CD8$^+$ intraepithelial lymphocytes (IELs) is the earliest pathological sign of CeD following gluten challenge[119,142]. In particular, induced CD8αβ$^+$TCRαβ$^+$ IELs destroy intestinal epithelial cells expressing stress-induced MHC class I polypeptide-related sequence antigens (MICAs) via NKG2D[143]. Chronic, unregulated activation of IELs is driven by IL-15 which is overexpressed in enterocytes of patients with active CeD [144], leading to epithelial damage and villous atrophy[119,142,145,146].

Dysbiosis has emerged recently as an important additional environmental modulator of risk in CeD, as supported by the association of CeD with neonatal infections[147], recurrent rotavirus infections[148,149], early life microbial alterations[150,151], and delivery mode[152]. Intestinal dysbiosis in CeD patients is characterized by a decrease in *Bifidobacterium* and *Lactobacilli*, and by an expansion of Gram-negative bacteria, such as *Bacteroides* spp. and *Escherichia coli*[153-155]. Expansion of Proteobacteria genera has been reported in the small intestine of patients with active CeD, and in patients who do not respond to the GFD[156]. We have shown that experimental expansion of Proteobacteria in the small intestine using antibiotics exacerbates gluten-induced immunopathology in NOD/DQ8 mice[93]. The underlying mechanisms remain unclear, but recent studies and unpublished data from our lab suggest multiple pathways are involved which include modification of gluten metabolism by bacteria[157] and direct effects by proteases produced by opportunistic pathogens (Caminero and McCarville, unpublished results).

1.5 Microbiota-targeting therapies

Given the number of GI diseases that have been associated with dysbiosis, there is great interest in understanding the underlying mechanisms to effectively develop therapies targeting the microbiota. Recent trials with fecal microbiota transplantation (FMT), in which the microbial community of a healthy donor is introduced into the colon of a patient, achieved remarkable results in patients with pseudomembranous colitis caused by recurrent infection with *C. difficile*[158]. The success of FMT in *C. difficile* infection has raised the exciting possibility for its application in treating other dysbiosis-associated diseases, such as IBD[159].

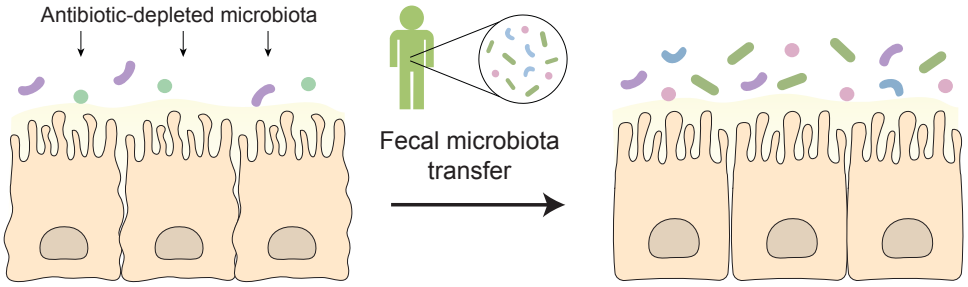


Figure 2.1. Fecal microbiota transfer. This microbiota-modulating method usually involves treating the patient with antibiotics to deplete their dysbiotic microbiota prior to reconstituting their gut with microbiota from a healthy donor.

Antibiotic therapy has been widely used in IBD based on data from randomized controlled trials. However, results from meta analyses have since disputed the effectiveness of antibiotic therapy for IBD[160-162], and antibiotics such as rifaximin have only been modestly effective in patients with IBS[163]. The use of antibiotics also risks further exacerbation of dysbiosis and reduced bacterial diversity[164].

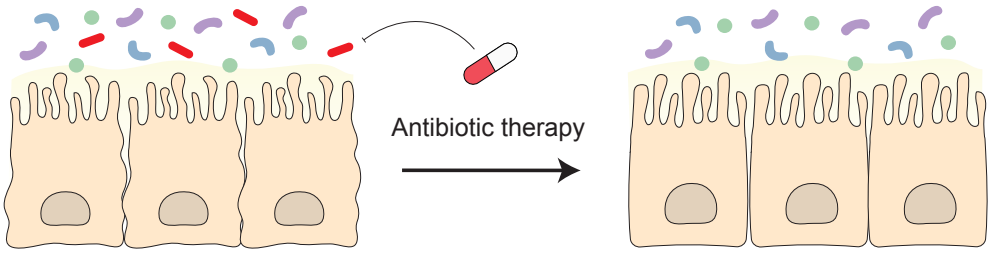


Figure 2.2. Antibiotic therapy. Targeted antibiotic therapy aims to reduce harmful microbial species that may be drivers of gut inflammation.

Prebiotics and probiotics are another way of therapeutic microbiome modulation, which aims to shift the microbiota composition for positive effects on host health. Dietary prebiotics involve nutritional interventions to selectively expand beneficial microbes, but evidence for their effectiveness is still inadequate[165, 166]. Administration of probiotics such as *Lactobacillus* and *Bifidobacterium* species, have been widely applied in various GI disorders with variable efficacy[44,167-172]. The health benefits conferred by these live

microbes on the host[173] may involve stabilization of microbial composition, colonization resistance, epithelial barrier fortification and immune modulation[174-177].

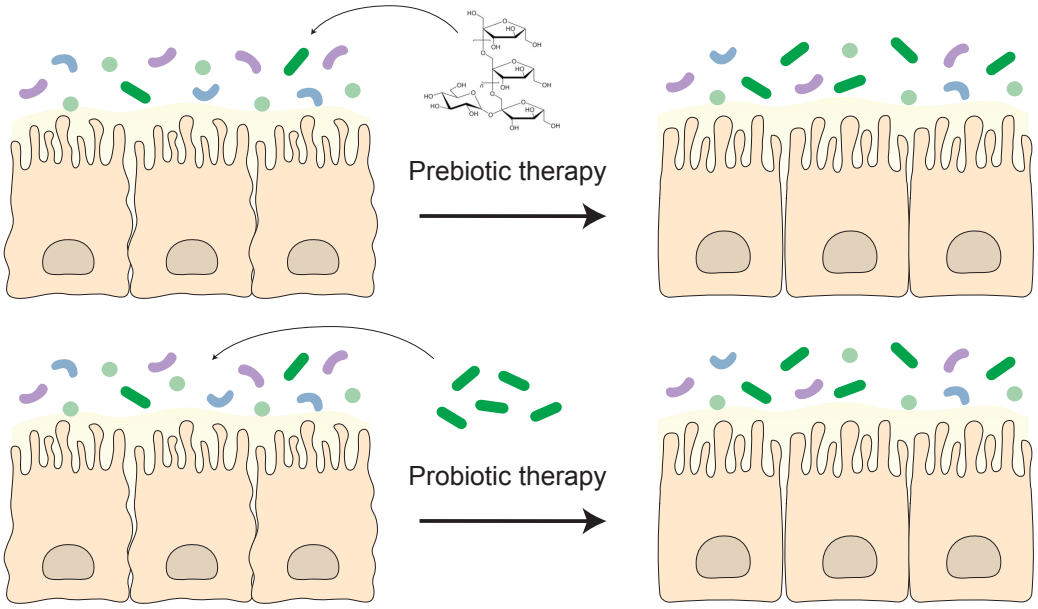


Figure 2.3. Prebiotic and probiotic therapy. Both prebiotics and probiotics are used to boost beneficial bacterial groups, either through nutritional intervention such as with inulin (top) or direct administration of live probiotic species (bottom).

Though many probiotic studies have been performed in CD, none have proven successful[178-182]. More favorable data exists for UC as certain strains have shown efficacy in inducing and maintaining remission[170,181,183,184]. For IBS, few randomized controlled trials lend support for probiotic use, however, larger sample sizes and improved designs are needed[185]. The probiotic *B. infantis* natren life start (NLS) super strain was able to alleviate symptoms in patients with active CeD[186], and a follow-up study demonstrated that administration of NLS modulated innate immunity[187]. Administration of probiotic species could dampen proinflammatory responses in CeD. Indeed, certain *Bifidobacterium* and *Lactobacillus* species exert protective effects in the small intestine against gliadin-induced damage by reducing the cytokines involved in CeD pathogenesis[188-191]. Overall, the insubstantial evidence for the efficacy of probiotics in microbiome-associated disorders may be due to the lack of mechanistic knowledge for probiotics. *In vitro* studies have introduced possible immunomodulatory mechanisms, for

instance by inducing expression of human β-defensin 2 in intestinal epithelial cells[192,193]. Few studies have examined the specific probiotic products that mediate their beneficial effects. Interestingly, the protease lactocepin produced by *Lactobacillus paracasei*, one of the strains in the extensively used probiotic mixture VSL#3, was found to selectively degrade the cytokine IL-10, and was necessary for reducing inflammation in an animal model of colitis[194]. As current therapies are unfocused with little knowledge of host-microbe interactions, more mechanistic studies are needed to develop targeted interventions against chronic GI inflammatory disorders.

1.6 Proteolytic balance in the gut: A potential mechanism through which dysbiosis can affect inflammatory conditions

The field of microbiome studies is shifting from descriptive studies on composition to more mechanistic studies, focusing on their functional contribution in the gastrointestinal tract. There has been a growing appreciation of the extremely diverse microbial metabolite and co-metabolite repertoire and its interactions with the host. For example, SCFAs generated from microbial fermentation can elicit a tolerogenic, anti-inflammatory immune phenotype[74,76]. Similarly, the microbiota also generates metabolites that can bind aryl hydrocarbon receptors on host immune cells and epithelial cells to regulate host immune responses. Microbial components such as the classically studied polysaccharide A from *B. fragilis*[78] are also capable of immune modulation. Lesser appreciated are interactions with the host and microbial proteins such as proteases and protease inhibitors. There is evidence that microbial proteases and protease inhibitors interact with host cells or host products, and thus influence immune responses and disease risk[195,196].

1.6.1 Host proteases and protease inhibitors

Proteases are found in all organisms and represent approximately 2-4% of encoded gene products[197,198]. Proteases regulate diverse processes such as cell death,

wound repair, coagulation, tissue turnover/remodelling, immune regulation, and digestion, their best-known role in the gut. They can either directly catalyze proteolysis of mediators or cleave the extracellular domain of G-protein-coupled cell-surface protease-activated receptors (PARs) to elicit a wide range of physiological and immune effects. Because proteases irreversibly cleave amide bonds of proteins and participate in numerous physiological and immune pathways, mechanisms have evolved to tightly regulate their activity. Proteases can be initially synthesized as inactive zymogens, which are subsequently activated by a series of upstream proteolysis events, pH changes, or dimerization before achieving its active conformation (177). Endogenous protease inhibitors also inactivate proteases through a variety of reversible or irreversible mechanisms. Proteases are broadly classified based on their catalytic residues as serine, cysteine, aspartate, threonine, and metalloproteinases, and their cognate protease inhibitors share this classification scheme. For example, tissue inhibitors of metalloproteinases (TIMPs) regulate metalloproteinase (MMP) activity[199], and serine protease inhibitors act as irreversible inhibitors of serine proteases[200].

Among the organ systems, the GI tract harbors the highest abundance of proteases, and luminal, circulating, secreted, intracellular, intramembrane, or pericellular proteases can all be detected[201]. Proteases have a significant impact on gut physiology, controlling the activity and availability of proteolytic substrates such as growth factors, cytokines, chemokines, and extracellular matrix components[198]. Further, PARs are ubiquitously expressed throughout the GI tract by intestinal epithelial cells, neurons, immune cells, and fibroblasts, through which a variety of signaling cascades such as ion exchange, nociception, secretion, and mucosal permeability are activated. Epithelial cells express PARs apically and basolaterally, and can therefore be activated by either tissue or luminal proteases including those from bacterial origin. Serine proteases from the intestinal lumen, neurons, fibroblasts, and immune cells can activate PARs. Downstream responses of PAR activation are still poorly understood, and depend on many factors such as the specific PAR, binding localization, cell type, and inhibitory factors.

The upper GI tract is dominated by pancreatic proteases including trypsin, chymotrypsin, and elastase which perform digestive functions. Intestinal epithelial cells produce matrix metalloproteinases, mesotrypsin, elastase, and trypsin-3[202,203]. Mast cells release tryptase, chymase, as well as cathepsin G and granzyme B, while resident macrophages are a source of matrix metalloproteinases, caspase, and cathepsins. Inflammatory cells that traffic to the gut are also a source of proteases which elicit tissue degradation, autophagy, and apoptosis. In particular, neutrophils release elastase, proteinase-3, and cathepsin G that cleave a range of substrates including components of the extracellular matrix. Proteinase-3 and cathepsin G can also cleave CXCL-8 and CXCL-5, respectively and increase their ability to chemotactically attract neutrophils[204]. Due to the vast physiological and immune effects of proteases in the gut, the balance between active and inactive proteases is fundamental in maintaining intestinal homeostasis.

1.6.2 Proteolytic imbalance in GI disorders

There is a growing interest in the role of proteases in inflammatory pathologies, such as asthma, kidney disease, and cardiovascular disease. It has been proposed that a perturbation in proteolytic balance, either through increased activity of proteases or decreased activity of inhibitors, may contribute to intestinal pathophysiology. Increased proteolytic activity can induce structural and functional changes in the intestinal barrier, ultimately leading to chronic inflammation. These physiological and immune effects can occur independently or precipitate each other to drive pathogenesis.

Increased proteolytic activity has been observed in stool of patients with UC, and increased elastolytic activity has specifically been shown in feces and colonic biopsies of IBD patients. Both increases in proteases and decreases in protease inhibitors have been described for GI inflammatory disorders. Increased levels of serine proteases, MMP, neutrophil elastase, and cathepsin have all been described in IBD[100, 101]. Increased MMP in IBD patients have been accompanied by decreases in TIMP levels. MMPs and TIMPs are central to the pathogenesis of IBD through digestion of the ECM in response to

inflammatory stimuli, and due to their immunomodulatory effects[205]. Decreased expression of the serine protease inhibitor, elafin, has been found in colonic biopsies of IBD patients[206, 207], as well as small intestinal biopsies of active CeD patients[208]. It is important to recognize that the source of these disease-associated increases in proteases has not been defined. In addition, it is unclear whether this proteolytic imbalance is an initiator or consequence of disease. Current studies assume that the majority of these proteases are host-derived, despite the massive amount of proteases that come from the intestinal microbiota.

1.6.3 Therapeutic potential of protease inhibitors

Some recent therapies have been directed at correcting dysregulated proteolytic balance in inflammatory disorders. Inhibition of neutrophil elastase has been investigated in preclinical models of lung, skin, and intestinal inflammation[209]. Serine protease inhibitors (serpins) are the most widely distributed superfamily of protease inhibitors, and their anti-inflammatory properties have been applied in inflammatory pathologies. Mammalian serine protease inhibitors such as elafin or secretory leukocyte protease inhibitor (SLPI) have previously been delivered to the intestinal mucosa using bacterial vectors[207,210]. Elafin is normally expressed throughout the gut epithelium, and is decreased in the mucosa and submucosal intestinal epithelium of patients with IBD. Decreased elafin expression corresponds to increased elastolytic activity in culture supernatants from inflamed and non-inflamed areas of biopsied colon. Delivery of elafin via a food-grade *Lactococcus lactis* protected mice from colonic inflammation, increased intestinal permeability, and attenuated colitis severity[207]. A similar therapeutic effect was achieved using SLPI delivered via the *Lactococcus lactis* vector, which resulted in significantly decreased intestinal inflammation. *L. lactis* expressing elafin was also able to attenuate gluten-induced enteropathy in the non-obese diabetic (NOD)/DQ8 mouse model of gluten sensitivity. Furthermore, mucosal delivery of elafin in gliadin-sensitized NOD/DQ8 mice protected against gliadin-induced enteropathy through improvements in small intestinal permeability, infiltration of intraepithelial lymphocytes, and villous

morphology. However, clinical use of *L. lactis* expressing elafin is hindered by the inherent instability of elafin in humans and *L. lactis* expressing elafin is a genetically modified organism (GMO), making it problematic for effective clinical use. Hence, there is a need to test other serine protease inhibitors or exploit endogenous elafin-like molecules in probiotic strains.

1.6.4 Microbial protease and protease inhibitors in the GI tract

The role of microbial proteases in the gut has been overlooked, partly due to limited methodologies for distinguishing host from microbial proteases, as well as the overwhelming presence of pancreatic proteases in the upper GI tract. Despite the abundance of host-derived pancreatic proteases, early studies define a significant contribution of proteases from the microbiota in the colon[211]. Certainly, the intestinal microbiota produces a wide repertoire of extracellular proteases which are utilized for their metabolism, development, and virulence[212, 213]. Depending on the species, intestinal bacteria vary in their ability to drive mucosal inflammation based on the proteases they produce. The opportunistic gut bacterium *Pseudomonas aeruginosa* secrete elastases which can degrade tight junctions[214-216]. Thus, bacterial elastases may contribute to and/or exacerbate this effect in CeD. Because proteases are traditionally studied as virulence factors, pathogen-derived proteases have been most thoroughly investigated for their effects in the GI tract. For example, protease release by pathogenic *E. coli*[217] or enterotoxigenic *Bacteroides fragilis* is necessary for their virulent life cycle[218]. Although *B. fragilis* and *B. thetaiotaomicron* are members of the human GI tract, they can cause opportunistic infections concurrent with the upregulation of certain proteases. Specifically, *B. fragilis* produces a metalloproteinase that is able to cleave the extracellular matrix component E-cadherin[219], and *B. thetaiotaomicron* encodes putative proteases with similar homology[220]. Proteases from the opportunistic pathogen *Clostridium perfringens* can target components of the extracellular matrix such as gelatin, type IV collagen, and mucin[221]. Thus, members of the intestinal microbiota can effectively degrade the mucosal barrier through protease secretion and potentiate

inflammation. More recently, the commensal bacterium *Enterococcus faecalis* was found to produce gelatinase which degrades E-cadherin, promoting colonic barrier impairment and increased colitis severity[222]. Especially in the context of a genetically susceptible or dysbiotic state in the host, these commensal-derived proteases may be important determinants in GI disease pathogenesis. Therefore, the dysregulation of microbial proteolytic balance could provide a mechanistic link for the association of dysbiosis and various GI disorders.

Production of proteinase inhibitors is much less common among bacteria. Instead, many microbes secrete proteases as inactive zymogens, as described with proteases from *P. aeruginosa* which must be cleaved to become active[223, 224]. Apart from protease inhibitors released by soil bacteria and some extremophile bacteria[225], very few microbial protease inhibitors have been identified[226-229]. Only recently have protease inhibitors produced by gut microbiota been considered. A serine protease inhibitor (*Bl0108*), or serpin, was identified in the genome of an infant-derived strain of *Bifidobacterium longum*. Biochemical characterization revealed that this prokaryotic serpin inhibits eukaryotic proteases, pancreatic and neutrophil elastase. It has been hypothesized that through the production of serpin, this commensal *B. longum* guards against host proteases in order to gain a competitive advantage in the gut. It is possible that serpin may give this probiotic *B. longum* strain immunomodulatory properties. Serpin-encoding genes have also since been identified in the genomes of other *Bifidobacterium* species including *B. breve*[230,231]. Moreover, two putative serpins from the human gut bacterium *Eubacterium sireaum* can inhibit human neutrophil elastase and proteinase-3 *in vitro*[232]. The relevance of these microbial protease inhibitors is poorly understood, and overall, there is a lack of investigation into the intestinal microbiota as contributors to proteolytic homeostasis in the gut.

CHAPTER 2
THESIS OUTLINE AND AIMS

Hypothesis and Aims

Proteolytic imbalance has been observed in colonic biopsies and feces from patients with various GI chronic inflammatory disorders. It has been assumed that only protease and protease inhibitors produced by the host are clinically important. Given the growing interest in the gut microbiota and its association with inflammatory conditions, it is surprising that the contribution of microbiota in intestinal proteolytic balance has not been more extensively investigated. Hence, the overall aim of this thesis is to **investigate the role of proteases and protease inhibitors from the commensal microbiota in GI pathogenesis**. The overall hypothesis is that **increased microbial proteolytic activity contributes to inflammation, and therapies could exploit microbial protease inhibitors to restore proteolytic balance**.

The findings of this thesis are organized into three aims and two chapters. In chapter three, we established a gnotobiotic mouse model of microbial proteolytic imbalance and explored pathogenic effects induced in the colon by the transfer of defined microbial communities with specific *in vitro* proteolytic phenotype. We then tested the ability of a protease inhibitor produced naturally by a commensal microbe to mitigate these pathogenic effects. In chapter four, we tested the therapeutic efficacy of the same microbial protease inhibitor in a mouse model of gluten sensitivity and small intestinal inflammation.

CHAPTER 3: Role of microbial proteases in colonic inflammation.

Aims

1. To characterize the effects of harboring a microbial community with high proteolytic activity in the colon.
2. To test the effect of microbial protease inhibitors in this system.

Hypothesis: Increased proteolytic activity from microbiota is a pathogenic mechanism which can targeted using a microbial protease inhibitor *B. longum* serpin.

CHAPTER 4: Therapeutic potential of a microbial serine protease inhibitor in a model of gluten sensitivity.

Aim

1. To test the therapeutic efficacy of a microbial protease inhibitor, serpin, produced by a commensal *B. longum* strain in the NOD/DQ8 mouse model of gluten sensitivity.

Hypothesis: In the NOD/DQ8 disease model, *B. longum* serpin has anti-inflammatory and barrier-protecting effects.

CHAPTER 3

ROLE OF MICROBIAL PROTEASES IN COLONIC INFLAMMATION

3.1 INTRODUCTION

Proteolytic imbalance, defined as an increase in proteases and/or a decrease in protease inhibitors, is associated with several GI disorders including IBD, IBS, colorectal cancer (CRC), and CeD[207,208,233,234]. Proteolytic imbalance can contribute to GI pathophysiology by inducing structural and functional changes in the intestinal barrier, ultimately leading to inflammation[235-237,238,239]. Excess production of proteases including MMP, neutrophil elastase, and cathepsin, have been observed in IBD patients[240,241], and increased proteolytic activity has been detected in stool of patients with IBD[100,101,207,242,243]. It is largely assumed that the excess proteases in GI diseases are produced by the host, while proteases produced by the gut microbiota are much less recognized. Both commensal and pathogenic microbes can produce a wide repertoire of extracellular proteases which function in their metabolism, development, and virulence[244]. In particular, those produced by the opportunistic gut pathogen *C. perfringens* can target components of the extracellular matrix such as gelatin, type IV collagen, and mucin[245]. Similarly, *B. fragilis* produces a metalloproteinase that is able to cleave the extracellular matrix component E-cadherin[246]. More recently, the commensal bacterium *E. faecalis* was found to produce gelatinase which degrades E-cadherin, promoting colonic barrier impairment and increased colitis severity[247]. Thus, these intestinal microbes can potentially degrade the mucosal barrier through protease secretion and drive inflammation. Because proteases have broad and pleiotropic effects, microbial proteases can also modify pathways affecting inflammation, wound healing, mucus cleavage, matrix remodelling, motility, etc. As such, microbial proteolytic balance may be an important contributor to gut homeostasis, which may be broken in a genetically susceptible or dysbiotic host. In chapter 3 of this thesis, we investigated the overlooked role of microbial proteases in the lower GI tract using a gnotobiotic mouse model.

3.2 MATERIALS & METHODS

Selection of microbiota for gnotobiotic colonizations

To elucidate the effects of microbial proteolytic activity on host immunity and physiology, we performed gnotobiotic colonizations using three bacterial communities, each consisting of four bacterial strains with differing proteolytic activities.

Bacterial strains were selected from a collection previously isolated from feces of a healthy volunteer and two patients with active UC experiencing an acute onset of severe symptoms[130]. Levels overall and elastolytic activity for each strain was measured *in vitro* as described below, and broadly categorized as low (+), medium (++), or high (+++) relative to each other. Based on information from this screen, we then selected strains based on their *in vitro* microbial proteolytic activity in order to create three bacterial communities with either high or low proteolytic activity (Figure 3A).

The UC patient-derived community with high proteolytic activity (HPA) consisted of *Clostridium perfringens* METW*, Pseudomonas aeruginosa* C4*, Enteroccocus faecalis* FAAJ*,* and *Bacteroides fragilis* BHI A. The low proteolytic activity community composed of UC-derived strains (LPA) included *Escherichia coli* K2 aer., *Ruminococcus gnavus* D5FAA1, *Enterococcus faecium* CNAG, and *Streptococcus salivarius* CAN K2. Another community with low proteolytic activity was created using HD-derived strains (HD), *Lactobacillus rhamnosus* LA2a*, L. fermentum* A17*, B. longum* A95*, and L. mucosae* A67.

A)

Community	Strain	Overall	Elastolytic
Low proteolytic activity from healthy patient (HD)	*Lactobacillus rhamnosus LA2a*	-	-
	L. fermentum A17	-	-
	L. mucosae A67	-	-
	B. longum A95	-	-
Low proteolytic activity from UC patient (LPA)	*E. coli K2 aer*	-	-
	Enterococcus faecium CNAG	-	-
	Streptococcus salivarius CAN K2	-	-
	Ruminococcus gnavus D5FAA1	-	-
High proteolytic activity from UC patient (HPA)	*Enterococcus faecalis FAAJ*	+++	+
	Pseudomonas aeruginosa C4	+++	+++
	Clostridium perfringens METW	+++	+
	Bacteroides fragilis BHI A	+++	-

B)

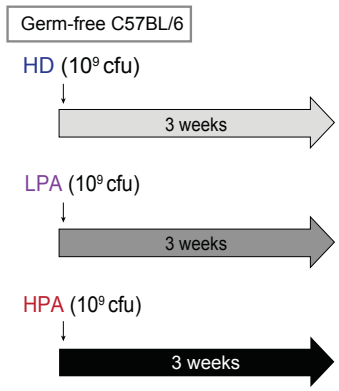


Figure 3. Bacterial communities and experimental design.

A) Each microbial community contains 4 bacterial strains. HD community contains low proteolytic activity bacterial strains derived from a healthy donor, and the LPA community contains strains with low proteolytic activity derived from a UC donor. HPA contains strains with high proteolytic activity isolated from a UC patient. **B)** Germ-free C57BL/6 mice were orally gavaged with 10E9 cfu of either HD, LPA, or HPA microbiota, and microbiota were allowed to stabilize over 3 weeks.

Preparation of bacterial strains for gnotobiotic colonizations

Individual strains were reconstituted from aliquots stored at -80°C by plating 10µl on agar containing BHI or MRS. Following incubation in aerobic or anaerobic conditions for 24h, an individual colony was selected from each plate and propagated overnight in liquid media.

Gnotobiotic colonization of mice

Male and female adult germ free C57BL/6 mice were obtained from the McMaster University Axenic Gnotobiotic Unit. Germ-free status was monitored regularly by bacterial culture and non-culture based techniques of fecal and cecal samples. Germ-free mice were kept on a 12-hour light/dark cycle, and housed in individual isolators with sterile bedding and autoclaved rodent diet with supplement. All experiments were conducted in accordance with McMaster University Animal Ethics Committee guidelines and Canadian Guidelines for Animal Research. Colonizations were performed in a level

II biological safety cabinet under sterile conditions by oral gavage with 0.1ml of bacterial suspension delivering 10^9 cfu of either HD, LPA, or HPA communities. Gnotobiotic mice were housed in a hermetically-sealed, positive pressured isocage system (Tecniplast, Montreal, QC), and microbiota was allowed to colonize and stabilize over 3 weeks prior to sacrifice (Figure 3B).

In vitro and *in vivo* proteolytic activity

Overall or trypsin-like proteolytic activity were measured in bacterial isolates and fecal samples from colonized mice using the azocasein method (Sigma-Aldrich), which utilizes a nonspecific chromogenic substrate that releases azo dye into the media upon cleavage. Fecal samples were centrifuged at 4000g for 15 min, and supernatants were diluted 1:10 with PBS before combining with substrate. 1% trichloroacetic acid (TCA) was added to the enzyme-substrate mixture to stop the reaction. Absorbance was read at 440 nm. Elastolytic activities were measured from cultures of bacterial supernatants using BHI media supplemented with elastin stained with congo red[248]. Positive proteolytic activity was determined by the presence of a hydrolytic halo surrounding the inoculation site on media containing respective substrates (eg. 1% gluten or 2% gelatin)[157]. Elastase activity of these bacteria and fecal samples from mice were also measured using Suc-Ala3-pNa, a specific colorimetric method by Sigma-Aldrich.

Nanostring gene expression analysis

Total RNA was extracted from colonic sections using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA purity and quality was verified using NanoDrop 2000 Spectrophotometer Bioanalyzer (Thermo Fisher), then reverse transcribed to complementary DNA using Reverse Transcriptase (Qiagen) according to the manufacturer's instructions. NanoString nCounter Gene Expression CodeSet for a custom NanoString Gene Expression CodeSet for selected genes were run according to the manufacturer's instructions (NanoString Technologies Inc.). Results were analyzed with the nSolver 2.5 software (NanoString Technologies) and heat maps

were generated based on hierarchical clustering of expression data. Log_2 ratios built from the expression data were analyzed using Ingenuity Pathway Analysis (Qiagen, Redwood City, CA, USA) in order to assess which canonical biological pathways were significantly altered. The network score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's exact test.

Bacterial translocation

Viable bacteria in tissues were detected using standard aerobic and anaerobic culture techniques[249]. MLN and spleen were aseptically isolated. Spleen tissues were diluted in ice-cold PBS to a concentration of 1 mg tissue per 10 μL, and MLN were diluted to 1 mg tissue per 100 μL. Diluted tissues were homogenized at 60 Hz for 3 min, and 75 μL of homogenate was cultured on either BHI or MRS for 48h at 37°C in aerobic and anaerobic conditions. Bacterial colonies were counted and normalized to tissue weight.

Serum LPS Detection

Levels of LPS were quantified in serum of mice using a limulus amebocyte lysate (LAL) Pierce chromogenic quantitation kit assay (ThermoFisher Scientific, Rockford, IL, USA) following the manufacturer's protocol. The reaction was measured on a microplate absorbance reader at 405 nm. The microplate was incubated at 37°C for 10 min before addition of chromogenic substrate solution. Acetic acid was used to stop the reaction after appearance of a colour change. Values are expressed as endotoxin units (EU) with 1 EU/ml serum.

***Ex vivo* Intestinal Permeability**

Intestinal permeability was evaluated *in vitro* by Ussing Chamber technique as previously described (World Precision Instruments, Sarasota, FL)[250]. Proximal colon samples were opened along the mesenteric border and mounted in the chambers, exposing

0.6cm of tissue. Kreb's buffer aerated (95% O_2, 5% CO_2) at 37°C, pH 7.3-7.4 bathed the tissues to maintain tissue viability. Glucose (10mM) was added to the serosal buffer as an energy substrate, and was osmotically balanced by 10mM of mannitol in the mucosal buffer. Potential difference across the tissue was measured via two Ag-AgCl electrodes in agar-salt bridges in close proximity to each side of the tissue. Two additional electrodes injected short circuit current (I_{sc}) to maintain voltage clamp conditions. Baseline PD and Isc were recorded after 20 minutes of equilibration. Paracellular permeability was evaluated by measuring the mucosal-to-serosal flux of the inert paracellular probe ^{51}Cr-EDTA. After baseline measurements, 6μCi/ml of ^{51}Cr-EDTA was added to the mucosal compartment, and a "hot" sample was obtained from the mucosal compartment. Samples from the serosal compartment were obtained every 30 minutes over 2 hours. ^{51}Cr-EDTA was quantified in samples using a liquid scintillation counter and expressed as % recovery/cm^2/hour.

Periodic acid-Schiff and Alcian blue staining

Colonic tissue samples were fixed in Carnoy's solution and embedded in paraffin. Acidic mucins were stained with 1% Alcian blue in 3% acetic acid (pH 2.5) for 15 min, and rinsed in tap water. Sections were then treated with 0.5% periodic acid for 5 min, and neutral mucins were stained magenta with Schiff's reagent for 10 min. Cells containing both acid and neutral mucins will stain purple. Tissue sections were then rinsed thoroughly with tap water. Nuclei were counterstained with hematoxylin. The number of goblet cells per crypt was calculated from an average of 10 crypts per tissue section for 7 mice.

β-defensin 3 expression in feces

Protein expression of mouse intestinal β-defensin 3 (homolog of human β-defensin 2) was analyzed using ELISA according to the manufacturer's protocol. Fecal samples were homogenized and diluted 1:10 in PBS with protease inhibitor. Absorbance

was read at 450 nm and values are expressed in nanograms of β-defensin 2 per mg of feces.

Quantification of polymorphonuclear infiltrate

Colonic tissue samples were fixed 10% buffered formalin then embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H&E), and assessed for inflammation in a blinded fashion. H&E-stained colonic sections were examined at 100x magnification in order to count the number of PMNs located in the intestinal epithelium. Counts were normalized to number of PMNs per 100 crypts.

Construction and preparation of *B. longum srp*(Con)

Strains were created and prepared as described under "Construction of Bacterial Strains" and "Preparation of *B. longum* biomass" in Materials & Methods of Chapter 4. Briefly, the constitutive promoter from the *B. longum* NCC2705 glyceraldehyde-3-phosphate dehydrogenase gene (*Bl1363*) was inserted in front of the *srp* gene (*Bl0108*) in the pMDY23 plasmid. This resulting plasmid constitutively expressing *srp* and was transformed into *B. longum* NCC2705, generating the *B. longum srp*(Con) recombinant strain.

Statistics

Statistical differences were assessed using ANOVA with Bonferroni post-hoc test or unpaired Mann-Whitney tests, when applicable, in GraphPad Prism 6.0. Outliers were identified using the ROUT method with a maximum false discovery rate of 1%. $p<0.05$ was considered significant.

3.3 RESULTS

In vitro microbial proteolytic activity phenotype is transferred to colonized mice

Colonization status was assessed by culturing bacteria from feces 3 weeks following colonization to recover the same morphologically distinct bacterial strains with which the mice were colonized. Each strain was present in all fecal cultures and approximately 10^9 cfu of bacteria per mg feces were recovered. Mice colonized with HPA strains had significantly higher overall proteolytic activity in fecal samples than LPA-colonized ($p<0.001$) and HD-colonized ($p<0.01$) mice. HPA-colonized mice also had significantly greater elastolytic activity in their feces compared to mice colonized with LPA microbiota ($p<0.01$) (Figure 4B). Finally, greater gelatin degradation was achieved by fecal supernatants of HPA-colonized mice compared to either HD- or LPA-colonized mice ($p<0.001$) (Figure 4).

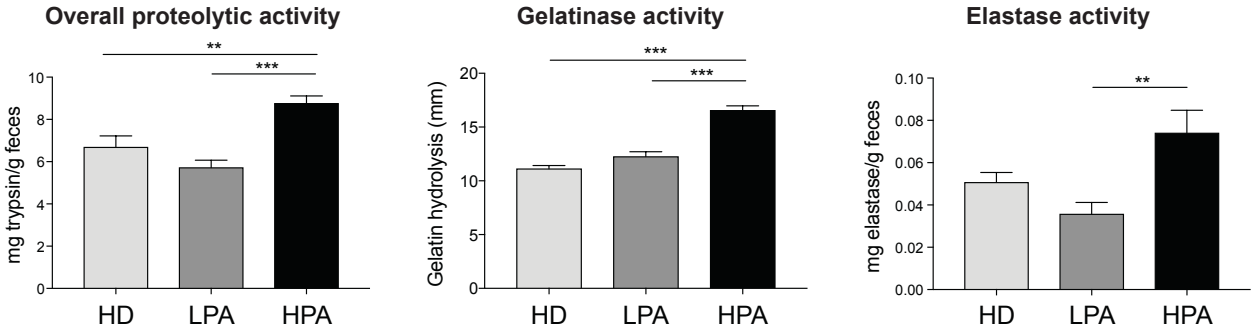


Figure 4. *In vitro* proteolytic activity from HD, LPA, and HPA microbiota are transferred to gastrointestinal tract of colonized mice. Overall, gelatinase, and elastase activity were measured in fecal supernatants. HPA-colonized mice had significantly higher overall proteolytic activity compared to HD-colonized mice ($p<0.01$) and compared to LPA-colonized mice ($p<0.001$). Similarly, gelatinase activity was significantly higher in HPA-colonized mice compared to HD-colonized ($p<0.001$) and LPA-colonized ($p<0.001$) mice. Elastase activity was also significantly higher in HPA-colonized mice compared to LPA-colonized mice ($p<0.01$). ** $p<0.01$; *** $p<0.001$.

Microbiota with high proteolytic activity is associated with disruptions in intestinal barrier function

We evaluated various features of intestinal barrier integrity in colonized mice. Viable bacterial translocation to the spleen was higher in HPA-colonized mice compared to those colonized with HD or LPA microbiota. Significantly more colony-forming units per mg of spleen were cultured from spleen homogenates compared with LPA-colonized ($p<0.05$) and HD-colonized ($p<0.05$) mice (Figure 5A). In accordance with this, levels of LPS in serum were higher in HPA-colonized mice compared to both HD-colonized ($p<0.05$) and LPA-colonized mice ($p<0.05$) (Figure 5B). We speculated that colonic paracellular permeability may be increased in HPA-colonized mice, however, ^{51}Cr-EDTA flux was not significantly different between groups (Figure 5C). Several barrier-related genes were differentially expressed in mice colonized with high proteolytic activity microbiota (Figure 5D). Expression of *Lyz1* was significantly upregulated in HPA-colonized mice compared to HD-colonized mice. Compared to LPA-colonized mice, *Lyz1, Hif1a, Cdh1, Tjp1, Cldn2*, and *Ttf3* were significantly upregulated in HPA-colonized mice. Since there were no significant differences in paracellular permeability between colonized mice, we next characterized the integrity of the colonic mucus layer using combined AB/PAS staining. There were significantly fewer PAS-stained colonic GCs in mice colonized with HPA compared to mice colonized with HD ($p<0.001$) and LPA ($p<0.01$) (Figure 5E).

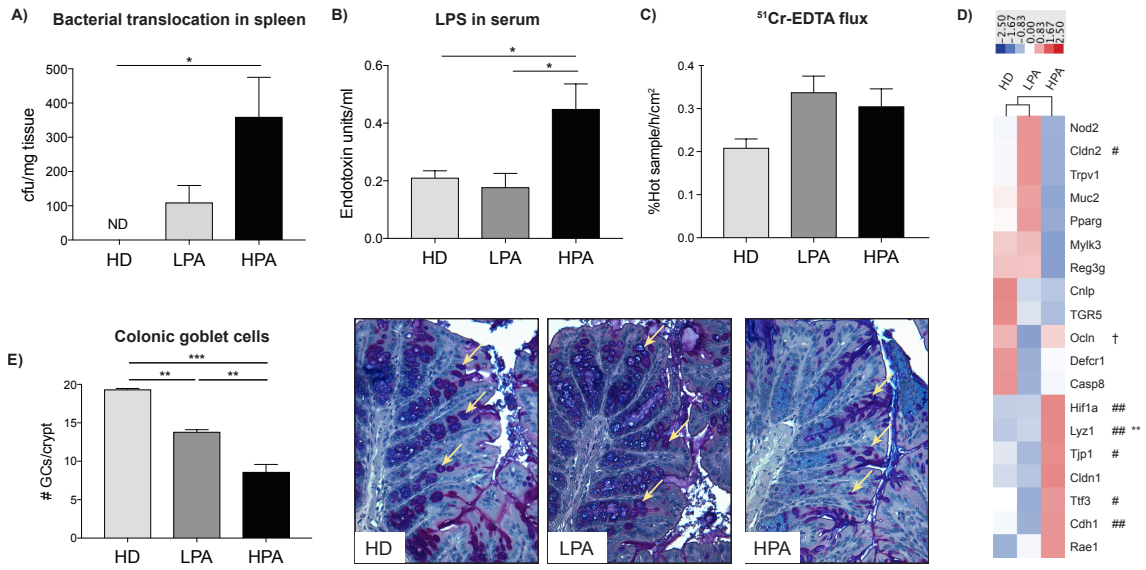


Figure 5. Microbiota with high proteolytic activity alters various components of the intestinal barrier.

A) HPA-colonized mice exhibited higher translocation of viable bacteria to the spleen compared to mice colonized with low proteolytic microbiota. **B)** Similarly, HPA-colonized mice had significantly higher levels of LPS in serum. **C)** Colonic paracellular permeability was not significantly increased between groups as measured *ex vivo* by ^{51}Cr-EDTA flux in Ussing chambers. **D)** Colonization with HD, LPA, and HPA resulted in differential expression of numerous genes involved in barrier function. * $p<0.05$ HD vs. HPA; **$p<0.01$ HD vs. HPA; # $p<0.05$ LPA vs. HPA; ## $p<0.01$ LPA vs. HPA; † $p<0.05$ HD vs. LPA. **E)** Colonic sections were stained with Periodic acid-Schiff and Alcian blue (PAS) in order to visualize mucus-filled GCs. Mice colonized with HPA microbiota had significantly lower numbers of PAS-stained GCs per crypt, as indicated by the yellow arrows, compared with those colonized with LPA ($p<0.01$) and HD microbiota ($p<0.001$) which had crypts filled with PAS-stained GCs. LPA-colonized mice also had less GCs per crypt compared to HD-colonized mice ($p<0.01$).

Microbiota with high proteolytic activity induces an inflammatory phenotype in the host

Inflammatory status was assessed in mice colonized with the three bacterial communities of varying proteolytic activities, HD, LPA, and HPA. We probed the

expression of various inflammation pathway genes, as well as the presence of inflammatory mediators in the colon. Colonization with high proteolytic activity HPA microbiota significantly upregulates pro-inflammatory genes compared to mice colonized with lower proteolytic activity HD or LPA microbiota. Expression of *CD11b* ($p<0.01$), *CCR2* and *IL-22ra2* ($p<0.05$) were significantly higher in HPA-colonized mice compared to LPA-colonized mice. *Cxcr4*, *CCR6*, and *CD11C* ($p<0.05$) were significantly upregulated in HPA-colonized mice compared to HD-colonized mice (Figure 6A). Ingenuity Pathway Analysis software revealed activation of a TRAF-dependent monocyte activation pathway given the gene expression changes in the Nanostring dataset (Supplementary figure 1). Furthermore, quantification of PMNs revealed greater inflammatory infiltrate in mice colonized with HPA microbiota (Figure 6C). Finally, levels of β-defensin are increased in the feces of HPA-colonized mice as measured by ELISA (Figure 6D).

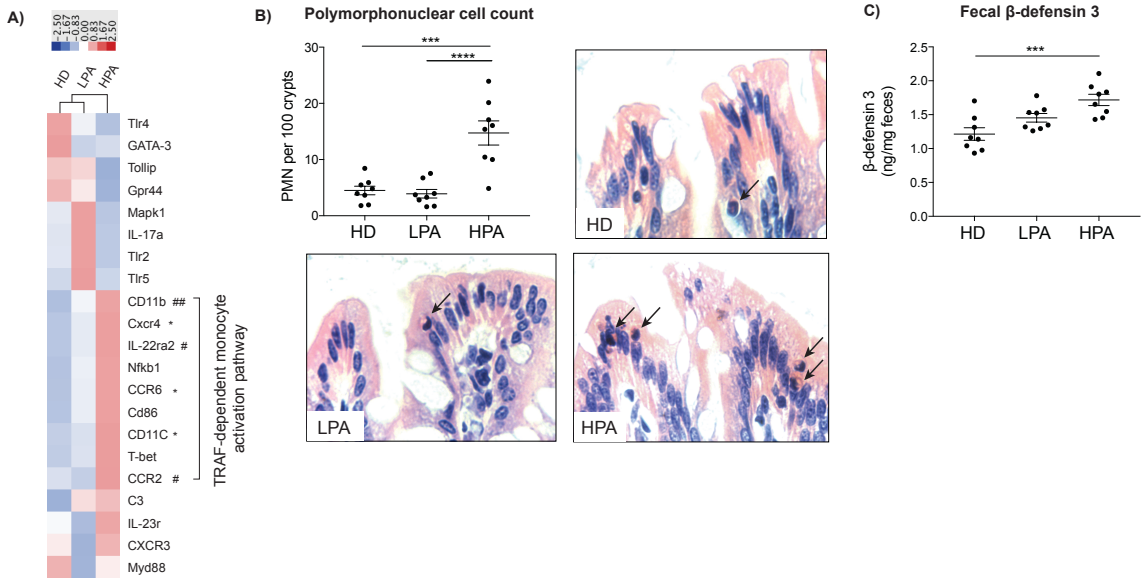


Figure 6. Microbiota with high proteolytic activity induces a pro-inflammatory phenotype. **A)** Several inflammatory genes were significantly upregulated in mice colonized with HPA microbiota. * $p<0.05$ HD vs. HPA; ** $p<0.01$ HD vs. HPA; # $p<0.05$ LPA vs. HPA; ## $p<0.01$

LPA vs. HPA; † $p<0.05$ HD vs. LPA. Upon analysis of gene expression data using Ingenuity Pathway Analysis software (Qiagen), upregulated genes were found to belong to a TRAF-dependent monocyte activation network (Supplementary figure 1). **B)** Colon of mice colonized with HPA had significantly greater numbers of PMN cells, as indicated in black arrows, within the epithelial layer compared to HD-colonized ($p<0.001$) and LPA-colonized ($p<0.0001$) mice. **C)** Levels of β-defensin 3 were significantly higher in feces of HPA-colonized mice compared to HD-colonized mice ($p<0.001$).

Administration of *B. longum* overexpressing serpin protects from intestinal barrier dysfunction and inflammation in HPA-colonized mice

To further investigate a causal role for microbial proteases, we inhibited microbial proteolytic activity from the HPA community using the bacterial protease inhibitor serpin constitutively expressed by *B. longum*. HPA-colonized mice were administered 10^9 cfu of either *B. longum* constitutively expressing serpin (*B. longum srp*(Con)) or vehicle (PBS-glycerol) each day for 2 weeks. *B. longum srp*(Con) treatment to HPA-colonized mice inhibited elastolytic activity ($p<0.01$) (Figure 7B). Further, some pathogenic effects of colonization with HPA were attenuated with *B. longum srp*(Con). Colonic paracellular permeability was measured via Ussing chambers, and HPA-colonized mice receiving *B. longum srp*(Con) intervention had lower ^{51}Cr-EDTA flux compared to those receiving PBS alone (Figure 7C). Furthermore, mice receiving *B. longum srp*(Con) had significantly less PMN cells in the colonic epithelium than mice receiving PBS-glycerol (Figure 7D).

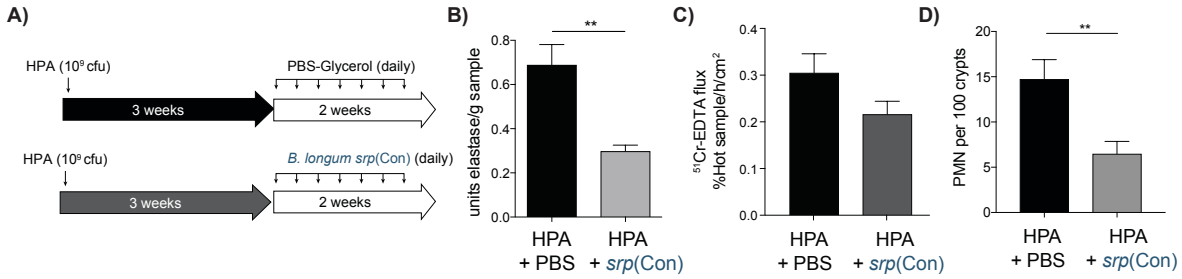


Figure 7. Effects of inhibiting microbial proteolytic activity in HPA-colonized mice. **A)** HPA-colonized mice were fed *B. longum srp*(Con) or PBS-glycerol (PBS) daily for two weeks by oral gavage. **B)** Administration of *B. longum srp*(Con) significantly decreased elastolytic activity in HPA-colonized mice ($p<0.01$). **C)** *B. longum srp*(Con) treatment resulted in decreased colonic paracellular ^{51}Cr-EDTA flux and **D)** PMN cell infiltrate in the colonic epithelium was significantly lower in HPA-colonized mice treated with *B. longum srp*(Con).

3.4 DISCUSSION

With the vast surface of the GI mucosa under constant antigenic challenge from the environment, the mucosal immune system must simultaneously defend the host against invading pathogens and maintain tolerance to dietary or innocuous antigens. Regulation of host-microbe interactions is critical for gut homeostasis and failure of normal regulatory mechanisms can lead to chronic inflammation[251]. Microbial proteases have classically been studied as adaptive factors for pathogens, soil bacteria, or extremophiles, but the concept that microbial proteases could serve as adaptive factors for inhabiting the GI tract is understudied.

Whether the increased proteolytic activity associated with various GI inflammatory disorders is due to host or microbial proteases/protease inhibitors has not been addressed, and therefore, this thesis aims to define a role for microbial proteases and protease inhibitors in overall gut homeostasis. We first explored the effects of increased microbial proteolytic activity by establishing a gnotobiotic mouse model of high microbial proteolytic activity. We found that mice colonized with high proteolytic activity

microbiota present with low-grade inflammation, increased live bacterial translocation, and increased serum endotoxin levels compared with mice colonized with low proteolytic activity microbiota. Inhibition of microbial proteolytic activity i*n vivo* using a commensal *B. longum* constitutively expressing serpin mitigated some of these pathogenic effects. Altogether, we demonstrate that an increase in proteolytic activity from a dysbiotic microbial community can mediate detrimental effects on the host.

3.4.1 In vivo model of high microbial proteolytic activity

We generated a reductionist *in vivo* model to test the effects of microbial proteolytic activity by colonizing mice with four strains of bacteria with either high or low proteolytic activity. Because most current studies examine changes in total mRNA and protein levels of host proteases, which is not necessarily reflective of proteolytic activity, we monitored total proteolysis via the cleavage of azocasein and elastase. These methods were used to verify transfer of the *in vitro* proteolytic activity of bacterial communities into the GI tract of colonized mice, and it was confirmed that HPA-colonized mice had the highest fecal proteolytic and elastolytic activity overall. Therefore, we successfully established an *in vivo* mouse model of microbial proteolytic imbalance in order to investigate potential effects on the host.

3.4.2 Intestinal barrier dysfunction

During homeostasis, the intestinal epithelium absorbs nutrients while effectively preventing translocation of intraluminal bacteria and their products. Disruption of intestinal barrier function allows viable bacteria and microbial products to translocate from the gut lumen to the systemic compartment. Mice colonized with high proteolytic activity bacteria exhibited greater barrier dysfunction compared to mice colonized with low proteolytic activity bacteria. Culturing MLN and spleen revealed significantly more translocation of viable bacteria to the spleen in HPA-colonized mice. Moreover, endotoxin levels in serum were significantly increased in the serum of HPA-colonized mice compared to either LPA- or HD-colonized mice. Circulating LPS bind to their

respective TLRs on various cells including macrophages and DCs, which results in activation of NFκB and increased expression of various pro-inflammatory cytokines that can exert deleterious systemic effects. It is important to note that although both LPA and HPA communities contain LPS-producing Gram-negative species, HPA-colonized mice had higher plasma levels of LPS. It would be interesting to examine the rate of LPS shedding of these species, as well as the potential variations in LPS composition which may alter their immunogenicity[79]. There are several possible mechanisms for excess leakage of bacteria and their products systemically, including defects in TJ function and the paracellular permeability pathway, the mucus barrier, structural injury, aberrant uptake of antigens via microfold cells of the Peyer's patches or direct sampling by DCs or macrophages. Normally, bacteria are transported to the MLN by DCs where a local immune response is induced, maintaining systemic immune ignorance[61, 252]. Indeed, similar numbers of bacteria were cultured from MLN of all colonized mice which may reflect this sampling process known to occurs after colonization in germ-free mice[59, 253]. However, in mice colonized with high proteolytic microbiota, spread of bacteria beyond the MLN occurred.

To probe the route of increased bacterial translocation, we assessed the expression of various barrier-related genes using Nanostring and found that several genes related to the maintenance and repair of gut barrier function were differentially regulated based on microbial proteolytic activity. Tight junction gene claudin-2 (*cldn2*) was downregulated, while ZO-1 (*Tjp1*), E-cadherin (*Cdh1*) and claudin-1 (*cldn1*) were upregulated in HPA-colonized mice. mRNA expression does not necessarily correlate with protein expression of these TJ proteins especially since degradation of TJ proteins may not be reflected at the mRNA level. Hence, we decided to investigate the overall net physiological effect of these variations in permeability genes by measuring paracellular permeability in the proximal colon of colonized mice via ^{51}Cr-EDTA flux. There was a trend of higher paracellular permeability in mice colonized with both LPA and HPA (derived from the UC patient) compared to the community with low proteolytic activity isolated from the

HD, suggesting that alterations in the paracellular permeability pathway may not be the mechanism underlying bacterial translocation in the model.

Apart from defects in the paracellular route, there are other mechanisms resulting in failure of the immunological barrier to compartmentalize intestinal microbiota. Transcellular epithelial translocation or direct sampling of luminal bacteria by resident cells (e.g. DC or M cell-mediated transport) can result in increased bacterial translocation without disrupting tight junction function. Interestingly, monocyte activation genes were upregulated in HPA-colonized mice which may indicate greater differentiation into macrophages. Dysregulated sampling of microbial antigens by DCs or macrophages may provide a conduit for the increased bacterial translocation and systemic LPS levels[254-256] in these mice colonized with high proteolytic microbiota.

Another possible mode of bacterial translocation into the periphery is through defects in the protective mucus layer covering the intestinal epithelial surface. Mucus layers are organized around the heavily glycosylated mucin, Muc2 produced by GCs, forming a net-like polymer[257]. Expression of *Muc2* was downregulated in HPA-colonized mice. Muc2 is the major component contained in GC granules, and in agreement, HPA-colonized mice also had significantly fewer intestinal GCs indicating GC depletion. Perhaps in HPA-colonized mice, this impairment permits bacteria to breach the epithelium and trigger inflammation. Along with decreased Muc2 and defects in mucus layer, depletion of GCs is frequently observed UC[258, 259]. The typical GC depletion seen in UC is used to assess disease activity and may reflect emptied GCs[11]. Furthermore, GC depletion is hypothesized to act as a host defence mechanism in response to pathogenic infection[260,261]. Since *C. perfringens* within in the HPA community possesses mucolytic activity, the host may be purposely downregulating GC numbers or mucus production by GCs in order to avert microbial access to the nutritional substrate mucin and control their invasion. Thus, the microbial signals from high proteolytic microbiota are inducing emptying by GCs or other GC functional changes in the host, which may be intertwined with the increased systemic translocation of microbes and other inflammatory phenomena.

Another component of the intestinal barrier related to immune defense is the production of AMPs. AMP production is induced by bacteria and promotes bacterial killing by permeabilizing bacterial cell walls. They are secreted by epithelial cells and retained in the mucus layer, where they prevent direct contact between microbes and the intestinal epithelium[206]. β-defensins are AMPs with broad-spectrum antimicrobial activity typically produced by epithelial cells. Its secretion is up-regulated in response to inflammatory and bacterial stimuli[262-264]. Furthermore, secretion of human β-Defensin 2 is increased in numerous gut inflammatory disorders such as the colon in CD[265,266] and the feces of patients with IBS[267,268]. We measured the mouse homologue of β-defensin 2, β-defensin 3, in feces of colonized mice and found a significant increase of β-defensin 3 in mice colonized with HPA compared to both HD or LPA. High proteolytic microbiota therefore induces a heightened barrier defensive response in the host, and such innate immune activation may be an antecedent to immune dysregulation seen in GI pathology.

3.4.3 Immune activation

After further characterizing the immune status in colonized mice, we found that increased microbial proteolytic activity in HPA-colonized mice is associated with a proinflammatory molecular signature and microscopic intestinal inflammation. When we assessed the expression of 21 inflammation-related genes in colonic tissue, 16 were significantly up-regulated in mice colonized with high proteolytic microbiota, including *CD11b, CD11c, CCR6, CCR2, CXCR4,* and *IL-22ra2*. *CCR2* encodes a receptor for monocyte chemoattractant protein-1 (CCL2), a cytokine that mediates monocyte chemotaxis. Immature dendritic cells express the receptor CCR6 which may regulate the migration and recruitment of DCs and T cells during inflammation. Both CD11b and CD11c are highly expressed on monocytes and macrophages[269]. Ingenuity pathway analysis was used to reveal biological networks related to cell-to-cell signaling and interaction that were up-regulated in the colonic tissue of mice colonized with high proteolytic microbiota. The TRAF-dependent monocyte activation network was most significantly activated in mice colonized with high proteolytic microbiota, consisting of

CD11b, CD11c (ITGAX), CCR6, CCR2, *CXCR4*, *CD86* and *LYZ*. It is possible that activated monocytes, potentially macrophages, recruit other immune cells such as neutrophils to the intestinal compartment. We are currently quantifying macrophage numbers in the colon through F4/80 immunostaining, and have quantified PMN recruitment in H&E-stained sections. The aberrant recruitment, activation, and accumulation of neutrophils is central to IBD pathogenesis, and indeed, there was a greater extent of PMN infiltrate in the colonic epithelium of HPA-colonized mice compared to mice colonized with low proteolytic microbiota. Both macrophage and neutrophil accumulation induced by a dysbiotic microbiota with high proteolytic activity may amplify inflammatory responses through recruitment of other immune cells.

Microbial proteases can activate the mucosal immune system indirectly via barrier defects or directly via cleavage of inflammatory mediators or PARs on immune cells[270]. Previous studies have established a correlation between circulating LPS and innate immune activation in the host, and serum LPS is a cause of HIV-related systemic immune activation[271]. Therefore, the induction of innate immune genes and elevated serum LPS in HPA-colonized mice support a pro-inflammatory role of high proteolytic microbiota.

3.4.4 Intestinal dysfunction

β-defensins are well-known for their role in mast cell activation, triggering their release of histamine. Increased levels of histamine have also been associated with CD[272], IBS[273], microscopic colitis[274], and UC[275], and plays a central role in disease pathogenesis. Patients with diarrhea-predominant IBS (IBS-D) have increased mast cell activation and hyperplasia throughout the small intestine[276]. Thus, our results raise the possibility that microbial protease activity contributes to mast cell dysfunction in IBS through increases in β-defensin. Although the pathophysiology of IBS is incompletely understood, several physiological functions that are dysregulated in IBS are known to be modified by proteases. Through PAR2 cleavage, proteases can cause visceral hypersensitivity and alter GI motility[238]. Furthermore, increased tryptase and trypsin/trypsin-like activity has been detected in colonic biopsies of IBS patients[203], and treatment with serine protease

inhibitors protected against nociception in mice sensitized with biopsy supernatants[233]. Patients with IBS-D also have increased serine protease activity which may contribute to colonic hypersensitivity. The pathophysiology of IBS-D includes rapid intestinal transit and increased luminal fluid secretion[277], and high proteolytic microbiota may causally contribute to these changes. We therefore measured colonic expression of various genes involved in the neural pathways, and found that serotonin (5-HT) was significantly upregulated in HPA-colonized mice compared to HD- or LPA-colonized mice (Supplementary figure 2). Serotonin is a key signaling molecule that targets enterocytes, smooth muscle, and enteric neurons in the gut. Abnormal increases in 5-HT have been described in a range of GI diseases including CeD, IBS, IBD and IBS-D[278]. Although the role of serotonin is controversial and polyfunctional, the release of 5-HT is known to stimulate local enteric nervous reflexes to increase secretion and propulsive motility. We measured ion secretion on colonic sections of mice by determining short-circuit current (I_{sc}) in Ussing chambers. Although it did not reach significance, I_{sc} was higher in HPA-colonized mice with a mean of 76 uA/cm^2 compared with 59 uA/cm^2 in HD-colonized mice, indicating potentially greater electrogenic ion movement by active transcellular transport. Serotonin-initiated electrogenic Cl^- secretion is important for dilution and neutralization of luminal contents, as well as secretory diarrhea in response to inflammation and for the elimination of luminal pathogens[279-281]. HPA-colonized mice showed increases in 5-HT mRNA, β-defensin, ion secretion, and perhaps faster colonic transit. Although preliminary, the results propose more careful characterization of microbial proteases in modulating neuromuscular intestinal function in our model.

3.4.5 Microbial protease inhibitors

Having established possible mechanisms wherein commensal microbiota with a high proteolytic activity phenotype could underscore disease pathogenesis, we investigated whether these effects could be attenuated by inhibiting proteolytic activity using a microbial protease inhibitor. Therefore, we administered *B. longum srp*(Con) to HPA-colonized mice each day for 2 weeks. Although differences in overall proteolytic

activity were less clear, *B. longum srp*(Con) treatment resulted in significant inhibition of elastolytic activity. Inhibition of elastolytic activity was associated with a lower PMN infiltrate to the colonic epithelium and decreased paracellular permeability in HPA-colonized mice. In the future, colonizations using a greater number of wild-type and mutant strains with and without proteolytic activity, respectively, will help to further define a pathogenic role for bacterial proteases.

3.4.6 Conclusions

Microbially-driven proteolytic imbalance can induce a low-grade inflammatory state in the host that can be blocked through administration of microbial proteases. We have demonstrated that microbiota with high proteolytic activity induces activation of innate immune pathways, as well as an altered intestinal barrier which is associated with increased translocation of viable bacteria and bacterial products (Figure 8). We can speculate a broad mechanism in which increased microbial proteolytic activity induces a host immune response characterized by the induction of β-defensin 3, GC depletion, and upregulation of pro-inflammatory genes, including those involved in monocyte activation. Despite these defensive responses, microbes and microbial products still breach the intestinal barrier gaining systemic access. Acute GC depletion may provide a gateway for bacterial translocation through a defective mucus layer[13,257]. This, coupled to increased differentiation of monocytes to macrophages may allow access of microbial antigens from the luminal to systemic compartment and/or recruitment of acute inflammatory cells such as PMNs to the colon. The accumulation of PMN in the colon may then amplify immune activation and contribute to chronic inflammation. More work is needed to complete this mechanistic picture and evaluate its relevance in patients with dysbiosis and gut inflammation. Nonetheless, the results provide evidence for microbially-driven proteolytic imbalance as a pathogenic mechanism which may contribute to the onset of gut inflammatory disorders.

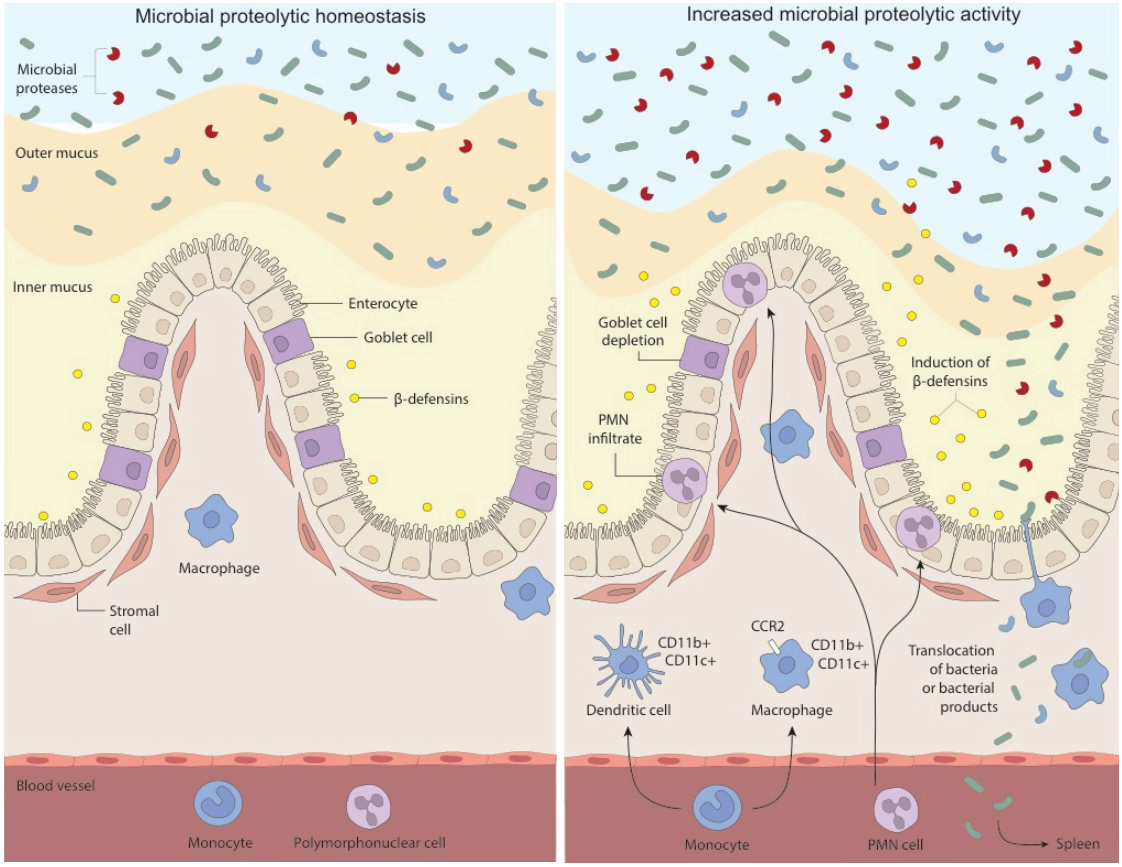


Figure 8. Summary of host effects observed in mice colonized with high proteolytic microbiota. Colonization with microbiota with high proteolytic activity results in distinct changes in both the intestinal barrier and immune system that are associated with increased translocation of bacteria and bacterial products into systemic circulation. In the absence of microbial proteolytic imbalance (left panel), there is minimal translocation of bacteria, and homeostatic processes are in place. With a dysbiotic microbiota producing high levels of proteolytic activity (right panel), there is increased production of β-defensin, depletion of GCs, and upregulation of monocyte activation pathway genes such as *CCR2* and *CD11b* and *CD11c*. As well, PMNs infiltrate and accumulate in the colonic epithelium, further indicating a pro-inflammatory phenotype in mice colonized with high proteolytic microbiota.

CHAPTER 4

THERAPEUTIC ROLE OF A MICROBIAL PROTEASE INHIBITOR

(Applied and Environmental Microbiology, submitted and in second revision)

A commensal *Bifidobacterium longum* strain improves gluten-related immunopathology in mice through expression of a serine protease inhibitor

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Running Title: A commensal *B. longum* prevents gluten pathology through expression of a serpin

Keywords: probiotic; microbiota; gluten; serpin, celiac

ABSTRACT

Microbiota-modulating strategies, including probiotic administration, have been tested for the treatment of chronic gastrointestinal diseases despite limited information regarding their mechanisms of action. We previously demonstrated that patients with active celiac disease have decreased duodenal expression of elafin, a human serine protease inhibitor, and supplementation of elafin by a recombinant *Lactococcus lactis* prevents gliadin-induced immunopathology in the NOD/DQ8 mouse model of gluten sensitivity. The commensal probiotic strain *Bifidobacterium longum* NCC2705 produces a serine protease inhibitor (Srp) that exhibits immune-modulating properties. Here, we demonstrate that *B. longum* NCC2705, but not a *srp* knockout mutant, attenuates gliadin-induced immunopathology and impacts intestinal microbial composition in NOD/DQ8 mice. Our results highlight the beneficial effects of a serine protease inhibitor produced by commensal *B. longum* strains.

IMPORTANCE:

Probiotic therapies have been widely used to treat gastrointestinal disorders with variable success and poor mechanistic insight. Delivery of specific anti-inflammatory molecules has been limited to the use of genetically modified organisms, which has raised some public and regulatory concerns. By examining a specific microbial product naturally expressed by a commensal bacterial strain, we provide insight into a mechanistic basis for the use of *B. longum* NCC2705 to help treat gluten-related disorders.

INTRODUCTION

Microbiota-modulating therapies have been tested for the treatment of chronic gastrointestinal diseases and disorders with inconsistent findings. Probiotics are live microorganisms, which when administered in adequate amounts, confer a health benefit on the host (Food and Agriculture Organization and World Health Organization, 2002). Specific strains have shown modest efficacy in irritable bowel syndrome (IBS) (1), complications of inflammatory bowel disease (IBD), such as pouchitis (2), and celiac disease (CeD), a chronic enteropathy caused by ingestion of gluten-containing cereals in genetically susceptible individuals (3). In particular, *B. longum* strains have been proposed as beneficial supplements for a wide range of health conditions. Depletions in bifidobacteria have been noted in patietns with CeD(4), and attempts have been made to supplement these strains as therapy (3, 5). However, despite great public interest in the clinical use of specific probiotic strains for intestinal disorders, there is insufficient mechanistic insight to rationalize consistent recommendations. Investigating therapeutic effects of specific molecules produced by probiotic strains may help bridge this gap.

Dysregulated proteolytic balance has been described in several gastrointestinal disorders (6-10). We have previously shown that the expression of the human serine protease inhibitor (serpin), elafin, is decreased in the duodenum of patients with active CeD (10, 11). Recombinant *Lactococcus lactis* expressing elafin has been shown i) to be protective in several murine colitis models (10) and ii) to prevent gluten immunopathology in the NOD/DQ8 mouse model of gluten sensitivity (11). However, given the concerns raised with the clinical application of such genetically modified

organisms (GMOs), we investigated the effect of a commensal bacterium that naturally expresses an elafin-like serpin. Although eukaryotic serpins such as elafin are known to possess anti-inflammatory properties (8), bacterially produced serpins have not been explored for their therapeutic capacity *in vivo*. The infant-derived commensal probiotic strain *Bifidobacterium longum* NCC2705 (*B. longum srp*$^+$) produces a serpin (Srp) encoded by the gene *BL0108* (*srp)*, in a non-constitutive manner. Expression of *srp* is induced in the murine intestinal tract, and Srp may exhibit anti-inflammatory properties as it inhibits both pancreatic and neutrophilic elastase *in vitro* (12). We tested the hypothesis that administration of the commensal *B. longum srp*$^+$ prevents immunopathology in the NOD/DQ8 mouse model of gluten sensitivity.

We show that both the wild-type *B. longum srp*$^+$ and a recombinant strain constitutively expressing *srp* (*B. longum srp* (Con)), prevents gliadin-induced immunopathology in NOD/DQ8 mice, while the *srp* knockout strain (*B. longum* Δ*srp*) does not. These results clearly suggest that the beneficial effect of *B. longum srp*$^+$ is mediated by Srp. This warrants clinical investigation of commensal *B. longum srp*$^+$ in managing CeD and non-celiac gluten/wheat sensitivity (NCG/WS), or chronic gastrointestinal conditions associated with proteolytic imbalance.

MATERIALS & METHODS

Construction of bacterial strains

B. longum NCC2705 (*B. longum srp*$^{+}$) was isolated at the Nestlé Research Center from the feces of a healthy infant (13). The strain is well-characterized at the molecular and biochemical levels. The full genome of 2.26 Mb has been sequenced, and it was demonstrated that *srp*, previously known as *Bl0108*, encodes a *bona fide* serine protease inhibitor with affinity and inhibitory activity to eukaryotic elastases (12).

Upstream and downstream sequences (3kb) of the *srp* gene of *B. longum srp*$^{+}$ were amplified by PCR and cloned into the pJH101 vector. The pJH101 vector is available at the German Collection of Microorganisms and Cell Cultures (DSMZ) and was initially designed for the construction of integrable plasmids in *B. subtilis*. pJH101 contains a chloramphenicol resistance gene and does not contain an origin of replication for *B. subtilis* nor *B. longum*. The resulting plasmid pMDY24 containing no coding sequences of *srp* was introduced into *B. longum srp*$^{+}$. Transformation was performed as described previously (14). Five transformants were obtained by plating on MRS medium supplemented with 0.05% cysteine (MRS-cys) containing chloramphenicol, and integration was confirmed by Southern blot. Transformants were cultivated for 100 generations on MRS-cys without chloramphenicol to clear the antibiotic resistance gene. Twelve chloramphenicol-sensitive isolates were confirmed to be *srp* knockout strains. One isolate was included in the Nestlé Culture Collection under *B. longum* NCC 9035 (*B. longum* Δ*srp*).

The plasmid pMDY25 was constructed by inserting a constitutive promoter from *Bl1363*, glyceraldehyde-3-phosphate dehydrogenase, in front of the *srp* gene in the pMDY23 plasmid which encodes spectinomycin resistance. In this recombinant strain, the level of synthesis of Srp no longer depends on any kind of induction (*B. longum srp*(Con)).

The *L. lactis* food-grade strain was engineered to express recombinant human elafin (*L. lactis*-elafin), whose expression was driven by a nisin-inducible promoter, as described in detail previously (10).

Preparation of *B. longum* biomass

B. longum srp$^+$ and *B. longum Δsrp* strains were inoculated at 2% from a fresh overnight culture in MRS-cys and grown anaerobically at 37°C for 16h. Bacteria were harvested by centrifugation, resuspended in sterile PBS containing 20% glycerol (PBS-20% glycerol) and stored in aliquots at -80°C. Viable counts for the *B. longum srp*$^+$ and *B. longum Δsrp* preparations were equal to 6.6×10^9 cfu/ml and 4.4×10^9 cfu/ml, respectively. *B. longum srp*(Con) was cultured and further processed as described above in the presence of 100 μg/ml of spectinomycin and grown for 48 h at 37°C. Levels of viable bacteria were equal to 1.5×10^9 cfu/ml.

***In vitro* inhibitory activity of *B. longum* strains against elastase.**

Enzymatic activity of human neutrophil elastase (HNE) was determined by cleavage of FITC-labeled elastin (FITC-elastin). Concentrations of HNE (1.5, 3.125,

6.25, 12.5, 25, and 50 mU/mL) were incubated with 10^8 cfu of *B. longum srp*$^+$, *B. longum srp*(Con) or *B. longum Δsrp* and 40 µL of buffer solution (50 mM Tris-HCL, 1mM $CaCl_2$, 50 mM NaCl, 0.25% Triton X100; pH 8.0) at 37°C for 30 min. 50 µL FITC-elastin substrate was added and fluorescence was measured at an excitation wavelength of 530 nm using a spectrophotometer (SpectraMax, Molecular Devices, San Leandro, CA).

Animals

All experiments were conducted with approval from the McMaster University Animal Care Committee. Female and male 8 to 12-week-old NOD/DQ8 transgenic mice (15) were fed a gluten-free diet for 2 generations (Harlan Laboratories, Indianapolis, IN) and housed in a specific pathogen-free colony at McMaster University. These mice lack all endogenous mouse MHC II molecules and express the DQ8 human transgene on a NOD background (15). Oral sensitization of NOD/DQ8 mice with peptic-tryptic (PT) digest of gliadin, one of the main protein fractions in gluten, and subsequent gliadin challenge induces moderate enteropathy, intraepithelial lymphocytosis, and barrier dysfunction (Figure 9A) as described previously (15).

Mucosal delivery of *B. longum* and gliadin sensitization.

Mice were orally gavaged with 500 µg PT-gliadin plus 25 µg cholera toxin as adjuvant (Sigma-Aldrich) once a week for 3 weeks. Non-sensitized mice (control) were gavaged with PBS plus 25 µg cholera toxin. Sensitized mice were then treated daily by oral gavage (10^9 cfu, 200µl/mouse) for two weeks with either *B. longum srp*$^+$, *B. longum*

Δsrp, or *B. longum srp* (Con) suspended in PBS-20% glycerol. During the probiotic treatment period, sensitized mice were orally challenged with gliadin (2 mg/mouse) dissolved in 0.02 M acetic acid (vehicle) three times per week. Vehicle-treated mice were simultaneously gavaged PBS-20% glycerol during the challenge period. Control mice were maintained on a gluten-free chow diet and gavaged with PBS-20% glycerol and 0.02 M acetic acid (Figure 9A).

Detection of *B. longum* strains.

Primers used for specificity towards *B. longum srp*$^+$ and derivatives were used to amplify DNA extracted from proximal small intestinal tissue and contents (Figure 14B). The primers were as follows: forward, 5'-TCCAGATCATTTCCGATTCC-3'; reverse, 5'-CGGCGTATTTCTATCGCATC-3' and amplified as previously described (16).

***srp* mRNA expression in *B. longum* strains.**

B. longum srp$^+$, *B. longum srp*(Con) and *B. longum Δsrp* were cultured as above for 8 h and cells were collected by centrifugation. Total RNA was extracted using the RNeasy mini kit (Qiagen) with additional DNAse treatment. Purity and quality was checked using QIAxcel RNA Quality Control Kit v2.0 (Qiagen). RNA level was quantified using the SuperScript III Platinium SYBR Green One-Step qRT-PCR kit (Invitrogen) and using standard PCR conditions described in the kit. *srp* primers were as follows: forward 5'-ACCAATCGCTGCTAAGTTCG-3', reverse 5'-TCGCTGGCAAGAGAGTAGTC-3'. The lactate dehydrogenase (*ldh*) housekeeping

gene was used for standardization. The following primers were used for *ldh*: forward, 5'-CGAACGCCATCTACATGCTC-3' and reverse, 5'-AAGATCTGGTTCTCTTGCAG-3'. Fold change of *srp* mRNA was calculated using the Pfaffl method (17).

***srp* detection *in vivo*.**

srp mRNA was measured in small intestinal contents and feces *of B. longum srp*$^{+}$-treated mice. Samples were collected fresh and flash frozen in liquid nitrogen. RNA was extracted from these samples using the PowerMicrobiome™ RNA Isolation kit (cat N° 26000-50, MoBio Laboratories Inc., Carlsbad, CA). RNA quality was checked using the Agilent 2100 Bioanalyzer system with the Agilent RNA 6000 nano kit. RNA was quantified using the Quant-it Ribogreen RNA kit. *srp* mRNA was measured by qRT PCR in two steps. RNA transcription to single stranded cDNA was performed using 1 μg of RNA in 20 μl of total reaction using qScript cDNA supermix and the following PCR conditions: 25°C 5 min; 42°C 30 min; 85°C 5 min and 4°C hold. *srp* was further amplified by real time PCR using the following primers: forward 5'-ACCAATCGCTGCTAAGTTCG-3', reverse 5'-TCGCTGGCAAGAGAGTAGTC-3'and probe FAM 5'-CCGAGATGAGCGCCGCGAACT-3'BHQ (Microsynth). cDNA (100 ng) was used in a total reaction of 20 μl with the TaqMan Universal PCR Master Mix and the following cycle: 50°C for 2 min; 95°C for 10 min; 95°C for 15 sec; 60°C for 1 min; repeated for 40 cycles.

Evaluation of small intestinal immunopathology.

Small intestinal cross-sections were fixed in 10% buffered formalin for 48 hours and embedded in paraffin, as previously described (15). Immunohistochemistry was performed on formalin-fixed, paraffin-embedded sections of proximal small intestine to visualize $CD3^+$ cells as described previously (15, 18). Slides were examined at 20X magnification using light microscopy in a blinded fashion. The number of $CD3^+$ IELs per 20 enterocytes were counted in five randomly chosen villous tips by a blinded observer as described, and expressed as IELs/100 enterocytes (18). Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) for histological evaluation of tissue morphology under light microscopy (Olympus, ON, Canada). Using the Image-Pro 6.3 software (Mediacybernetics, MD, USA), enteropathy was quantified in a blinded fashion by measuring villus-to-crypt ratios (V:C) as previously described (15). Intestinal paracellular permeability was evaluated *ex vivo* by Ussing Chamber technique as previously described (World Precision Instruments, Sarasota, FL) (19). Paracellular permeability of proximal small intestinal samples was evaluated by measuring the mucosal-to-serosal flux of the inert paracellular probe ^{51}Cr-EDTA. ^{51}Cr-EDTA was quantified in samples using a liquid scintillation counter and expressed as % recovery/cm^2/hour, or ^{51}Cr-EDTA flux.

Microbiota compositional analysis.

Fecal and small intestinal contents were collected and flash frozen on dry ice. DNA was extracted from samples as previously described (20) and amplified for the

hypervariable V3 region of the 16S rRNA gene for sequencing on the Illumina MiSeq platform (Illumina, San Diego, CA). Analysis of data was performed as previously described (20). Briefly, sequences were trimmed using Cutadapt software (version 1.2.1) (21) and aligned through the PANDAseq software (version 2.8) (22). Operational taxonomic units selected using AbundantOTU (23) were assigned taxonomy according to the Greengenes reference database (24). Principal coordinate analysis (PCoA) plots were generated using R (R Foundation for Statistical Computing, Vienna, Austria). Pairwise UniFrac distances were calculated among microbial communities, and both relative abundance data (weighted) and presence/absence information (unweighted).

Statistical Analysis

Data were analyzed in GraphPad Prism 6.0, QIIME, R and SPSS software. Normal data were analyzed by ANOVA followed by Bonferroni Post-hoc analysis. HNE inhibition statistics were performed using Mann-Whitney test compared to buffer. Microbiota β-diveristy statistics were performed using PERMANOVA. Microbiota abundances were analyzed in SPSS via Kruskal-Wallis followed by FDR ($q<0.05$). All significant genera presented passed FDR.

RESULTS

B. longum srp^{+} and *L. lactis*-elafin are equally effective in preventing gliadin immunopathology in mice.

We initially compared the efficacy of *B. longum srp*$^{+}$ with elafin delivery by recombinant *L. lactis*, previously shown to prevent intraepithelial lymphocytosis in NOD/DQ8 mice sensitized with gliadin (25). Mice treated with *L. lactis*-elafin or *B. longum srp*$^{+}$ had lower CD3^{+} IEL counts in the small intestine compared with mice receiving vehicle and gliadin ($p<0.05$) (Figure 9B and 9C).

B. longum constitutively expressing *srp* exhibits an increased inhibitory capacity towards human neutrophil elastase *in vitro*.

To characterize *B. longum srp*$^{+}$, *B. longum Δsrp,* and *B. longum srp*(Con), we first measured the expression of *srp in vitro*. *B. longum srp*(Con) expressed 452-fold more *srp* mRNA *in vitro* than *B. longum srp*$^{+}$, and no *srp* mRNA was detected in *B. longum Δsrp* (Figure 10A). We then quantified the ability of *B. longum srp*$^{+}$ and *B. longum srp*(Con) to inhibit human neutrophil elastase (HNE) activity *in vitro*, as pure Srp from *B. longum srp*$^{+}$ was previously shown to inhibit HNE. *B. longum Δsrp* did not inhibit proteolysis of elastin by HNE, as RFU produced from cleavage of FITC-elastin was similar at all concentrations of HNE added. Compared to *B. longum Δsrp*, *B. longum srp*$^{+}$ inhibited elastin degradation by HNE at 1.5 mU/ml ($p<0.01$) resulting in a lower RFU value. *B. longum srp*(Con) inhibited HNE activity at all concentrations of HNE

compared to *B. longum Δsrp* ($p<0.01$ at 3.125, 6.25, 50 mU/ml; $p<0.05$ at 1.5, 12.5, 25 mU/ml) (Figure 10B).

***B. longum srp* mediates the protective effect observed in mice.**

We tested the capacity of *B. longum* strains to prevent gliadin immunopathology using *B. longum srp*$^+$, *B. longum Δsrp* (positive control), and *B. longum srp*(Con), NOD/DQ8 mice sensitized to gliadin and treated with *B. longum Δsrp* had higher IEL counts compared to non-sensitized mice (controls; $p<0.0001$) or to mice receiving *B. longum srp*$^+$ ($p<0.0001$) or *B. longum srp*(Con) ($p<0.0001$) (Figure 11A). Mice receiving *B. longum Δsrp* had reduced V:C ratios compared with controls ($p<0.05$) and *B. longum srp*(Con)-treated mice ($p<0.05$) (Figure 11B). Lastly, mice treated with *B. longum Δsrp*, but not mice receiving *B. longum srp*$^+$ or *B. longum srp*(Con), had increased paracellular permeability in the proximal small intestine compared with controls ($p<0.05$) (Figure 11C).

Gliadin and *B. longum srp* expression shift fecal microbiota profiles in mice

Both the small intestinal and fecal contents of controls and gliadin-sensitized *B. longum*-treated NOD/DQ8 mice were sequenced using 16s Illumina technology. The small intestinal microbiota profiles were similar between all groups (Supplementary Figures 5, 6). However, in both weighted (Bray-Curtis Dissimilarity) and unweighted (Unifrac) β-diversity parameters, shifts in fecal microbiota profiles were observed between controls and all gliadin-sensitized mice (Figure 12, Supplementary Figure 7).

Moreover, gliadin-sensitized mice treated with *B. longum srp*$^+$ and *B. longum srp*(Con) clustered separately from mice receiving *B. longum Δsrp*, and this difference in β-diversity was significant (Figure 12). Relative abundances of Actinomycetales were lower in all gliadin-sensitized mice compared with controls. *B. longum srp*(Con) administration was associated with elevated levels of *Akkermansia*. An unknown Clostridiaceae was increased in mice treated with *B. longum srp*(Con) compared with those given *B. longum srp*$^+$ or no probiotic. Relative abundance of an unknown Clostridiales Family XIII was increased in gliadin-treated mice given *B. longum Δsrp* and *B. longum srp*$^+$ (Figure 13B).

B. longum strains and _B. longum srp_ are detected in the gastrointestinal tract of treated mice

We next determined whether *B. longum srp*$^+$ and the mutant strains, *B. longum srp*(Con) and *B. longum Δsrp,* were present in the small intestinal lumen of treated mice via PCR amplification. There was no difference in relative abundances of total Bifidobacteria in the small intestine between mice receiving vehicle or any of the *B. longum* strains, as measured by 16 Illumina sequencing (Figure 14A). However, strain-specific primers for *B. longum srp*$^+$ revealed that *B. longum srp*$^+$ and its derivatives were present in the small intestine of treated mice (Figure 14B). Furthermore, *srp* mRNA was detected in the feces and/or intestinal content of 2/4 mice treated with *B. longum srp*$^+$ and 3/4 mice treated with *B. longum srp*(Con). In contrast, *srp* mRNA was not detected in any sample from controls or *B. longum Δsrp*-treated mice.

DISCUSSION

There is a spectrum of clinical conditions caused by adverse reactions to gluten, and its constitutive proteins, such as gliadin. These include the well-characterized autoimmune enteropathy CeD, wheat allergy, as well as NCGS/NCWS which overlaps symptomatically with IBS (26). The only effective management for CeD is a life-long gluten-free diet (GFD), which has several limitations including poor compliance, accidental contaminations and slow resolution of mucosal inflammation (27). Patients with NCGS/NCWS also improve symptomatically on a GFD, but it is unknown whether these patients will tolerate less restrictive avoidance or could be successfully treated with other therapies. Since patients with active CeD and non-responders to the GFD, have been found to harbor dysbiotic intestinal communities (4, 28-30), probiotics have been proposed as potential candidates to restore gut microbial homeostasis. Smecuol *et al*. (2013) found that administration of the *Bifidobacterium* Natren life start (NLS) attenuated symptoms in CeD patients on a gluten-containing diet (31), and administration of NLS was shown to modulate innate immunity in a follow-up study (3). In another clinical trial, children with newly diagnosed CeD that received encapsulated *B. longum* CECT 7347, showed moderate changes in inflammatory markers and microbiota, but no symptomatic improvement beyond those achieved with the concomitant GFD (32). Although these studies raise the possibility that certain probiotics may be beneficial, adjuvant to the GFD, in CeD and perhaps other gluten-related disorders, their use was not guided by pathophysiological rationale and the mechanisms of action remain unclear.

Our study addresses the efficiency of a specific bacterial serpin (Srp), expressed naturally by *B. longum srp*$^{+}$, in the prevention of inflammation induced by gliadin in a genetically susceptible mouse model (11, 15, 33) with previously determined well-defined endpoints. We have previously shown that the severity of gluten immunopathology in NOD/DQ8 mice is influenced by the microbiota with which these mice are colonized, and that administration of recombinant *L. lactis* expressing elafin can attenuate the inflammatory response of the host towards gluten (25, 34, 35). Serpins are produced by a wide range of organisms and play a key role in maintaining immune homeostasis (36, 37). In the gut, serpins are expressed at mucosal surfaces and are involved in regulating barrier function (38, 39). Srp inhibits eukaryotic serine proteases *in vitro*, including both neutrophilic and pancreatic elastase. The inhibition of neutrophil elastase, which is a driver of intestinal tissue damage and a biomarker of intestinal inflammation (40), represents an immunomodulatory capacity for Srp that may be relevant in treating gastrointestinal inflammatory conditions (12). We confirmed that the recombinant *B. longum srp*(Con) expresses higher levels of *srp* than *B. longum srp*$^{+}$ *in vitro* (Figure 10A), and that *srp* expression is undetectable in the mutant strain *B. longum Δsrp in vitro*. Since purified Srp from *B. longum srp*$^{+}$ has been demonstrated to inhibit human neutrophil elastase (HNE) (12), we tested HNE inhibition by the three strains expressing *srp* at various levels *in vitro*. Indeed, *B. longum* strains expressing *srp*, but not *B. longum Δsrp*, inhibited HNE, suggesting that *B. longum* Srp has potential anti-inflammatory properties. Compared to *B. longum Δsrp*, *B. longum srp*(Con) inhibited HNE across all concentrations. Difference in elastase inhibition may have been even

greater with longer incubations of *B. longum* strains with elastase, in line with the greater differences in *srp* expression between *B. longum* strains measured after 8 hours incubation. Further, it was previously shown that elastase was capable of inducing serpin mRNA levels in wildtype *B. longum* strains (41). Such induction could explain why a difference between the two strains cannot be observed in this experiment. The innate immune response is a key component in the development of atrophy in CeD (42, 43), and has been proposed to be involved in the pathogenesis of NCGS/NCWS (44, 45). The influx and release of neutrophil components is increased in patients with CeD (46), and by inhibiting HNE activity, Srp may specifically target a mechanism that contributes to gluten-related disorders.

Using the NOD/DQ8 model of gluten sensitivity, we examined the therapeutic potential of *B. longum srp*$^{+}$ *in vivo*. As a quality control, we confirmed the presence of *B. longum* in the small intestine of probiotic-treated mice (Figure 14B), and confirmed *srp* expression only in mice receiving *B. longum srp*$^{+}$ and *B. longum srp*(Con). Oral administration of *B. longum srp*$^{+}$ and *B. longum srp*(Con) for 2 weeks protected mice from developing gliadin-induced immunopathology. Because these effects were not achieved in mice receiving *B. longum Δsrp*, *srp* expression is important for the protective mechanism. This may be related to immune regulation, maintenance of barrier function, overall beneficial shifts in gut microbiota or inhibition of elastase released during inflammation (47, 48).

Probiotic-based therapies have been advocated to restore the balance of a "dysbiotic" or disease-promoting microbiota (5). Proteobacteria overgrowth in the small

intestine has been reported in patients with active CeD and in those with persistent symptoms after gluten withdrawal (27, 34). We have shown that experimental Proteobacteria expansion in the small intestine of NOD/DQ8 worsens gluten immunopathology (27, 34). We therefore measured small intestinal and fecal microbial β-diversity and relative abundances of bacterial groups (Figures 12-13, Supplementary figures 5-6). We found no significant shifts in the small intestinal microbiota between the separate groups, suggesting that Srp from *B. longum* is unlikely to act through modification of compositional changes of the upper gastrointestinal tract microbiota (49-52). On the other hand, mild shifts in β-diversity were observed in fecal microbiota of mice treated with *B. longum srp*$^{+}$ and *B. longum srp*(Con) compared to *B. longum Δsrp*. Although most differences in relative abundances of genera between groups are difficult to interpret, *Akkermansia* spp. were exclusively increased in *B. longum srp*(Con)-treated mice compared to all other groups. The commensal *Akkermansia muciniphila* is considered to be anti-inflammatory and beneficial for the intestinal mucus layer and barrier integrity in some models of inflammatory disorders (53, 54), and decreased levels of *A. muciniphila* have been observed in patients with IBD and metabolic disorders (55, 56). This raises the hypothesis that a significant level of Srp delivery, as that provided by *B. longum srp*(Con), may improve the overall mucosal barrier and immune function of the gut, in part, through increases in *Akkermansia* species. Because the role of *Akkermansia* is somewhat controversial based on a recent study (57), the implications of this finding in our model must be further tested to draw conclusions.

In conclusion, *B. longum srp*$^{+}$ is a commensal bacterium that expresses a serpin, in a non-constitutive manner, that is effective in preventing gliadin-induced immunopathology in NOD/DQ8 mice. As a commensal bacterium, *B. longum srp*$^{+}$ circumvents controversy surrounding the use of GMOs for the delivery of anti-inflammatory molecules, which may facilitate its translation for human consumption. This study provides mechanistic insight and pathophysiological rationale to explore the efficacy of *B. longum srp*$^{+}$ as an adjunctive therapy in gluten-related disorders or other gastrointestinal inflammatory conditions associated with proteolytic imbalance. Future studies should address the host mechanisms behind protection of gluten-induced pathology by protease inhibitors, or exacerbation due to excess luminal proteases.

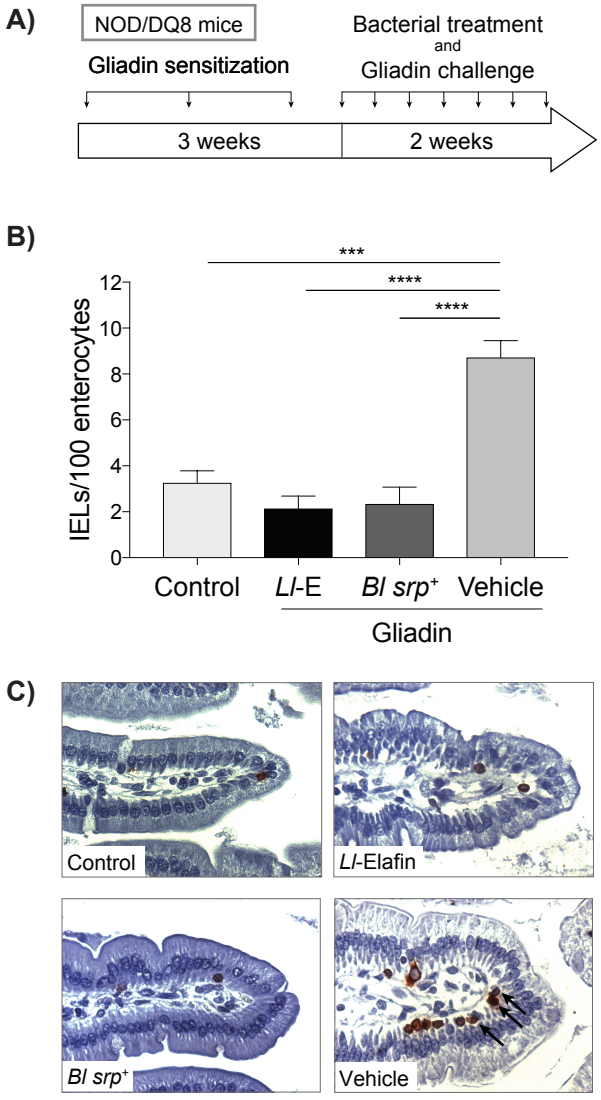

Figure 9. *B. longum srp*$^{+}$ and *L. lactis*-elafin are equally effective in preventing gliadin immunopathology in mice.

A) NOD/DQ8 mice were sensitized with cholera toxin and pepsin-trypsin digested gliadin 1x per week for 3 weeks. Non-sensitized mice (controls) received cholera toxin alone. Subsequently, mice were treated daily with *B. longum srp*$^{+}$, *L. lactis*-elafin, or PBS-20% glycerol and simultaneously challenged with gliadin 3x per week for 2 weeks. Control mice received no bacterial treatment. **B)** CD3^{+} intraepithelial lymphocytes in small intestinal villi tips were

quantified and expressed as IELs per 100 enterocytes. Mice treated with *srp*-expressing *B. longum* *srp*$^+$ had significantly lower numbers of IELs compared to gliadin-sensitized mice receiving no bacterial treatment. Further, *B. longum srp*$^+$ treatment resulted in similar numbers of IELs as those treated with *L. lactis* expressing elafin. **C)** Representative images were captured at 40X magnification. Data shown as mean ± standard error of the mean (SEM). Statistical significance was performed by ANOVA followed by Bonferroni post-hoc analysis, *$p<0.05$. Non-sensitized, no treatment (Control); gliadin + WT *B. longum* NCC2705 (*Bl srp*$^+$); gliadin + *L. lactis* expressing elafin (*Ll*-E); gliadin, no treatment (Vehicle) (n=3-6/group).

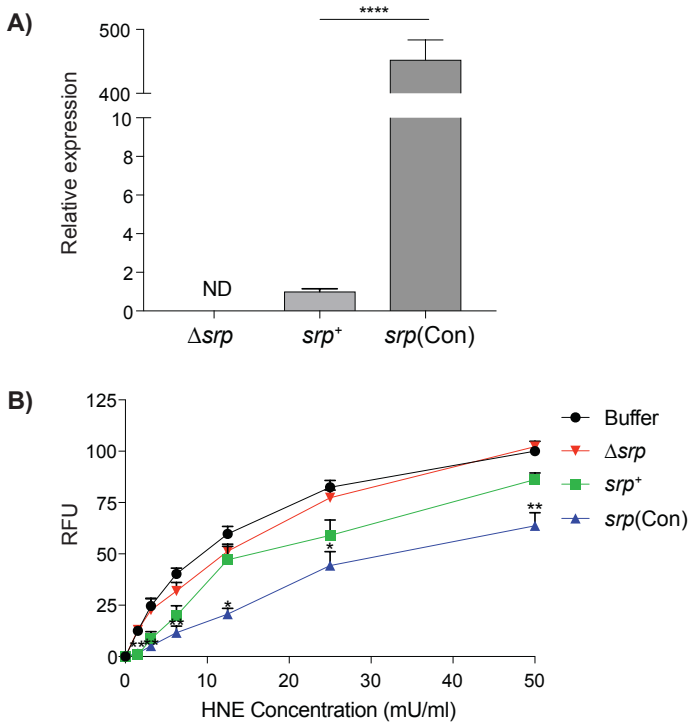

Figure 10. *B. longum* constitutively expressing *srp* inhibits human neutrophil elastase activity *in vitro*.

A) *srp* mRNA levels were quantified from various *B. longum* strains. Expression of *srp* was higher in *B. longum srp*(Con) than *B. longum srp*$^+$(**** $p<0.0001$). No *srp* mRNA was detected in *B. longum Δsrp* (n=4). **B)** Inhibitory capacities of various *B. longum* strains were tested *in vitro*, as measured by fluorescence produced via cleavage of FITC-elastin substrate at 1.5, 3.125, 6.25, 12.5, 25, and 50 mU/mL, expressed as relative fluorescence units (RFU) (n=3/group). Compared to *B. longum Δsrp*, *B. longum srp*$^+$ inhibited cleavage of elastin by human neutrophil elastase (HNE) *in vitro* at 1.5 mU/mL ($p<0.01$), resulting in lower RFU. In the same assay, *B. longum srp*(Con) further inhibited HNE across all concentrations of HNE compared to *B. longum Δsrp*, resulting in lower RFU. As well, *B. longum Δsrp* did not inhibit HNE, as cleavage of elastin-FITC determined by RFU produced was not significantly different between *B. longum Δsrp* and buffer

alone at any concentration of HNE added. HNE alone (buffer); *B. longum srp*$^{+}$ (*srp*$^{+)}$; *B. longum Δsrp* (Δ*srp*); *B. longum srp*(Con) (*srp*(Con)). ND, not detectable. Data shown as mean ± SEM. Statistical significance was performed using Kruskal-Wallis, * p<0.05 vs. *B. longum Δsrp*; ** p<0.01 vs. *B. longum Δsrp*.

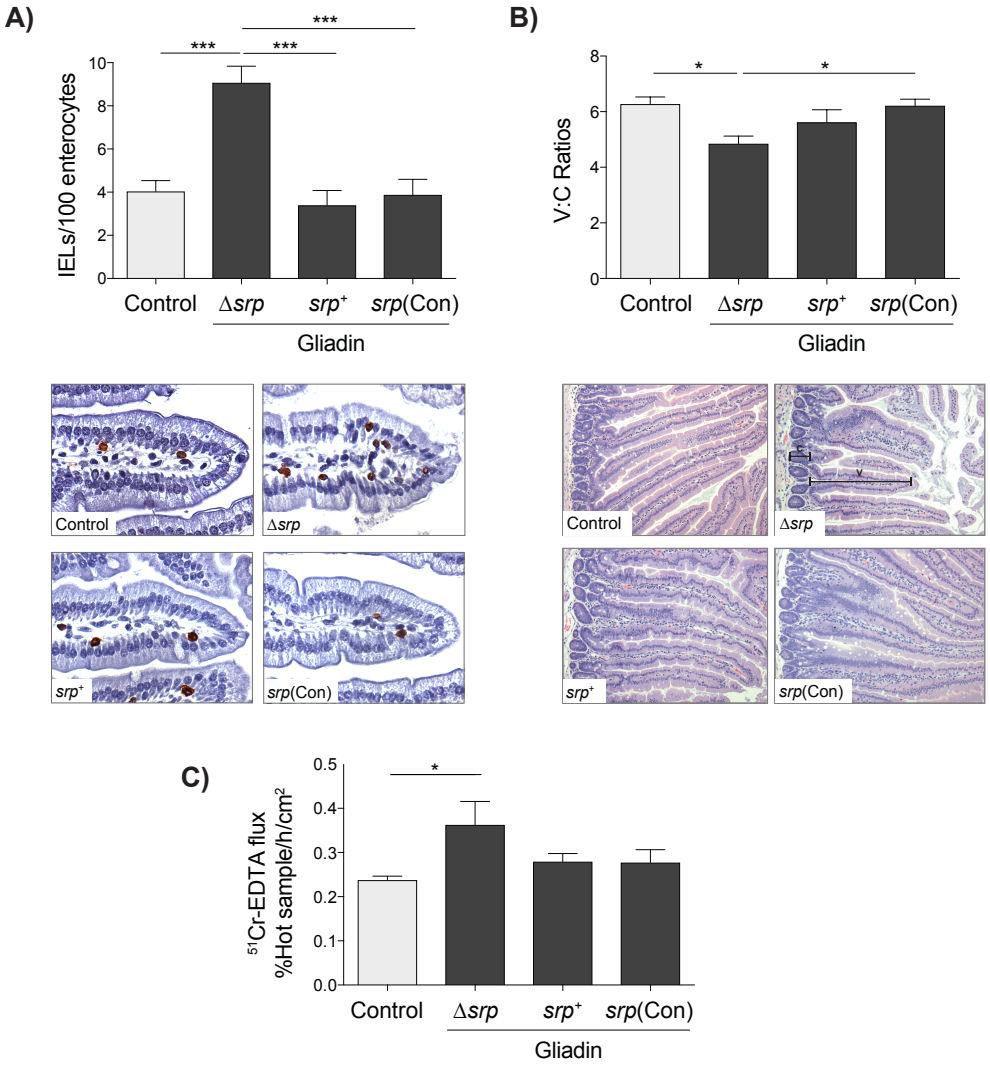

Figure 11. *B. longum srp* mediates the protective effect observed in mice.

NOD/DQ8 mice were sensitized with cholera toxin and pepsin-trypsin digested gliadin 1x per week for 3 weeks. Non-sensitized mice (controls) received cholera toxin alone. Subsequently,

sensitized mice were treated daily with either *B. longum srp*$^+$ (*srp*$^+$), *B. longum Δsrp* (*Δsrp*), *B. longum srp*(Con) (*srp*(Con)), and simultaneously challenged with gliadin 3x per week for 2 weeks. Control mice received PBS-20% glycerol. **A)** CD3$^+$ intraepithelial lymphocytes in small intestinal villi tips were quantified and expressed as IELs per 100 enterocytes. Mice treated with *srp*-expressing *B. longum srp*$^+$ or *B. longum srp*(Con) had significantly lower numbers of IELs than *B. longum Δsrp*-treated mice. Representative images were captured at 40X magnification (n=10-11/group). **B)** Small intestinal sections were H&E stained, and villus (V) and crypt (C) lengths were measured via light microscopy, expressed as V:C ratios. *B. longum srp*(Con) treatment in gliadin-sensitized mice resulted in significantly higher V:C ratios than *B. longum Δsrp* treatment. Representative images were captured at 10X magnification (n=10-11/group). **C)** Paracellular permeability was restored in sensitized NOD/DQ8 mice treated with *B. longum* strains expressing *srp*, *B. longum srp*$^+$ and *B. longum srp*(Con). Proximal small intestinal sections were mounted on Ussing chambers to measure *ex vivo* paracellular permeability, expressed as ^{51}Cr-EDTA flux (n=7-8/group). Data is shown as mean ± SEM. Non-sensitized, no treatment (Control); *B. longum srp*$^+$ (*srp*$^+$); *B. longum Δsrp* (*Δsrp*); *B. longum srp*(Con) (*srp*(Con)). Statistical significance was performed by ANOVA followed by Bonferroni post-hoc analysis, ***$p<0.001$, *$p<0.05$.

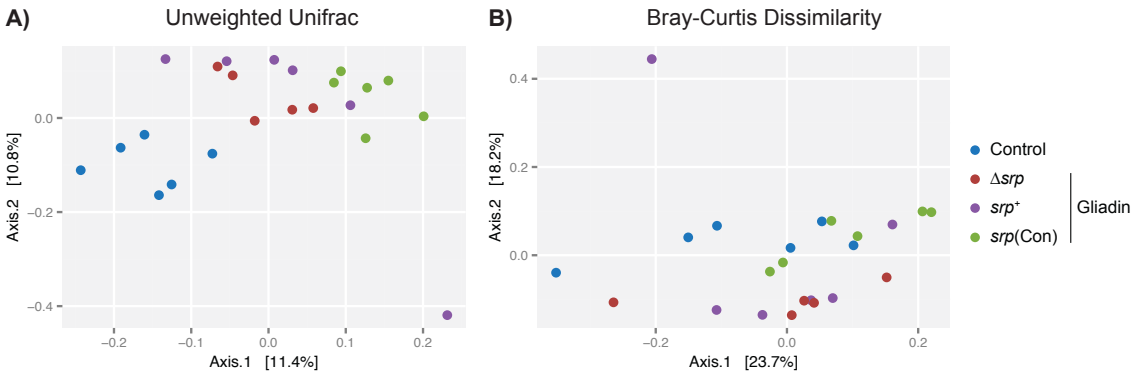

Figure 12. Gliadin and treatment with *srp*-expressing *B. longum* shift fecal microbiota profiles.

Principal coordinate analysis plots of 16S data in NOD/DQ8 mice. **A)** Gliadin induces a shift in β-diversity calculated using Unifrac unweighted distance ($p<0.001$). Microbial compositions are different between mice receiving *B. longum Δsrp* and *B. longum srp*$^+$ ($p<0.05$); *B. longum Δsrp* and *B. longum srp*(Con) ($p<0.001$); and *B. longum srp*$^+$ and *B. longum srp*(Con) ($p<0.001$) (n=5-6/group). **B)** Gliadin also shifts β-diversity when assessed using Bray-Curtis dissimilarity parameters ($p<0.005$). Microbial compositions are significantly different between *B. longum Δsrp* and *B. longum srp*$^+$ ($p<0.05$); *B. longum Δsrp* and *B. longum srp*(Con) ($p<0.005$); *B. longum srp*$^+$ and *B. longum srp*(Con) ($p<0.005$). Each circle represents an individual fecal sample. Non-sensitized, no treatment (Control); *B. longum srp*$^+$ (*srp*$^+$); *B. longum Δsrp* (*Δsrp*); *B. longum srp*(Con) (*srp*(Con)). Statistics were performed via PERMANOVA in QIIME. Plots were constructed in R.

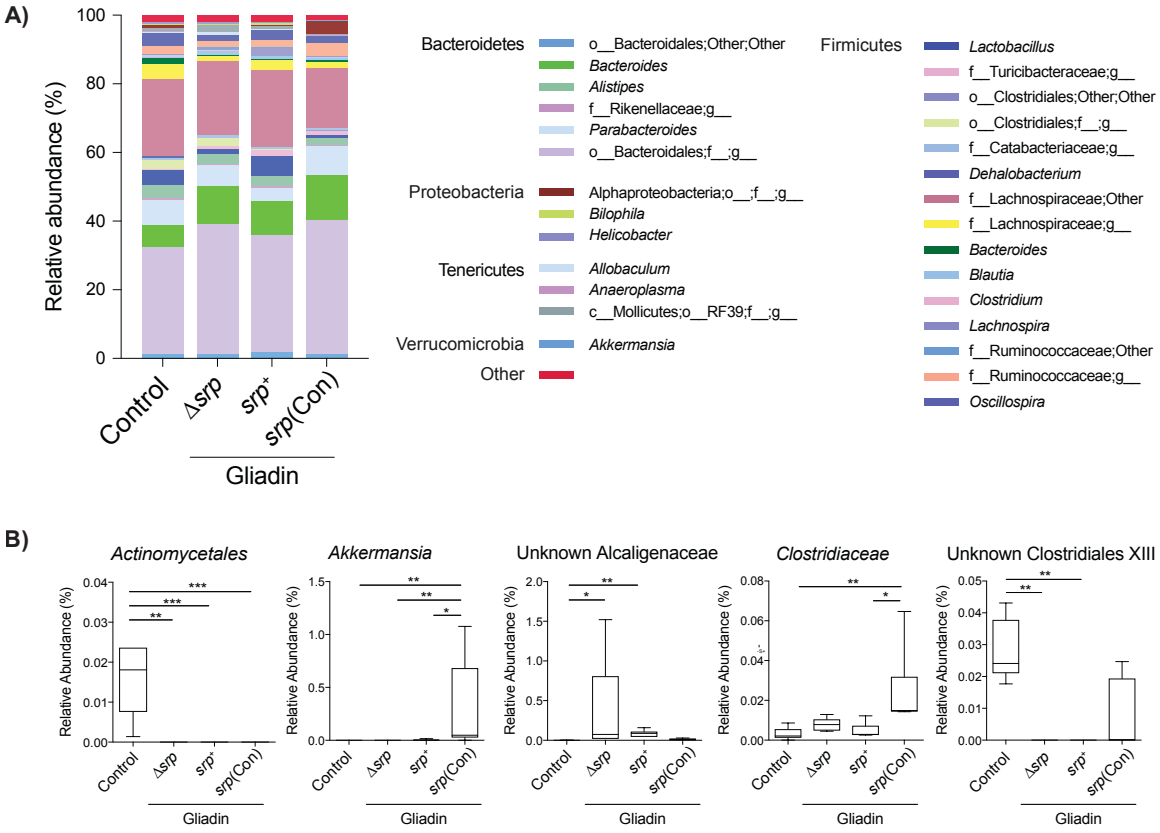

Figure 13. Fecal genera affected by *B. longum* expressing *srp*.

A) Genus-level composition of fecal microbiota after *B. longum* treatment in NOD/DQ8 mice are depicted as an average percentage of each group in stacked column charts. **B)** Genera significantly differing in relative abundances between groups. Data shown as box and whisker plots. (*p<0.05; **p<0.01; ***p<0.001). Non-sensitized, no treatment (Control); *B. longum srp*$^{+}$ (*srp*$^{+}$); *B. longum Δsrp* (*Δsrp*); *B. longum srp*(Con) (*srp*(Con)) (n=5-6/group). Statistics were performed via Kruskal-Wallis followed by FDR (q<0.05).

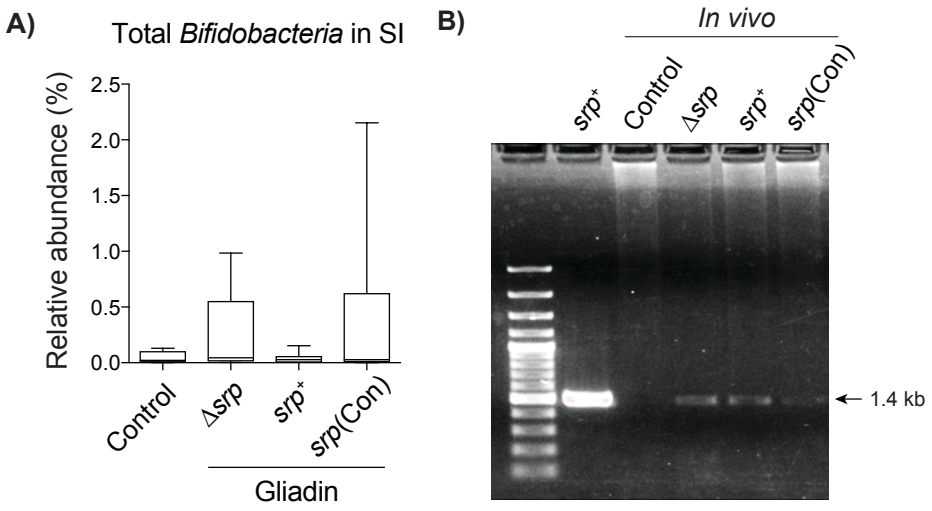

Figure 14. *B. longum* NCC2705 is detected in the gastrointestinal tract of treated mice.

A) Relative abundance of Bifidobacteria genus members as determined by 16S rRNA sequencing is similar between all groups of NOD/DQ8 mice (n=5-6/group). **B)** Strain-specific primers detected *B. longum Δsrp*, *B. longum srp*$^+$, and *B. longum* srp(Con) in small intestinal DNA extracted from all bacterially-treated mice. Non-sensitized, no treatment (Control); *B. longum srp*$^+$ (*srp*$^+$); *B. longum Δsrp* (*Δsrp*); *B. longum srp*(Con) (*srp*(Con)) (n=5/group). Statistics were performed via Kruskal-Wallis followed by FDR ($q<0.05$).

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References

1. Moayyedi P, Ford AC, Talley NJ, Cremonini F, Foxx-Orenstein AE, Brandt LJ, Quigley EM. 2010. The efficacy of probiotics in the treatment of irritable bowel syndrome: a systematic review. Gut 59:325-332.
2. Mimura T, Rizzello F, Helwig U, Poggioli G, Schreiber S, Talbot I, Nicholls R, Gionchetti P, Campieri M, Kamm M. 2004. Once daily high dose probiotic therapy (VSL# 3) for maintaining remission in recurrent or refractory pouchitis. Gut 53:108-114.
3. Pinto-Sanchez MI, Smecuol EC, Temprano MP, Sugai E, Gonzalez A, Moreno ML, Huang X, Bercik P, Cabanne A, Vazquez H, Niveloni S, Mazure R, Maurino E, Verdu EF, Bai JC. 2016. *Bifidobacterium infantis* NLS Super Strain Reduces the Expression of alpha-Defensin-5, a Marker of Innate Immunity, in the Mucosa of Active Celiac Disease Patients. J Clin Gastroenterol.
4. Golfetto L, de Senna FD, Hermes J, Beserra BT, Franca Fda S, Martinello F. 2014. Lower bifidobacteria counts in adult patients with celiac disease on a gluten-free diet. Arq Gastroenterol 51:139-43.
5. Quagliariello A, Aloisio I, Bozzi Cionci N, Luiselli D, D'Auria G, Martinez-Priego L, Perez-Villarroya D, Langerholc T, Primec M, Micetic-Turk D, Di Gioia D. 2016. Effect of Bifidobacterium breve on the Intestinal Microbiota of Coeliac Children on a Gluten Free Diet: A Pilot Study. Nutrients 8.
6. Bustos D, Negri G, De Paula J. 1998. Colonic proteinases: increased activity in patients with ulcerative colitis. Medicina 58:262-264.
7. Gecse K, Roka R, Ferrier L. 2008. Increased faecal serine protease activity in diarrhoeic IBS patients: a colonic lumenal factor impairing colonic permeability and sensitivity. Gut 57:591-599.
8. Roka R, Rosztoczy A, Leveque M. 2007. A pilot study of fecal serine-protease activity: a pathophysiologic factor in diarrhea- predominant irritable bowel syndrome. Clin Gastroenterol Hepatol 5:550-555.
9. Annaházi A, Molnár T, Farkas K, Rosztóczy A, Izbéki F, Gecse K, Inczefi O, Nagy F, Földesi I, Szűcs M. 2012. Fecal MMP‐9: A new noninvasive differential diagnostic and activity marker in ulcerative colitis. Inflammatory bowel diseases.
10. Motta J, Bermúdez-Humarán L, Deraison C, Martin L, Rolland C, Rousset P, Boue J, Dietrich G, Chapman K, Kharrat P, Vinel J, Alric L, Mas E, Sallenave J, Langella P, Vergnolle N. 2012. Food-grade bacteria expressing elafin protect against inflammation and restore colon homeostasis. Sci Transl Med 4:158ra144.
11. Galipeau HJ, Wiepjes M, Motta JP, Schulz JD, Jury J, Natividad JM, Pinto-Sanchez I, Sinclair D, Rousset P, Martin-Rosique R, Bermudez-Humaran L, Leroux JC, Murray JA, Smecuol E, Bai JC, Vergnolle N, Langella P, Verdu EF. 2014. Novel role of the serine protease inhibitor elafin in gluten-related disorders. Am J Gastroenterol 109:748-56.
12. Ivanov D, Emonet C, Foata F, Affolter M, Delley M, Fisseha M, Blum-Sperisen S, Kochhar S, Arigoni F. 2006. A serpin from the gut bacterium *Bifidobacterium*

longum inhibits eukaryotic elastase-like serine proteases. J Biol Chem 281:17246-52.
13. Schell MA, Karmirantzou M, Snel B, Vilanova D, Berger B, Pessi G, Zwahlen MC, Desiere F, Bork P, Delley M, Pridmore DR, Arigoni F. 2002. The genome sequence of Bifidobacterium longum reflect its adaptation to the human gastrointestinal tract. Proceedings of the National Academy of Sciences 99:14422-14427.
14. Argnani A, Leer RJ, van Luijk N, Pouwels PH. 1996. A convenient and reproducible method to genetically transform bacteria of the genus Bifidobacterium. Microbiology 142 (Pt 1):109-14.
15. Galipeau H, Rulli N, Jury J, Huang X, Araya R, Murray J, David C, Chirdo F, McCoy K, Verdu E. 2011. Sensitization to gliadin induces moderate enteropathy and insulitis in nonobese diabetic-DQ8 mice. J Immunol 187:4338-46.
16. Berger B, Moine D, Mansourian R, Arigoni F. 2010. HspR mutations are naturally selected in Bifidobacterium longum when successive heat shock treatments are applied. J Bacteriol 192:256-63.
17. Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res 29:e45-e45.
18. Biagi F, Luinetti O, Campanella J, Klersy C, Zambelli C, Villanacci V, Lanzini A, Corazza GR. 2004. Intraepithelial lymphocytes in the villous tip: do they indicate potential coeliac disease? J Clin Pathol 57:835-9.
19. Anonymous. !!! INVALID CITATION !!! .
20. Whelan FJ, Verschoor CP, Stearns JC, Rossi L, Luinstra K, Loeb M, Smieja M, Johnstone J, Surette MG, Bowdish DM. 2014. The loss of topography in the microbial communities of the upper respiratory tract in the elderly. Annals of the American Thoracic Society 11:513-521.
21. Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet journal 17:pp. 10-12.
22. Masella AP, Bartram AK, Truszkowski JM, Brown DG, Neufeld JD. 2012. PANDAseq: paired-end assembler for illumina sequences. BMC Bioinformatics 13:31.
23. Ye Y. 2011. Identification and Quantification of Abundant Species from Pyrosequences of 16S rRNA by Consensus Alignment. Proceedings (IEEE Int Conf Bioinformatics Biomed) 2010:153-157.
24. DeSantis TZ, Jr., Hugenholtz P, Keller K, Brodie EL, Larsen N, Piceno YM, Phan R, Andersen GL. 2006. NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. Nucleic Acids Res 34:W394-9.
25. Galipeau H, Wiepjes M, Motta J, Schulz J, Jury J, Natividad J, Pinto-Sanchez I, Sinclair D, Rousset P, Martin-Rosique R, Bermudez-Humaran L, Leroux J, Murray J, Smecuol E, Bai J, Vergnolle N, Langella P, Verdu E. 2014. Novel Role of the Serine Protease Inhibitor Elafin in Gluten-Related Disorders. American Journal of Gastroenterology 109:748-756.

26. Verdu EF, Armstrong D, Murray JA. 2009. Between celiac disease and irritable bowel syndrome: the “no man's land” of gluten sensitivity. Am J Gastroenterol 104:1587-1594.
27. Wacklin P, Kaukinen K, Tuovinen E, Collin P, Lindfors K, Partanen J, Mäki M, Mättö J. 2013. The duodenal microbiota composition of adult celiac disease patients is associated with the clinical manifestation of the disease. Inflamm Bowel Dis 19:934-941.
28. D'Argenio V, Casaburi G, Precone V, Pagliuca C, Colicchio R, Sarnataro D, Discepolo V, Kim SM, Russo I, Del Vecchio Blanco G, Horner DS, Chiara M, Pesole G, Salvatore P, Monteleone G, Ciacci C, Caporaso GJ, Jabri B, Salvatore F, Sacchetti L. 2016. Metagenomics Reveals Dysbiosis and a Potentially Pathogenic N. flavescens Strain in Duodenum of Adult Celiac Patients. Am J Gastroenterol 111:879-90.
29. Marild K, Ye W, Lebwohl B, Green PH, Blaser MJ, Card T, Ludvigsson JF. 2013. Antibiotic exposure and the development of coeliac disease: a nationwide case-control study. BMC Gastroenterol 13:109.
30. Decker E, Engelmann G, Findeisen A, Gerner P, Laass M, Ney D, Posovszky C, Hoy L, Hornef MW. 2010. Cesarean delivery is associated with celiac disease but not inflammatory bowel disease in children. Pediatrics 125:e1433-40.
31. Smecuol E, Hwang HJ, Sugai E, Corso L, Chernavsky AC, Bellavite FP, Gonzalez A, Vodanovich F, Moreno ML, Vazquez H, Lozano G, Niveloni S, Mazure R, Meddings J, Maurino E, Bai JC. 2013. Exploratory, randomized, double-blind, placebo-controlled study on the effects of *Bifidobacterium infantis* natren life start strain super strain in active celiac disease. J Clin Gastroenterol 47:139-47.
32. Olivares M, Castillejo G, Varea V, Sanz Y. 2014. Double-blind, randomised, placebo-controlled intervention trial to evaluate the effects of Bifidobacterium longum CECT 7347 in children with newly diagnosed coeliac disease. British Journal of Nutrition 112:30-40.
33. Galipeau HJ, McCarville JL, Huebener S, Litwin O, Meisel M, Jabri B, Sanz Y, Murray JA, Jordana M, Alaedini A, Chirdo FG, Verdu EF. 2015. Intestinal microbiota modulates gluten-induced immunopathology in humanized mice. Am J Pathol 185:2969-82.
34. Galipeau HJ, McCarville JL, Huebener S, Litwin O, Meisel M, Jabri B, Sanz Y, Murray JA, Jordana M, Alaedini A. 2015. Intestinal microbiota modulates gluten-induced immunopathology in humanized mice. The American journal of pathology 185:2969-2982.
35. Caminero A, Galipeau HJ, McCarville JL, Johnston CW, Bernier SP, Russell AK, Jury J, Herran AR, Casqueiro J, Tye-Din JA, Surette MG, Magarvey NA, Schuppan D, Verdu EF. 2016. Duodenal Bacteria From Patients With Celiac Disease and Healthy Subjects Distinctly Affect Gluten Breakdown and Immunogenicity. Gastroenterology 151:670-83.

36. Heit C, Jackson BC, McAndrews M, Wright MW, Thompson DC, Silverman GA, Nebert DW, Vasiliou V. 2013. Update of the human and mouse SERPIN gene superfamily. Human Genomics 7:22.
37. Kaiserman D, Whisstock JC, Bird PI. 2006. Mechanisms of serpin dysfunction in disease. Expert Rev Mol Med 8:1-19.
38. Hasnain SZ, McGuckin MA, Grencis RK, Thornton DJ. 2012. Serine protease(s) secreted by the nematode Trichuris muris degrade the mucus barrier. PLoS Negl Trop Dis 6:e1856.
39. Uchiyama K, Naito Y, Takagi T, Mizushima K, Hirai Y, Hayashi N, Harusato A, Inoue K, Fukumoto K, Yamada S. 2012. Serpin B1 protects colonic epithelial cell via blockage of neutrophil elastase activity and its expression is enhanced in patients with ulcerative colitis. American Journal of Physiology-Gastrointestinal and Liver Physiology 302:G1163-G1170.
40. Langhorst J, Elsenbruch S, Koelzer J, Rueffer A, Michalsen A, Dobos GJ. 2008. Noninvasive markers in the assessment of intestinal inflammation in inflammatory bowel diseases: performance of fecal lactoferrin, calprotectin, and PMN-elastase, CRP, and clinical indices. Am J Gastroenterol 103:162-169.
41. Turroni F, Foroni E, Motherway MOC, Bottacini F, Giubellini V, Zomer A, Ferrarini A, Delledonne M, Zhang Z, van Sinderen D. 2010. Characterization of the serpin-encoding gene of *Bifidobacterium breve* 210B. Appl Environ Microbiol 76:3206-3219.
42. Jabri B, Sollid LM. 2009. Tissue-mediated control of immunopathology in coeliac disease. Nature Reviews Immunology 9:858-870.
43. Sollid LM, Jabri B. 2013. Triggers and drivers of autoimmunity: lessons from coeliac disease. Nature Reviews Immunology 13:294-302.
44. Araya RE, Gomez Castro MF, Carasi P, McCarville JL, Jury J, Mowat AM, Verdu EF, Chirdo FG. 2016. Mechanisms of innate immune activation by gluten peptide p31-43 in mice. American Journal of Physiology-Gastrointestinal and Liver Physiology 311:G40-9.
45. Junker Y, Zeissig S, Kim S-J, Barisani D, Wieser H, Leffler DA, Zevallos V, Libermann TA, Dillon S, Freitag TL. 2012. Wheat amylase trypsin inhibitors drive intestinal inflammation via activation of toll-like receptor 4. J Exp Med 209:2395-2408.
46. Hällgren R, Colombel JF, Dahl R, Fredens K, Kruse A, Jacobsen NO, Venge P, Rambaud JC. 1989. Neutrophil and eosinophil involvement of the small bowel in patients with celiac disease and Crohn's disease: studies on the secretion rate and immunohistochemical localization of granulocyte granule constituents. The American journal of medicine 86:56-64.
47. Nikolaus S, Bauditz J, Gionchetti P, Witt C, Lochs H, Schreiber S. 1998. Increased secretion of pro-inflammatory cytokines by circulating polymorphonuclear neutrophils and regulation by interleukin 10 during intestinal inflammation. Gut 42:470-476.
48. Kessenbrock K, Fröhlich L, Sixt M, Lämmermann T, Pfister H, Bateman A, Belaaouaj A, Ring J, Ollert M, Fässler R. 2008. Proteinase 3 and neutrophil

elastase enhance inflammation in mice by inactivating antiinflammatory progranulin. J Clin Invest 118:2438-2447.
49. Kristensen NB, Bryrup T, Allin KH, Nielsen T, Hansen TH, Pedersen O. 2016. Alterations in fecal microbiota composition by probiotic supplementation in healthy adults: a systematic review of randomized controlled trials. Genome Med 8:52.
50. Grimm V, Radulovic K, Riedel CU. 2015. Colonization of C57BL/6 Mice by a Potential Probiotic Bifidobacterium bifidum Strain under Germ-Free and Specific Pathogen-Free Conditions and during Experimental Colitis. PLoS One 10:e0139935.
51. Behnsen J, Deriu E, Sassone-Corsi M, Raffatellu M. 2013. Probiotics: properties, examples, and specific applications. Cold Spring Harb Perspect Med 3:a010074.
52. Eloe-Fadrosh EA, Brady A, Crabtree J, Drabek EF, Ma B, Mahurkar A, Ravel J, Haverkamp M, Fiorino AM, Botelho C, Andreyeva I, Hibberd PL, Fraser CM. 2015. Functional dynamics of the gut microbiome in elderly people during probiotic consumption. MBio 6.
53. Reunanen J, Kainulainen V, Huuskonen L, Ottman N, Belzer C, Huhtinen H, de Vos WM, Satokari R. 2015. Akkermansia muciniphila adheres to enterocytes and strengthens the integrity of the epithelial cell layer. Appl Environ Microbiol 81:3655-3662.
54. Derrien M, Belzer C, de Vos WM. 2016. Akkermansia muciniphila and its role in regulating host functions. Microb Pathog.
55. Rajilic-Stojanovic M, Shanahan F, Guarner F, de Vos WM. 2013. Phylogenetic analysis of dysbiosis in ulcerative colitis during remission. Inflamm Bowel Dis 19:481-488.
56. Png CW, Lindén SK, Gilshenan KS, Zoetendal EG, McSweeney CS, Sly LI, McGuckin MA, Florin TH. 2010. Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria. Am J Gastroenterol 105:2420-2428.
57. Seregin SS, Golovchenko N, Schaf B, Chen J, Pudlo NA, Mitchell J, Baxter NT, Zhao L, Schloss PD, Martens EC, Eaton KA, Chen GY. 2017. NLRP6 Protects Il10-/- Mice from Colitis by Limiting Colonization of Akkermansia muciniphila. Cell Rep 19:733-745.

CHAPTER 5
OVERALL DISCUSSION

5.1 Summary

In chapter 3, we demonstrated a role for the microbiota in maintaining proteolytic balance in the gut, as mice harboring a dysbiotic microbiota with high proteolytic activity exhibited low-grade inflammation and alterations in intestinal barrier function. To further implicate the role of microbial proteolytic activity, we inhibited microbial proteolytic activity using a microbial protease inhibitor (*B. longum* serpin). We demonstrated anti-inflammatory and barrier-fortifying effects of *B. longum* serpin in mice colonized with high proteolytic microbiota. In chapter 4, we carried out a more in-depth exploration into the effects of *B. longum* serpin on gut homeostasis, and demonstrated its pre-clinical therapeutic potential in a mouse model of upper GI inflammatory disease.

5.2 Microbial proteases and protease inhibitors

The balance of proteases and their inhibitors is crucial for maintaining gut homeostasis, and it has become increasingly clear that proteases contribute to the pathology of many GI diseases such as CeD, IBD, IBS, and CRC. These proteases, and the effects they mediate, are assumed to originate from the host. Meanwhile, the role of proteases and even protease inhibitors abundantly produced by the microbiota have been dismissed for unclear reasons. Findings in this thesis support the idea that microbial proteases or protease inhibitors can serve as an axis for host-microbe interactions to impact host physiology and immunity. We put forth possible avenues through which microbial proteases could drive pathogenic effects in the host and potentiate disease. It follows that microbial protease inhibitors may have therapeutic potential by mitigating dysregulated pathways in various GI diseases.

There are several reasons why the contribution of microbiota to proteolytic activity in the gut has been overlooked. Many of the proteases linked to GI disease states such as mast cell tryptase, neutrophil elastase, cathepsins, and matriptases[282] have well-defined involvement in IBD as part of the inflammatory milieu and wound repair

response in response to intestinal injury. However, the increase in tryptase specifically from mast cells has been inferred based on associations between increased tryptase expression, increased proteolytic activity, and increased number of mast cells[283,284]. These separate observations do not rule out the contribution of microbial proteases to the observed increased proteolytic activity. Rather, most of these studies have simply ignored the role of microbial proteases in gut inflammation, as methods used in studies dismissing microbial proteolytic activity cannot define whether proteolytic activity is host or microbial. The lack of attention towards microbial proteolytic activity in the gut has also been inferred from the overwhelming abundance of host pancreatic proteases present in the gut[285]. It has been argued that the abundance of host pancreatic proteases, especially in the upper GI tract, would negate the effects of microbial proteases. An early study showed that pancreatic proteases are inhibited through degradation or inactivation by the increasing abundance of microbiota along the GI tract[285,286]. Thus, recognizing that protease inhibition is mediated by microbiota actually emphasizes the influence they play, especially in the lower gut. Few studies, since then, have examined microbial proteolytic activity within the GI tract despite studies of the human microbiome continuing to intensify. Results from this thesis define a pro-inflammatory role for microbial proteases in the colon using a gnotobiotic approach, and incites further investigation into the relevance of microbial proteolytic activity in GI diseases.

It is important to consider protease inhibitors produced from the intestinal microbiota, as they directly impact proteolytic homeostasis and are able to interact with host factors. Serpin from a probiotic *B. longum* mediated protection against gliadin-induced immunopathology in a CeD-like mouse model. NOD/DQ8 mice treated with *B. longum* expressing serpin were protected from small intestinal intraepithelial lymphocytosis, villous shortening, and increased paracellular permeability induced by gluten sensitization. The beneficial effects of *B. longum* serpin in the small intestine suggest that microbial protease inhibitors may be an important participant in upper GI tract homeostasis, in spite of the abundant host pancreatic proteases that are commonly believed to overwhelm the effects of microbial proteases and protease inhibitors[285].

Furthermore, because *B. longum* serpin induced shifts in microbiota composition in feces, but not in the small intestine, a therapeutic mechanism for *B. longum* serpin may involve modulating microbiota of the lower GI tract. In future experiments, it would be interesting to determine whether these compositional shifts have any effect on proteolytic balance in the GI tract of treated mice.

The effects of microbial proteases and protease inhibitors in the gut demand further investigation to better understand the mechanisms involved in host-microbe interactions. Based on the results of this thesis, dysregulated microbial proteolytic balance could provide one mechanism for the association of dysbiosis and GI disorders, and inhibition of microbial protease activity may represent a novel approach in microbiota-targeted treatments for intestinal inflammatory disease.

5.3 Limitations and future directions

Several avenues have been opened within this thesis for more specific mechanistic studies into the contribution of microbial proteases and protease inhibitors. To extend our experiments wherein we inhibit microbial proteolytic activity in HPA-colonized mice using serpin-expressing *B. longum*, we could compare mice colonized with wild-type isolates of HPA microbes compared with their protease mutant strains. This would implicate specifically proteases in the pathogenic processes observed here. Because we observed that microbial serpin administered *in vivo* induces a measurable decline in elastolytic activity, we could proceed to narrow our scope to examine effects of inhibiting elastolytic activity by utilizing *P. aeruginosa* and a transposon mutant (*P. aeruginosa* Δ*lasB*) which lacks elastase activity to perform monocolonizations in germ-free mice. To further explore the molecular signaling mechanisms involved, PAR receptor pathways can be investigated. PARs are activated by these microbial proteases, and we have recently obtained PAR2-/- mice that will be derived germ-free to then colonize with selected bacterial strains or their mutants. It would also be interesting to uncover how microbial proteolytic activity disrupts the intestinal barrier, leading to the translocation of

bacteria and bacterial products into the periphery. If this is mediated by resident cells such as DCs or monocytes, perhaps in harboring a high proteolytic microbiota, the host may receive aberrant immune education and thus become predisposed to GI pathology. Therefore, it would be instructive to study how microbial proteolytic activity activates immune pathways, and whether this modifies susceptibility to acute or chronic intestinal inflammation. More immunohistochemistry could be performed to quantify immune cells such as neutrophils, mast cells, and $CD3^+$ intraepithelial lymphocytes in the samples obtained during my studies. Further characterization of the mucus layer in colonized mice would also provide clues as to whether microbial proteases alter mucus composition or integrity, facilitating bacterial translocation. In addition to reduced thickness of the mucus barrier, alterations in mucin sulphation and glycosylation has been associated with intestinal inflammation[287-289].

Another potential effect of microbial proteolytic activity raised during our experiments was the possibility that it contributes to neuromuscular dysfunction and altered bowel habits, which could have implications in IBS. Indeed, HPA-colonized mice exhibited markedly softer stools, and seemed to have increased colonic transit time as the rate of fecal expulsion was noticeably faster. Unfortunately, we were unable to quantify this observation, as it was only noticed at the time of sacrifice. Studies employing videofluoroscopy as previously described[268] or quantification of fecal water content, or bead transit measurement would be interesting to perform. Because of the known role of PAR2 in ion transport and gut motility[238], these physiological effects are a promising area of exploration. Eventually, it would be interesting to induce intestinal inflammation in our gnotobiotic model to see whether HD, LPA, and HPA-colonized mice differ in their susceptibility to a second inflammatory or injury hit. Perhaps after the 3-week stabilization period following colonization, inducing low-grade, subclinical inflammation with a low dose of dextran sodium sulfate may reveal differences in disease severity depending on the proteolytic activity of the microbiota. Induction of experimental colitis would also shed light on whether high proteolytic bacteria behave differently compared to

low proteolytic bacteria during conditions of intestinal inflammation comparable to a flare in a patient with IBD.

We performed *de novo* colonizations with bacterial communities in ex-germ-free mice, but it would also be of interest to induce a dysbiotic shift within a pre-existing microbial community. We have done some preliminary work with supplementing either HD, LPA, or HPA communities to mice harboring the simplified Altered Schaedler Flora (ASF) community composed of 8 benign strains. This scenario mimics a dysbiotic event such as infection or antibiotic use which shifts the microbiota towards one of higher proteolytic activity. We again achieved successful transfer of the *in vitro* microbial proteolytic activity to the mouse gut (Supplementary figure 3A) and from here, can examine similar outcomes such as barrier function and inflammatory status. Preliminary results indicate significantly greater translocation of live bacteria to the spleen in ASF mice supplemented with HPA compared with HD and LPA communities (Supplementary figure 3B), with no significant changes in paracellular permeability (Supplementary figure 3C&D).

Although we have demonstrated anti-inflammatory properties of *B. longum* serpin in gliadin-sensitized NOD/DQ8 mice, an upper GI disease model, we currently lack a complete understanding of the mechanism behind the therapeutic effects of the protease inhibitor serpin from *B. longum.* Perhaps *B. longum* serpin exerts its benefits by restoring proteolytic balance in NOD/DQ8 mice, thereby preventing gliadin-induced immunopathology. Serpin may interfere with several pathways in gluten-induced pathogenesis, such as neutrophil influx or the deamidation of gluten peptides by TG2. Implications of the compositional shift in microbiota are also incompletely understood, but hold interesting potential in reducing inflammation or positively affecting intestinal barrier.

Since bacterial strains from the HPA community were derived from a patient with active UC, the pro-inflammatory effects of colonization with HPA may be driven by the severe inflammatory conditions from which the bacterial strains were isolated. Nevertheless, our findings are relevant to exacerbation of inflammation during a flare,

though we must also question whether these strains would mediate the same effects if isolated prior to a flare under non-inflammatory conditions. We hypothesize that effects in the murine host would be similar using the same strains from a non-active UC patient, as the active UC strains were both grown for several generations under *in vitro* conditions and colonized in a naïve host. Despite these opportunities to lose any pro-inflammatory phenotype acquired due to an inflammatory UC host, the microbial proteolytic activity phenotype remained stable. Certainly, this concept requires further investigation, in addition to the regulation of microbial protease or protease inhibitor expression and whether levels of protease expression within the same bacterial strain differs between IBD patients.

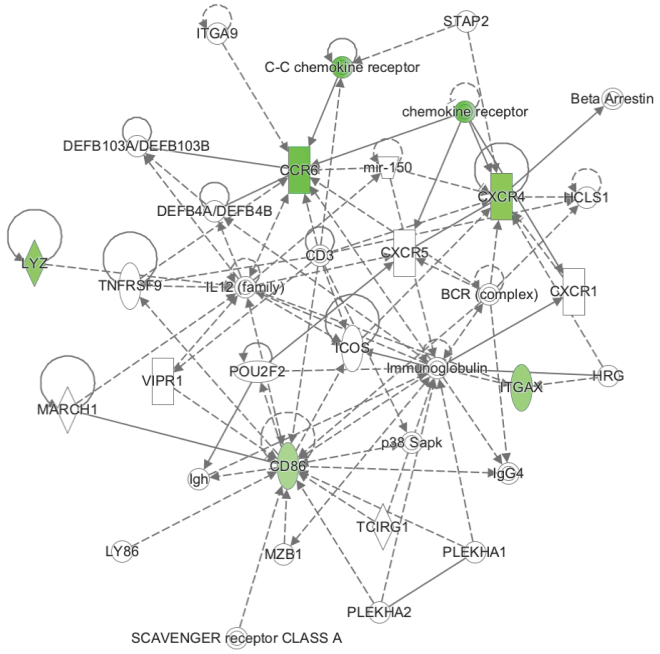
The influence of proteases in GI health is a complex matter, due to their multitude of functions, diverse signaling networks and receptors. Bioactive fragments generated from proteolytic digestion could also mediate novel functions and further complicate the in vivo picture. Although we show that microbial proteolytic activity is a potential driver of intestinal inflammation, a potential for proteases or their substrate fragments to dampen inflammation has been described. For example, degradation of thrombin by neutrophil elastase generates a C-terminal peptide that can downregulate inflammation[290]. Interestingly, microbial elastase (Las) from *P. aeruginosa* can elicit similar anti-inflammatory effects through cleavage of thrombin whose fragments prevent TLR activation in response to LPS. This *P. aeruginosa* elastase can also reduce production of inflammatory cytokines *in vivo*[291]. Albeit through unclear mechanisms, there is previous evidence that *P. aeruginosa* elastase can degrade cytokines[292]. A fascinating future challenge will be teasing apart the interactions between host and microbial proteases, protease inhibitors and other factors. Because current methodologies are not readily able to discern host from microbial proteolytic activity, we are actively collaborating with Dr. Nathalie Vergnolle's lab in order to visualize elastolytic activity of our colonized mice *in situ*. Based on localization of proteolytic activity in the epithelium versus the lumen, we can infer origin of proteolytic activity as host or microbial, respectively. This may also offer clues as to whether there is cross-regulation of host and microbial proteases and

their inhibitors, as our preliminary observations indicate that the microbial protease inhibitor serpin from *B. longum* can downregulate elastase activity as well as gluten-degrading activity (Supplementary figure 4). Since this would have implications in gluten-related disorders, it would be interesting to evaluate elastase and glutenasic activity in the gut of NOD/DQ8 mice treated with *B. longum* strains expressing various levels of serpin, to potentially establish a connection with the therapeutic action of *B. longum* serpin uncovered in Chapter 4.

5.4 Conclusions

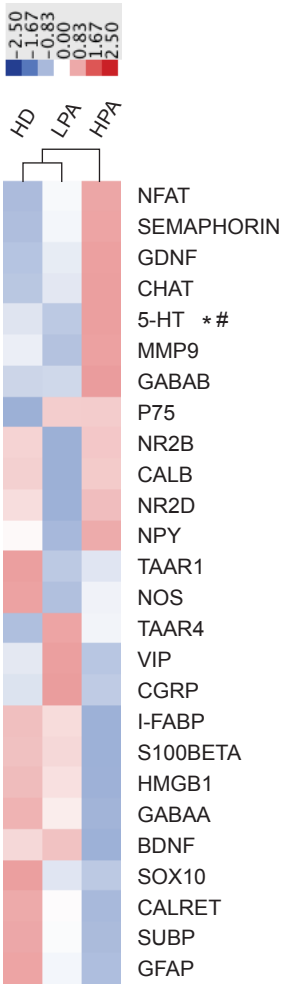
Dysbiotic microbiota with high proteolytic activity can induce pathogenic changes in the host that may contribute to the pathogenesis of chronic inflammation in the gastrointestinal tract. This thesis explored the contribution of both microbial proteases and protease inhibitors to the overall proteolytic activity in the gut, and carves out their relevance in gut homeostasis. These results open a new therapeutic opportunity for upper and lower gastrointestinal inflammation, based on the administration of microbial protease inhibitors as a targeted probiotic therapy.

SUPPLEMENTARY DATA



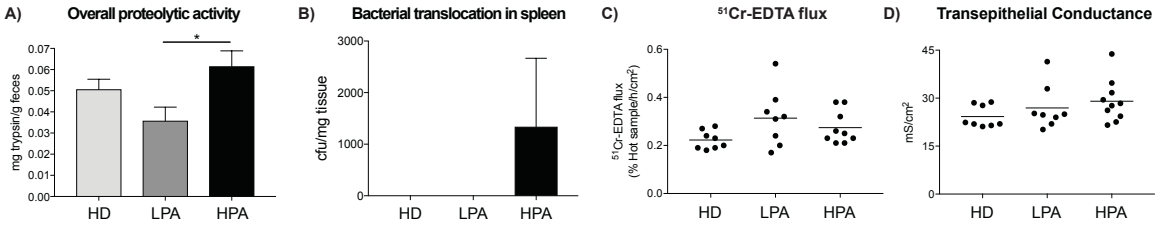
Supplementary figure 1. TRAF-dependent monocyte activation pathway.

Based on Nanostring gene expression data, Ingenuity Pathway Analysis identified the TRAF-dependent monocyte activation pathway to be activated in HPA-colonized mice.



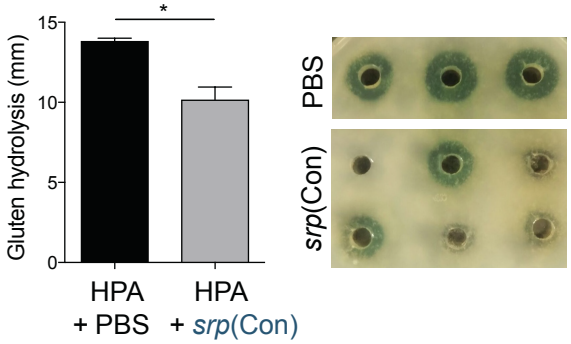
Supplementary figure 2. Expression of 5-HT is significantly upregulated in HPA-colonized mice.

RNA was isolated from colonized mice, reverse transcribed to cDNA, and expression of neural pathway genes were assessed using Nanostring. HPA-colonized mice had significantly higher levels of *5-HT* expression compared to HD-colonized mice (* $p<0.05$) and LPA-colonized mice (# $p<0.05$).

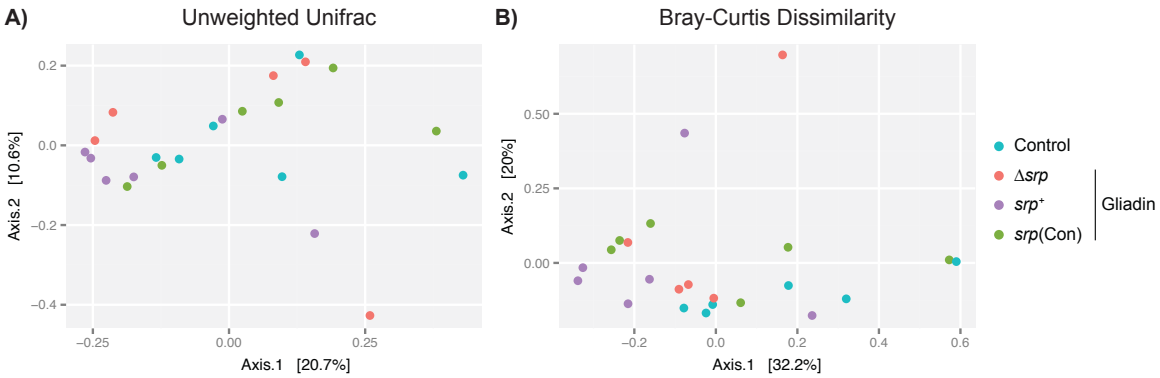


Supplementary figure 3. *In vivo* model of proteolytic activity in ASF mice.

A) *In vitro* proteolytic activity of HD, LPA, and HPA bacterial communities are transferred into colonized mice. **B)** Higher bacterial translocation is seen in ASF mice colonized with HPA microbiota compared to those colonized with HD or LPA microbiota. **C)** Colonic paracellular permeability does not significantly differ between colonized mice. **D)** Transepithelial conductance progressively increasesr in HD, LPA, then HPA-colonized mice which exhibits the highest average conductance.

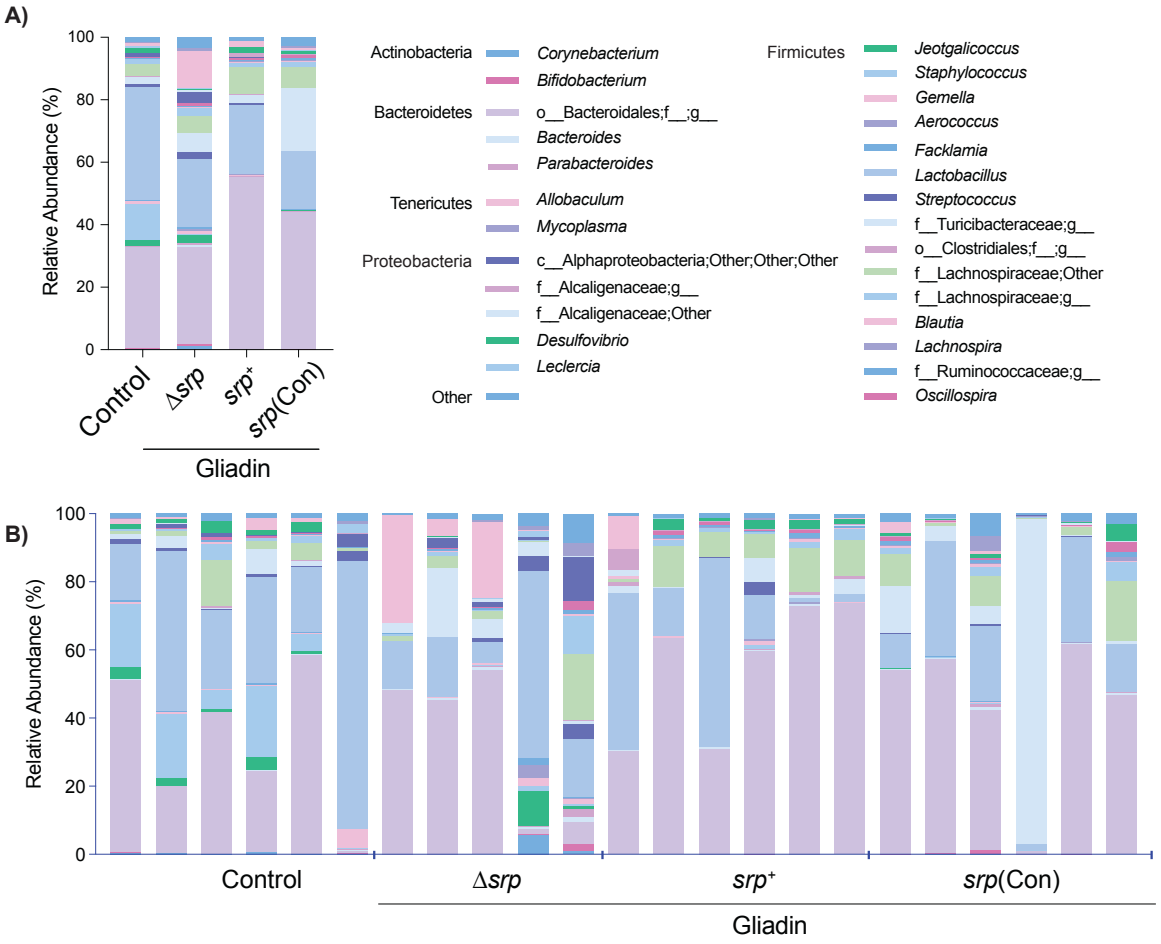


Supplementary figure 4. Treatment with *B. longum* expressing serpin inhibited gluten degradation in HPA-colonized mice. Fecal supernatants from mice were spotted on agar containing 1% gluten, and zone of clearance was measured to indicate degradation of gluten. *B. longum srp*(Con)-treated mice had significantly lower glutenasic activity in feces than mice given PBS vehicle ($p<0.05$).



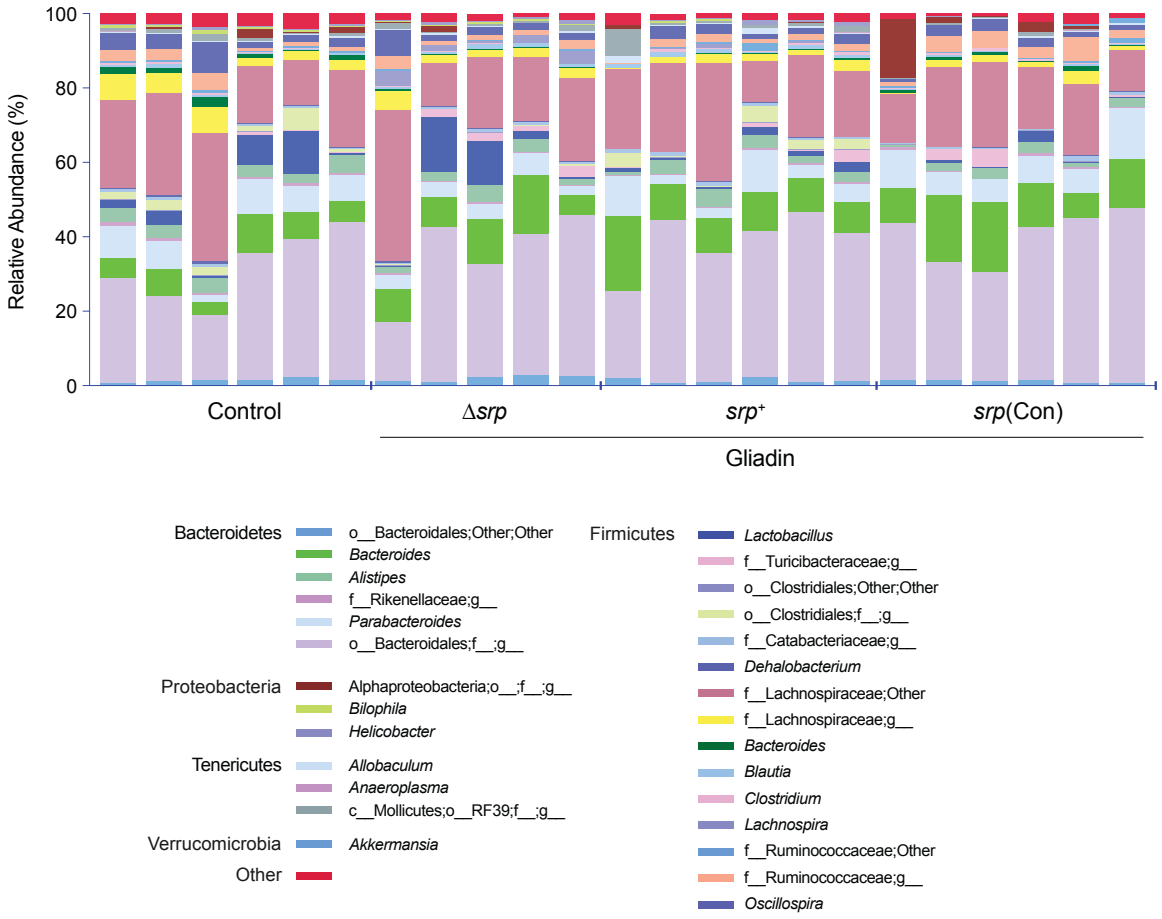
Supplementary figure 5. Small intestinal microbiota profiles.

Principal coordinate analysis plots representing β-diversity using both **A)** Bray-Curtis Dissimilarity and **B)** Unifrac Unweighted parameters revealed no significant differences in small intestinal microbiota (n=5-6/group). Non-sensitized, no treatment (Control); *B. longum srp*$^{+}$ (*srp*$^{+}$); *B. longum Δsrp* (*Δsrp*); *B. longum srp*(Con) (*srp*(Con)). Statistics were performed via PERMANOVA in QIIME. Plots were constructed in R.



Supplementary figure 6. Relative abundances in small intestinal microbiota profiles at genus level.

Small intestinal microbiota was sequenced via 16s miSeq Illumina technology. Operational taxonomic units at relative abundances ≥ 1% are presented as **A)** average of each group and **B)** per mouse (n=5-6). No changes in relative abundances were found. Non-sensitized, no treatment (Control); *B. longum srp*$^{+}$ (*srp*$^{+}$); *B. longum Δsrp* (*Δsrp*); *B. longum srp*(Con) (*srp*(Con)). Statistics were performed via Kruskal-Wallis followed by FDR ($q<0.05$).



Supplementary figure 7. Relative abundances of fecal microbiota for individual mice at genus level.

Fecal microbiota was sequenced via miSeq Illumina technology by amplification of the 16S rRNA gene, and relative abundances are represented at a genus level in stacked column charts (n=5-6/group). Non-sensitized, no treatment (Control); *B. longum srp*$^{+}$ (*srp*$^{+}$); *B. longum Δsrp* (*Δsrp*); *B. longum srp*(Con) (*srp*(Con)). Statistics were performed via Kruskal-Wallis followed by FDR ($q<0.05$).

REFERENCES

1. Maldonado-Contreras, A. et al. Structure of the human gastric bacterial community in relation to *Helicobacter pylori* status. *ISME journal* **5**, 574-579 (2011).
2. van der Flier, L.G. et al. Transcription factor achaete scute-like 2 controls intestinal stem cell fate. *Cell* **136**, 903-912 (2009).
3. Camilleri, M., Madsen, K., Spiller, R., Van Meerveld, B. & Verne, G. Intestinal barrier function in health and gastrointestinal disease. *Neurogastroenterology & Motility* **24**, 503-512 (2012).
4. Odenwald, M.A. & Turner, J.R. Intestinal permeability defects: is it time to treat? *Clinical Gastroenterology and Hepatology* **11**, 1075-1083 (2013).
5. Zhao, C., Wang, I. & Lehrer, R.I. Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells. *FEBS Letters* **396**, 319-322 (1996).
6. O'Neil, D.A. et al. Expression and regulation of the human β-defensins hBD-1 and hBD-2 in intestinal epithelium. *The Journal of Immunology* **163**, 6718-6724 (1999).
7. Fehlbaum, P., Rao, M., Zasloff, M. & Anderson, G.M. An essential amino acid induces epithelial β-defensin expression. *Proceedings of the National Academy of Sciences* **97**, 12723-12728 (2000).
8. García, J.-R. et al. Identification of a novel, multifunctional ß-defensin (human ß-defensin 3) with specific antimicrobial activity. *Cell and Tissue Research* **306**, 257-264 (2001).
9. Bensch, K.W., Raida, M., Mägert, H.-J., Schulz-Knappe, P. & Forssmann, W.-G. hBD-1: a novel β-defensin from human plasma. *FEBS Letters* **368**, 331-335 (1995).
10. Mowat, A.M. & Agace, W.W. Regional specialization within the intestinal immune system. *Nature Reviews Immunology* **14**, 667-685 (2014).
11. Johansson, M.E. et al. Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. *Gut*, gutjnl-2012-303207 (2013).
12. Vaishnava, S. et al. The antibacterial lectin RegIIIγ promotes the spatial segregation of microbiota and host in the intestine. *Science* **334**, 255-258 (2011).
13. Johansson, M.E. & Hansson, G.C. Keeping bacteria at a distance. *Science* **334**, 182-183 (2011).
14. Clevers, H.C. & Bevins, C.L. Paneth cells: maestros of the small intestinal crypts. *Annual Review of Physiology* **75**, 289-311 (2013).
15. Finkelman, F.D. et al. Interleukin‐4 and interleukin‐13‐mediated host protection against intestinal nematode parasites. *Immunological reviews* **201**, 139-155 (2004).
16. Steenwinckel, V. et al. IL-9 promotes IL-13-dependent paneth cell hyperplasia and up-regulation of innate immunity mediators in intestinal mucosa. *The Journal of Immunology* **182**, 4737-4743 (2009).

17. Oeser, K., Schwartz, C. & Voehringer, D. Conditional IL-4/IL-13-deficient mice reveal a critical role of innate immune cells for protective immunity against gastrointestinal helminths. *Mucosal immunology* **8**, 672-682 (2015).
18. Hasnain, S.Z. et al. IL-10 promotes production of intestinal mucus by suppressing protein misfolding and endoplasmic reticulum stress in goblet cells. *Gastroenterology* **144**, 357-368. e9 (2013).
19. Turner, J.-E., Stockinger, B. & Helmby, H. IL-22 mediates goblet cell hyperplasia and worm expulsion in intestinal helminth infection. *PLoS Pathog* **9**, e1003698 (2013).
20. Mostov, K.E. Transepithelial transport of immunoglobulins. *Annual review of immunology* **12**, 63-84 (1994).
21. Perrier, C., Sprenger, N. & Corthésy, B. Glycans on secretory component participate in innate protection against mucosal pathogens. *Journal of Biological Chemistry* **281**, 14280-14287 (2006).
22. Mantis, N.J., Rol, N. & Corthésy, B. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal immunology* **4**, 603-611 (2011).
23. Kadaoui, K.A. & Corthésy, B. Secretory IgA mediates bacterial translocation to dendritic cells in mouse Peyer's patches with restriction to mucosal compartment. *The Journal of Immunology* **179**, 7751-7757 (2007).
24. Lecocq, M., Detry, B., Guisset, A. & Pilette, C. FcαRI-mediated inhibition of IL-12 production and priming by IFN-γ of human monocytes and dendritic cells. *The Journal of Immunology* **190**, 2362-2371 (2013).
25. Baumann, J., Park, C.G. & Mantis, N.J. Recognition of secretory IgA by DC-SIGN: implications for immune surveillance in the intestine. *Immunology Letters* **131**, 59-66 (2010).
26. Duerksen, D., Wilhelm-Boyles, C. & Parry, D. Intestinal permeability in long-term follow-up of patients with celiac disease on a gluten-free diet. *Digestive Diseases and Sciences* **50**, 785-790 (2005).
27. Watts, T. et al. Role of the intestinal tight junction modulator zonulin in the pathogenesis of type I diabetes in BB diabetic-prone rats. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 2916-2921 (2005).
28. Förster, C. Tight junctions and the modulation of barrier function in disease. *Histochemistry and cell biology* **130**, 55-70 (2008).
29. Brandtzaeg, P., Kiyono, H., Pabst, R. & Russell, M. Terminology: nomenclature of mucosa-associated lymphoid tissue. *Mucosal Immunology* **1**, 31-37 (2008).
30. Coombes, J.L. et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-β–and retinoic acid–dependent mechanism. *Journal of Experimental Medicine* **204**, 1757-1764 (2007).
31. Iwasaki, A. & Kelsall, B.L. Unique functions of CD11b+, CD8α+, and double-negative Peyer's patch dendritic cells. *The Journal of Immunology* **166**, 4884-4890 (2001).
32. Denning, T.L., Wang, Y.-c., Patel, S.R., Williams, I.R. & Pulendran, B. Lamina propria macrophages and dendritic cells differentially induce regulatory and

interleukin 17–producing T cell responses. *Nature Immunology* **8**, 1086-1094 (2007).
33. Murai, M. et al. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nature immunology* **10**, 1178-1184 (2009).
34. Krause, P. et al. IL-10-producing intestinal macrophages prevent excessive antibacterial innate immunity by limiting IL-23 synthesis. *Nature communications* **6** (2015).
35. Rothenberg, M.E. Eosinophilia. *New England Journal of Medicine* **338**, 1592-1600 (1998).
36. Jung, Y. & Rothenberg, M.E. Roles and regulation of gastrointestinal eosinophils in immunity and disease. *The Journal of Immunology* **193**, 999-1005 (2014).
37. Kolaczkowska, E. & Kubes, P. Neutrophil recruitment and function in health and inflammation. *Nature Reviews Immunology* **13**, 159-175 (2013).
38. Kühl, A.A. et al. Aggravation of different types of experimental colitis by depletion or adhesion blockade of neutrophils. *Gastroenterology* **133**, 1882-1892 (2007).
39. Targan, S.R., Deem, R.L., Liu, M., Wang, S. & Nel, A. Definition of a lamina propria T cell responsive state. Enhanced cytokine responsiveness of T cells stimulated through the CD2 pathway. *The Journal of Immunology* **154**, 664-675 (1995).
40. Cheroutre, H., Lambolez, F. & Mucida, D. The light and dark sides of intestinal intraepithelial lymphocytes. *Nature Reviews Immunology* **11**, 445-456 (2011).
41. Benckert, J. et al. The majority of intestinal IgA+ and IgG+ plasmablasts in the human gut are antigen-specific. *The Journal of Clinical Investigation* **121**, 1946-1955 (2011).
42. Sekirov, I., Russell, S.L., Antunes, L.C.M. & Finlay, B.B. Gut microbiota in health and disease. *Physiological Reviews* **90**, 859-904 (2010).
43. Consortium, H.M.P. Structure, function and diversity of the healthy human microbiome. *Nature* **486**, 207-214 (2012).
44. O'Hara, A.M. & Shanahan, F. Mechanisms of Action of Probiotics in Intestinal Diseases. *The Scientific World Journal* **7** (2007).
45. Donaldson, G.P., Lee, S.M. & Mazmanian, S.K. Gut biogeography of the bacterial microbiota. *Nature Reviews Microbiology* **14**, 20-32 (2016).
46. Hollister, E.B., Gao, C. & Versalovic, J. Compositional and functional features of the gastrointestinal microbiome and their effects on human health. *Gastroenterology* **146**, 1449-1458 (2014).
47. Pflughoeft, K.J. & Versalovic, J. Human microbiome in health and disease. *Annual Review of Pathology: Mechanisms of Disease* **7**, 99-122 (2012).
48. Koenig, J.E. et al. Succession of microbial consortia in the developing infant gut microbiome. *Proceedings of the National Academy of Sciences* **108**, 4578-4585 (2011).

49. Yu, Z.-T. et al. The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota. *Glycobiology* **23**, 169-177 (2013).
50. Yatsunenko, T. et al. Human gut microbiome viewed across age and geography. *Nature* **486**, 222-227 (2012).
51. O'Hara, A.M. & Shanahan, F. The gut flora as a forgotten organ. *EMBO reports* **7**, 688-693 (2006).
52. Zoetendal, E.G. et al. The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. *The ISME journal* **6**, 1415-1426 (2012).
53. Gu, S. et al. Bacterial community mapping of the mouse gastrointestinal tract. *PloS one* **8**, e74957 (2013).
54. Swidsinski, A., Loening-Baucke, V., Lochs, H. & Hale, L.P. Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence in situ hybridization study in mice. *World J Gastroenterol* **11**, 1131-1140 (2005).
55. Pédron, T. et al. A crypt-specific core microbiota resides in the mouse colon. *MBio* **3**, e00116-12 (2012).
56. Swidsinski, A. et al. Viscosity gradient within the mucus layer determines the mucosal barrier function and the spatial organization of the intestinal microbiota. *Inflammatory bowel diseases* **13**, 963-970 (2007).
57. Geuking, M.B. et al. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity* **34**, 794-806 (2011).
58. Round, J. & Mazmanian, S. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* **9**, 313-323 (2009).
59. Hapfelmeier, S. et al. Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. *Science* **328**, 1705-1709 (2010).
60. Kabat, A.M., Srinivasan, N. & Maloy, K.J. Modulation of immune development and function by intestinal microbiota. *Trends in immunology* **35**, 507-517 (2014).
61. Macpherson, A.J. & Uhr, T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* **303**, 1662-1665 (2004).
62. Macpherson, A.J. et al. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* **288**, 2222-2226 (2000).
63. Jostins, L. et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* **491**, 119-124 (2012).
64. Kawai, T. & Akira, S. The roles of TLRs, RLRs and NLRs in pathogen recognition. *International immunology* **21**, 317-337 (2009).
65. Kawai, T. & Akira, S. TLR signaling. *Cell Death & Differentiation* **13**, 816-825 (2006).
66. Wen, L. et al. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature* **455**, 1109-1113 (2008).
67. Frantz, A. et al. Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with downregulation of polymeric immunoglobulin receptor, mucin-2, and antibacterial peptides. *Mucosal immunology* **5**, 501-512 (2012).

68. Couturier-Maillard, A. et al. NOD2-mediated dysbiosis predisposes mice to transmissible colitis and colorectal cancer. *The Journal of Clinical Investigation* **123**, 700-711 (2013).
69. Petnicki-Ocwieja, T. et al. Nod2 is required for the regulation of commensal microbiota in the intestine. *Proceedings of the National Academy of Sciences* **106**, 15813-15818 (2009).
70. Rehman, A. et al. Nod2 is essential for temporal development of intestinal microbial communities. *Gut* **60**, 1354-1362 (2011).
71. Frank, D.N. et al. Disease phenotype and genotype are associated with shifts in intestinal‐associated microbiota in inflammatory bowel diseases. *Inflammatory bowel diseases* **17**, 179-184 (2011).
72. Li, E. et al. Inflammatory bowel diseases phenotype, *C. difficile* and NOD2 genotype are associated with shifts in human ileum associated microbial composition. *PloS one* **7**, e26284 (2012).
73. Peterson, D.A., McNulty, N.P., Guruge, J.L. & Gordon, J.I. IgA response to symbiotic bacteria as a mediator of gut homeostasis. *Cell host & microbe* **2**, 328-339 (2007).
74. Usami, M. et al. Butyrate and trichostatin A attenuate nuclear factor κB activation and tumor necrosis factor α secretion and increase prostaglandin E 2 secretion in human peripheral blood mononuclear cells. *Nutrition research* **28**, 321-328 (2008).
75. Cummings, J., Pomare, E., Branch, W., Naylor, C. & Macfarlane, G. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* **28**, 1221-1227 (1987).
76. Vinolo, M.A., Rodrigues, H.G., Nachbar, R.T. & Curi, R. Regulation of inflammation by short chain fatty acids. *Nutrients* **3**, 858-876 (2011).
77. Lamas, B. et al. CARD9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands. *Nature medicine* **22**, 598-605 (2016).
78. Mazmanian, S.K., Round, J.L. & Kasper, D.L. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* **453**, 620-625 (2008).
79. Vatanen, T. et al. Variation in microbiome LPS immunogenicity contributes to autoimmunity in humans. *Cell* **165**, 842-853 (2016).
80. Moon, C. et al. Vertically transmitted faecal IgA levels determine extra-chromosomal phenotypic variation. *Nature* **521**, 90-93 (2015).
81. Kassinen, A. et al. The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. *Gastroenterology* **133**, 24-33 (2007).
82. Powrie, F. T cells in inflammatory bowel disease: protective and pathogenic roles. *Immunity* **3**, 171-174 (1995).
83. Harper, P., Lee, E., Kettlewell, M., Bennett, M. & Jewell, D. Role of the faecal stream in the maintenance of Crohn's colitis. *Gut* **26**, 279-284 (1985).
84. Levy, M., Kolodziejczyk, A.A., Thaiss, C.A. & Elinav, E. Dysbiosis and the immune system. *Nature Reviews Immunology* **17**, 219-232 (2017).

85. Turnbaugh, P.J. et al. An obesity-associated gut microbiome with increased capacity for energy harvest. *nature* **444**, 1027-131 (2006).
86. Vrieze, A. et al. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology* **143**, 913-916. e7 (2012).
87. Le Roy, T. et al. Intestinal microbiota determines development of non-alcoholic fatty liver disease in mice. *Gut* **62**, 1787-1794 (2013).
88. Buffie, C.G. et al. Precision microbiome reconstitution restores bile acid mediated resistance to *Clostridium difficile*. *Nature* **517**, 205-208 (2015).
89. Hsiao, E.Y. et al. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* **155**, 1451-1463 (2013).
90. Martín, R. et al. The commensal bacterium *Faecalibacterium prausnitzii* is protective in DNBS-induced chronic moderate and severe colitis models. *Inflammatory bowel diseases* **20**, 417-430 (2014).
91. Shaw, S.Y., Blanchard, J.F. & Bernstein, C.N. Association between the use of antibiotics in the first year of life and pediatric inflammatory bowel disease. *The American Journal of Gastroenterology* **105**, 2687-2692 (2010).
92. Stefka, A.T. et al. Commensal bacteria protect against food allergen sensitization. *Proceedings of the National Academy of Sciences* **111**, 13145-13150 (2014).
93. Galipeau, H.J. et al. Intestinal microbiota modulates gluten-induced immunopathology in humanized mice. *The American journal of pathology* **185**, 2969-2982 (2015).
94. Murphy, E. et al. Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. *Gut* **59**, 1635-1642 (2010).
95. Håkansson, Å. et al. Immunological alteration and changes of gut microbiota after dextran sulfate sodium (DSS) administration in mice. *Clinical and experimental medicine* **15**, 107-120 (2015).
96. Khor, B., Gardet, A. & Xavier, R.J. Genetics and pathogenesis of inflammatory bowel disease. *Nature* **474**, 307-317 (2011).
97. Mankertz, J. & Schulzke, J.-D. Altered permeability in inflammatory bowel disease: pathophysiology and clinical implications. *Current opinion in gastroenterology* **23**, 379-383 (2007).
98. Mokry, M. et al. Many inflammatory bowel disease risk loci include regions that regulate gene expression in immune cells and the intestinal epithelium. *Gastroenterology* **146**, 1040-1047 (2014).
99. Bustos, D., Negri, G. & De Paula, J. Colonic proteinases: increased activity in patients with ulcerative colitis. *Medicina* **58**, 262-264 (1998).
100. Gecse, K., Roka, R. & Ferrier, L. Increased faecal serine protease activity in diarrhoeic IBS patients: a colonic lumenal factor impairing colonic permeability and sensitivity. *Gut* **57**, 591-599 (2008).
101. Roka, R., Rosztoczy, A. & Leveque, M. A pilot study of fecal serine-protease activity: a pathophysiologic factor in diarrhea- predominant irritable bowel syndrome. *Clin Gastroenterol Hepatol.* **5**, 550-555 (2007).

102. O'Sullivan, S., Gilmer, J.F. & Medina, C. Matrix metalloproteinases in inflammatory bowel disease: an update. *Mediators of inflammation* **2015** (2015).
103. Coskun, M. Intestinal epithelium in inflammatory bowel disease. *Frontiers in medicine* **1** (2014).
104. Brannigan, A.E. et al. Neutrophil apoptosis is delayed in patients with inflammatory bowel disease. *Shock* **13**, 361-366 (2000).
105. Carter, L. & Wallace, J.L. Alterations in rat peripheral blood neutrophil function as a consequence of colitis. *Digestive Diseases and Sciences* **40**, 192-197 (1995).
106. Palmen, M. et al. Non‐lymphoid and lymphoid cells in acute, chronic and relapsing experimental colitis. *Clinical & Experimental Immunology* **99**, 226-232 (1995).
107. Kamada, N. et al. Unique CD14+ intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-γ axis. *The Journal of clinical investigation* **118**, 2269-2280 (2008).
108. Kumar, N.B., Nostrant, T.T. & Appelman, H.D. The histopathologic spectrum of acute self-limited colitis (acute infectious-type colitis). *The American journal of surgical pathology* **6**, 523-530 (1982).
109. Kvedaraite, E. et al. Tissue-infiltrating neutrophils represent the main source of IL-23 in the colon of patients with IBD. *Gut* **65**, 1632-1641 (2016).
110. Demir, A.K. et al. The relationship between the neutrophil–lymphocyte ratio and disease activity in patients with ulcerative colitis. *The Kaohsiung Journal of Medical Sciences* **31**, 585-590 (2015).
111. Celikbilek, M. et al. Neutrophil–lymphocyte ratio as a predictor of disease severity in ulcerative colitis. *Journal of Clinical Laboratory Analysis* **27**, 72-76 (2013).
112. Buell, M.G. & Berin, M.C. Neutrophil-independence of the initiation of colonic injury. *Digestive Diseases and Sciences* **39**, 2575-2588 (1994).
113. Natsui, M. et al. Selective depletion of neutrophils by a monoclonal antibody, RP‐3, suppresses dextran sulphate sodium‐induced colitis in rats. *Journal of gastroenterology and hepatology* **12**, 801-808 (1997).
114. Ogura, Y. et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* **411**, 603-606 (2001).
115. Hugot, J.-P. et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* **411**, 599-603 (2001).
116. Eckburg, P.B. et al. Diversity of the human intestinal microbial flora. *science* **308**, 1635-1638 (2005).
117. Frank, D.N. et al. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proceedings of the National Academy of Sciences* **104**, 13780-13785 (2007).
118. Mangin, I. et al. Molecular inventory of faecal microflora in patients with Crohn's disease. *FEMS microbiology ecology* **50**, 25-36 (2004).
119. Walker, A.W. et al. High-throughput clone library analysis of the mucosa-associated microbiota reveals dysbiosis and differences between inflamed and

non-inflamed regions of the intestine in inflammatory bowel disease. *BMC microbiology* **11**, 7 (2011).
120. Sokol, H. et al. Low counts of Faecalibacterium prausnitzii in colitis microbiota. *Inflammatory bowel diseases* **15**, 1183-1189 (2009).
121. Seksik, P. et al. Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon. *Gut* **52**, 237-242 (2003).
122. Manichanh, C., Borruel, N., Casellas, F. & Guarner, F. The gut microbiota in IBD. *Nature Reviews Gastroenterology and Hepatology* **9**, 599-608 (2012).
123. Sokol, H. et al. Specificities of the fecal microbiota in inflammatory bowel disease. *Inflammatory bowel diseases* **12**, 106-111 (2006).
124. Scanlan, P.D. et al. Culture‐independent analysis of the gut microbiota in colorectal cancer and polyposis. *Environmental microbiology* **10**, 789-798 (2008).
125. Mukhopadhya, I., Hansen, R., El-Omar, E.M. & Hold, G.L. IBD—what role do Proteobacteria play? *Nature Reviews Gastroenterology and Hepatology* **9**, 219-230 (2012).
126. Sartor, R.B. & Mazmanian, S.K. Intestinal microbes in inflammatory bowel diseases. *The American Journal of Gastroenterology Supplements* **1**, 15-21 (2012).
127. Lepage, P. et al. Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis. *Gastroenterology* **141**, 227-236 (2011).
128. Nishikawa, J., Kudo, T., Sakata, S., Benno, Y. & Sugiyama, T. Diversity of mucosa-associated microbiota in active and inactive ulcerative colitis. *Scandinavian journal of gastroenterology* **44**, 180-186 (2009).
129. Machiels, K. et al. A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. *Gut*, gutjnl-2013-304833 (2013).
130. Natividad, J.M. et al. Ecobiotherapy rich in firmicutes decreases susceptibility to colitis in a humanized gnotobiotic mouse model. *Inflammatory bowel diseases* **21**, 1883-1893 (2015).
131. Marietta, E. et al. A new model for dermatitis herpetiformis that uses HLA-DQ8 transgenic NOD mice. *The Journal of clinical investigation* **114**, 1090-1097 (2004).
132. Kilander, A., Nilsson, L. & Gillberg, R. Serum antibodies to gliadin in coeliac disease after gluten withdrawal. *Scand J Gastroenterol* **22**, 29-34 (1987).
133. MT, B. et al. Coeliac disease: a histological follow-up study. *Histopathology* **50**, 465-71 (2007).
134. Tuire, I. et al. Persistent Duodenal Intraepithelial Lymphocytosis Despite a Long-Term Strict Gluten-Free Diet in Celiac Disease. *Am J Gastroenterol 2012* **107**, 1563–1569 (2012).
135. Saturni, L., Ferretti, G. & Bacchetti, T. The Gluten-Free Diet: Safety and Nutritional Quality. *Nutrients* **2**, 16-34 (2010).
136. Schuppan, D., Junker, Y. & Barsani, D. Celiac Disease: From Pathogenesis to Novel Therapies. *Gastroenterology* **137**, 1912-1933 (2009).

137. Harris, L.A., Park, J.Y., Voltaggio, L. & Lam-Himlin, D. Celiac disease: clinical, endoscopic, and histopathologic review. *Gastrointestinal Endoscopy* **76**, 625-640 (2012).
138. Abadie, V., Sollid, L.M., Barreiro, L.B. & Jabri, B. Integration of genetic and immunological insights into a model of celiac disease pathogen- esis. *Annu Rev Immunol* **29**, 493-525 (2011).
139. Henderson, K.N. et al. The production and crystallization of the human leukocyte antigen class II molecules HLA-DQ2 and HLA-DQ8 complexed with deamidated gliadin peptides implicated in coeliac disease. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun* **63** (2007).
140. Bergseng, E., Xia, J., Kim, C.Y., Khosla, C. & Sollid, L.M. Main chain hydrogen bond interactions in the binding of proline-rich gluten peptides to the celiac disease-associated HLA-DQ2 molecule. *J Biol Chem* **280** (2005).
141. Koning, F. Celiac disease: quantity matters. *Semin Immunopathol.* **34**, 541-549 (2012).
142. Collin, P., Wahab, P. & Murray, J. Intraepithelial lymphocytes and coeliac disease. *Best Practice & Research Clinical Gastroenterology* **19**, 341-350 (2005).
143. Hüe, S. et al. A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. *Immunity* **21**, 367-377 (2004).
144. Meresse, B. et al. Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity* **21**, 357-366 (2004).
145. Abadie, V., Discepolo, V. & Jabri, B. Intraepithelial lymphocytes in celiac disease immunopathology. *Semin Immunopathol.* **34**, 551-66 (2012).
146. Cheroutre, H. IELs: enforcing law and order in the court of the intestinal epithelium. *Immunol Rev* **206**, 114-31 (2005).
147. Myléus, A. et al. Early infections are associated with increased risk for celiac disease: an incident case-referent study. *BMC pediatrics* **12**, 194 (2012).
148. Stene, L.C. et al. Rotavirus infection frequency and risk of celiac disease autoimmunity in early childhood: a longitudinal study. *The American journal of gastroenterology* **101**, 2333-2340 (2006).
149. Pavone, P., Nicolini, E., Taibi, R. & Ruggieri, M. Rotavirus and celiac disease. *The American journal of gastroenterology* **102**, 1831 (2007).
150. Collado, M.C., Donat, E., Ribes-Koninckx, C., Calabuig, M. & Sanz, Y. Specific duodenal and faecal bacterial groups associated with paediatric coeliac disease. *Journal of Clinical Pathology* **62**, 264-269 (2009).
151. De Palma, G. et al. Intestinal dysbiosis and reduced immunoglobulin-coated bacteria associated with coeliac disease in children. *BMC Microbiology* **10**, 63 (2010).
152. Decker, E. et al. Cesarean delivery is associated with celiac disease but not inflammatory bowel disease in children. *Pediatrics* **125**, e1433-40 (2010).
153. Sanz, Y., De Palma, G. & Laparra, M. Unraveling the Ties between Celiac Disease and Intestinal Microbiota. *International Reviews of Immunology* **30**, 207-218 (2011).

154. Collado, M.C., Donat, E., Ribes-Koninck, C., Calabuig, M. & Sanz, Y. Imbalances in faecal and duodenal Bifidobacterium species composition in active and non-active coeliac disease. *BMC Microbiology* **8** (2008).
155. Nadal, I., Donant, E., Ribes-Koninck, C., Calabuig, M. & Sanz, Y. Imbalance in the composition of the duodenal microbiota of children with coeliac disease. *Journal of Medical Microbiology* **56**, 1669-1674 (2007).
156. Wacklin, P. et al. The duodenal microbiota composition of adult celiac disease patients is associated with the clinical manifestation of the disease. *Inflammatory bowel diseases* **19**, 934-941 (2013).
157. Caminero, A. et al. Duodenal Bacteria From Patients With Celiac Disease and Healthy Subjects Distinctly Affect Gluten Breakdown and Immunogenicity. *Gastroenterology* **151**, 670-83 (2016).
158. van Nood, E. et al. Duodenal infusion of donor feces for recurrent *Clostridium difficile*. *New England Journal of Medicine* **368**, 407-415 (2013).
159. Moayyedi, P. et al. Fecal microbiota transplantation induces remission in patients with active ulcerative colitis in a randomized controlled trial. *Gastroenterology* **149**, 102-109. e6 (2015).
160. Khan, K.J. et al. Antibiotic therapy in inflammatory bowel disease: a systematic review and meta-analysis. *The American journal of gastroenterology* **106**, 661-673 (2011).
161. Huang, J., Liao, C., Wu, L., Cao, Y. & Gao, F. A meta‐analysis of randomized controlled trials comparing antibacterial therapy with placebo in Crohn’s disease. *Colorectal Disease* **13**, 617-626 (2011).
162. Su, J.W., Ma, J.J. & Zhang, H.J. Use of antibiotics in patients with Crohn's disease: A systematic review and meta‐analysis. *Journal of digestive diseases* **16**, 58-66 (2015).
163. Pimentel, M. et al. Rifaximin therapy for patients with irritable bowel syndrome without constipation. *New england journal of medicine* **364**, 22-32 (2011).
164. Ianiro, G., Tilg, H. & Gasbarrini, A. Antibiotics as deep modulators of gut microbiota: between good and evil. *Gut* **65**, 1906-1915 (2016).
165. Roberfroid, M. et al. Prebiotic effects: metabolic and health benefits. *British Journal of Nutrition* **104**, S1-S63 (2010).
166. Laurell, A. & Sjöberg, K. Prebiotics and synbiotics in ulcerative colitis. *Scandinavian Journal of Gastroenterology* **52**, 477-485 (2017).
167. O'Hara, A.M. et al. Functional modulation of human intestinal epithelial cell responses by Bifidobacterium infantis and Lactobacillus salivarius. *Immunology* **118**, 202–215 (2006).
168. Biagi, F. et al. Intraepithelial lymphocytes in the villous tip: do they indicate potential coeliac disease? *Journal of Clinical Pathology* **57**, 835-9 (2004).
169. Otte, J.M. & Podolsky, D.K. Functional modulation of enterocytes by gram-positive and gram-negative microorganisms. *Am. J. Physiol. Gastrointest. Liver Physiol.* **286**, G613–626 (2004).

170. Bibiloni, R. et al. VSL# 3 probiotic-mixture induces remission in patients with active ulcerative colitis. *The American Journal of Gastroenterology* **100**, 1539-1546 (2005).
171. Jonkers, D., Penders, J., Masclee, A. & Pierik, M. Probiotics in the management of inflammatory bowel disease. *Drugs* **72**, 803-823 (2012).
172. Moayyedi, P. et al. The efficacy of probiotics in the treatment of irritable bowel syndrome: a systematic review. *Gut* **59**, 325-332 (2010).
173. Joint, F. WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food. Guidelines for the evaluation of probiotics in food: report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food, London, Ontario, Canada, April 30 and May 1, 2002. *ftp. fao. org/es/esn/food/wgreport2. pdf. Accessed* **16** (2007).
174. Wolvers, D. et al. Guidance for substantiating the evidence for beneficial effects of probiotics: prevention and management of infections by probiotics. *The Journal of nutrition* **140**, 698S-712S (2010).
175. Vanderpool, C., Yan, F. & Polk, D.B. Mechanisms of probiotic action: implications for therapeutic applications in inflammatory bowel diseases. *Inflammatory bowel diseases* **14**, 1585-1596 (2008).
176. Carroll, I.M. et al. Anti-inflammatory properties of *Lactobacillus gasseri* expressing manganese superoxide dismutase using the interleukin 10-deficient mouse model of colitis. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **293**, G729-G738 (2007).
177. Fernandez, E.M. et al. Anti-inflammatory capacity of selected lactobacilli in experimental colitis is driven by NOD2-mediated recognition of a specific peptidoglycan-derived muropeptide. *Gut*, gut. 2010.232918 (2011).
178. Bousvaros, A. et al. A randomized, double‐blind trial of *Lactobacillus GG* versus placebo in addition to standard maintenance therapy for children with Crohn's disease. *Inflammatory Bowel Diseases* **11**, 833-839 (2005).
179. Gupta, P., Andrew, H., Kirschner, B.S. & Guandalini, S. Is *Lactobacillus* GG helpful in children with Crohn's disease? Results of a preliminary, open-label study. *Journal of pediatric gastroenterology and nutrition* **31**, 453-457 (2000).
180. Kato, K. et al. Randomized placebo‐controlled trial assessing the effect of bifidobacteria‐fermented milk on active ulcerative colitis. *Alimentary pharmacology & therapeutics* **20**, 1133-1141 (2004).
181. Wildt, S., Nordgaard, I., Hansen, U., Brockmann, E. & Rumessen, J.J. A randomised double-blind placebo-controlled trial with *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* subsp. *lactis* BB-12 for maintenance of remission in ulcerative colitis. *Journal of Crohn's and Colitis* **5**, 115-121 (2011).
182. Rahimi, R. et al. A meta-analysis on the efficacy of probiotics for maintenance of remission and prevention of clinical and endoscopic relapse in Crohn's disease. *Digestive diseases and sciences* **53**, 2524-2531 (2008).
183. Zocco, M. et al. Efficacy of *Lactobacillus* GG in maintaining remission of ulcerative colitis. *Alimentary pharmacology & therapeutics* **23**, 1567-1574 (2006).

184. Furrie, E. et al. Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. *Gut* **54**, 242-9 (2005).
185. O'Mahony, L. et al. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. *Gastroenterology* **128**, 541-51 (2005).
186. Smecuol, E. et al. Exploratory, randomized, double-blind, placebo-controlled study on the effects of *Bifidobacterium infantis* natren life start strain super strain in active celiac disease. *Journal of clinical gastroenterology* **47**, 139-47 (2013).
187. Pinto-Sanchez, M.I. et al. *Bifidobacterium infantis* NLS Super Strain Reduces the Expression of alpha-Defensin-5, a Marker of Innate Immunity, in the Mucosa of Active Celiac Disease Patients. *Journal of clinical gastroenterology* (2016).
188. Medina, M., De Palma, G., Ribes-Koninck, C., Calabuig, M. & Sanz, Y. Bifidobacterium strains suppress in vitro the pro-inflammatory milieu triggered by the large intestinal microbiota of coeliac patients. *Journal of Inflammation* **5** (2008).
189. Laparra, J., Olivares, M., Gallina, O. & Sanz, Y. Bifidobacterium longum CECT 7347 modulates immune responses in a gliadin-induced enteropathy animal model. *PLoS ONE* **7** (2012).
190. De Palma, G., Cinova, J., Stepankova, R., Tuckova, L. & Sanz, Y. Pivotal Advance: Bifidobacteria and Gram-negative bacteria differentially influence immune responses in the proinflammatory milieu of celiac disease. *Journal of Leukocyte Biology* **87**, 765-778 (2010).
191. Lindfors, K. et al. Live probiotic Bifidobacterium lactis bacteria inhibit the toxic effects induced by wheat gliadin in epithelial cell culture. *Clinical Exp Immunology* **152**, 1365-2249 (2008).
192. Vora, P. et al. β-defensin-2 expression is regulated by TLR signaling in intestinal epithelial cells. *The Journal of Immunology* **173**, 5398-5405 (2004).
193. Wehkamp, J. et al. NF-κB-and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by *Escherichia coli* Nissle 1917: a novel effect of a probiotic bacterium. *Infection and immunity* **72**, 5750-5758 (2004).
194. von Schillde, M.-A. et al. Lactocepin secreted by *Lactobacillus* exerts anti-inflammatory effects by selectively degrading proinflammatory chemokines. *Cell host & microbe* **11**, 387-396 (2012).
195. Oido-Mori, M. et al. *Porphyromonas gingivalis* gingipain-R enhances interleukin-8 but decreases gamma interferon-inducible protein 10 production by human gingival fibroblasts in response to T-cell contact. *Infection and immunity* **69**, 4493-4501 (2001).
196. Ivanov, D. et al. A serpin from the gut bacterium *Bifidobacterium longum* inhibits eukaryotic elastase-like serine proteases. *Journal of Biological Chemistry* **281**, 17246-52 (2006).
197. Turk, B. Targeting proteases: successes, failures and future prospects. *Nature reviews Drug discovery* **5**, 785-799 (2006).

198. Farady, C.J. & Craik, C.S. Mechanisms of macromolecular protease inhibitors. *Chembiochem* **11**, 2341-2346 (2010).
199. Nagase, H., Visse, R. & Murphy, G. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovascular research* **69**, 562-573 (2006).
200. Antalis, T.M. & Lawrence, D.A. Serpin mutagenesis. *Methods* **32**, 130-140 (2004).
201. Antalis, T.M., Shea-Donohue, T., Vogel, S.N., Sears, C. & Fasano, A. Mechanisms of disease: protease functions in intestinal mucosal pathobiology. *Nat Clin Pract Gastroenterol Hepatol* **4**, 393-402 (2007).
202. Knecht, W. et al. Trypsin IV or mesotrypsin and p23 cleave protease-activated receptors 1 and 2 to induce inflammation and hyperalgesia. *Journal of Biological Chemistry* **282**, 26089-26100 (2007).
203. Rolland-Fourcade, C. et al. Epithelial expression and function of trypsin-3 in irritable bowel syndrome. *Gut*, gutjnl-2016-312094 (2017).
204. Padrines, M., Wolf, M., Walz, A. & Baggiolini, M. Interleukin-8 processing by neutrophil elastase, cathepsin G and proteinase-3. *FEBS letters* **352**, 231-235 (1994).
205. Heuschkel, R. et al. Imbalance of stromelysin-1 and TIMP-1 in the mucosal lesions of children with inflammatory bowel disease. *Gut* **47**, 57-62 (2000).
206. Meyer-Hoffert, U. et al. Secreted enteric antimicrobial activity localises to the mucus surface layer. *Gut* **57**, 764-771 (2008).
207. Motta, J. et al. Food-grade bacteria expressing elafin protect against inflammation and restore colon homeostasis. *Science translational medicine* **4**, 158ra144 (2012).
208. Galipeau, H.J. et al. Novel role of the serine protease inhibitor elafin in gluten-related disorders. *American journal of gastroenterology* **109**, 748-56 (2014).
209. Henriksen, P.A. The potential of neutrophil elastase inhibitors as anti-inflammatory therapies. *Current opinion in hematology* **21**, 23-28 (2014).
210. Bermúdez-Humarán, L.G. et al. Serine protease inhibitors protect better than IL-10 and TGF-β anti-inflammatory cytokines against mouse colitis when delivered by recombinant lactococci. *Microbial Cell Factories* **14**, 26 (2015).
211. Macfarlane, G., Allison, C., Gibson, S. & Cummings, J. Contribution of the microflora to proteolysis in the human large intestine. *Journal of applied bacteriology* **64**, 37-46 (1988).
212. Rzychon, M., Sabat, A., Kosowska, K., Potempa, J. & Dubin, A. Staphostatins: an expanding new group of proteinase inhibitors with a unique specificity for the regulation of staphopains, Staphylococcus spp. cysteine proteinases. *Molecular microbiology* **49**, 1051-1066 (2003).
213. Travis, J. & Potempa, J. Bacterial proteinases as targets for the development of second-generation antibiotics. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology* **1477**, 35-50 (2000).
214. Azghani, A., Gray, L. & Johnson, A. A bacterial protease perturbs the paracellular barrier function of transporting epithelial monolayers in culture. *Infect Immun* **61**, 2681-2686 (1993).

215. Azghani, A., Miller, E. & Peterson, B. Virulence factors from Pseudomonas aeruginosa increase lung epithelial permeability. *Lung* **178**, 261-269 (2000).
216. Nomura, K. et al. Pseudomonas aeruginosa elastase causes transient disruption of tight junctions and downregulation of PAR-2 in human nasal epithelial cells. *Respir Res* **15**, 15-21 (2014).
217. Grys, T.E., Siegel, M.B., Lathem, W.W. & Welch, R.A. The StcE protease contributes to intimate adherence of enterohemorrhagic *Escherichia coli* O157: H7 to host cells. *Infection and immunity* **73**, 1295-1303 (2005).
218. Sears, C.L. Enterotoxigenic *Bacteroides fragilis*: a rogue among symbiotes. *Clinical Microbiology Reviews* **22**, 349-369 (2009).
219. Wu, S., Lim, K.-C., Huang, J., Saidi, R.F. & Sears, C.L. Bacteroides fragilis enterotoxin cleaves the zonula adherens protein, E-cadherin. *Proceedings of the National Academy of Sciences* **95**, 14979-14984 (1998).
220. Thornton, R.F., Murphy, E.C., Kagawa, T.F., O'Toole, P.W. & Cooney, J.C. The effect of environmental conditions on expression of *Bacteroides fragilis* and *Bacteroides thetaiotaomicron* C10 protease genes. *BMC microbiology* **12**, 190 (2012).
221. Pruteanu, M., Hyland, N.P., Clarke, D.J., Kiely, B. & Shanahan, F. Degradation of the extracellular matrix components by bacterial‐derived metalloproteases: Implications for inflammatory bowel diseases. *Inflammatory bowel diseases* **17**, 1189-1200 (2011).
222. Maharshak, N. et al. *Enterococcus faecalis* gelatinase mediates intestinal permeability via protease-activated receptor 2. *Infection and immunity* **83**, 2762-2770 (2015).
223. Park, S. & Galloway, D. Purification and characterization of LasD: a second staphylolytic proteinase produced by *Pseudomonas aeruginosa*. *Molecular microbiology* **16**, 263-270 (1995).
224. Braun, P., Bitter, W. & Tommassen, J. Activation of *Pseudomonas aeruginosa* elastase in *Pseudomonas putida* by triggering dissociation of the propeptide–enzyme complex. *Microbiology* **146**, 2565-2572 (2000).
225. Tanaka, S.-i., Koga, Y., Takano, K. & Kanaya, S. Inhibition of chymotrypsin-and subtilisin-like serine proteases with Tk-serpin from hyperthermophilic archaeon *Thermococcus kodakaraensis*. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics* **1814**, 299-307 (2011).
226. Chung, C., Ives, H., Almeda, S. & Goldberg, A. Purification from *Escherichia coli* of a periplasmic protein that is a potent inhibitor of pancreatic proteases. *Journal of Biological Chemistry* **258**, 11032-11038 (1983).
227. Massimi, I. et al. Identification of a Novel Maturation Mechanism and Restricted Substrate Specificity for the SspB Cysteine Protease of *Staphylococcus aureus*. *Journal of Biological Chemistry* **277**, 41770-41777 (2002).
228. Hiraga, K., Suzuki, T. & Oda, K. A novel double-headed proteinaceous inhibitor for metalloproteinase and serine proteinase. *Journal of Biological Chemistry* **275**, 25173-25179 (2000).

229. Wee, K.E., Yonan, C.R. & Chang, F. A new broad-spectrum protease inhibitor from the entomopathogenic bacterium *Photorhabdus luminescens*. *Microbiology* **146**, 3141-3147 (2000).
230. Turroni, F. et al. Characterization of the serpin-encoding gene of *Bifidobacterium breve* 210B. *Applied and environmental microbiology* **76**, 3206-3219 (2010).
231. Alvarez-Martin, P. et al. A two-component regulatory system controls autoregulated serpin expression in *Bifidobacterium breve* UCC2003. *Applied and Environmental Microbiology* **78**, 7032-7041 (2012).
232. Mkaouar, H. et al. Siropins, novel serine protease inhibitors from gut microbiota acting on human proteases involved in inflammatory bowel diseases. *Microbial cell factories* **15**, 201 (2016).
233. Cenac, N. et al. Role for protease activity in visceral pain in irritable bowel syndrome. *The Journal of Clinical Investigation* **117**, 636-647 (2007).
234. Von Lampe, B., Barthel, B., Coupland, S., Riecken, E. & Rosewicz, S. Differential expression of matrix metalloproteinases and their tissue inhibitors in colon mucosa of patients with inflammatory bowel disease. *Gut* **47**, 63-73 (2000).
235. Biancheri, P., Sabatino, A.D., Corazza, G.R. & MacDonald, T.T. Proteases and the gut barrier. *Cell Tissue Res* **351**, 269-280 (2013).
236. Pender, S.L. & MacDonald, T.T. Matrix metalloproteinases and the gut—new roles for old enzymes. *Current opinion in pharmacology* **4**, 546-550 (2004).
237. Sengupta, N. & MacDonald, T. The role of matrix metalloproteinases in stromal/epithelial interactions in the gut. *Physiology* **22**, 401-409 (2007).
238. Vergnolle, N. Clinical relevance of proteinase activated receptors (pars) in the gut. *Gut* **54**, 867-874 (2005).
239. Hyun, E., Andrade-Gordon, P., Steinhoff, M. & Vergnolle, N. Protease-activated receptor-2 activation: a major actor in intestinal inflammation. *Gut* **57**, 1222-1229 (2008).
240. Menzel, K. et al. Cathepsins B, L and D in inflammatory bowel disease macrophages and potential therapeutic effects of cathepsin inhibition in vivo. *Clinical & Experimental Immunology* **146**, 169-180 (2006).
241. Ravi, A., Garg, P. & Sitaraman, S.V. Matrix metalloproteinases in inflammatory bowel disease: boon or a bane? *Inflammatory bowel diseases* **13**, 97-107 (2007).
242. Annaházi, A. et al. Fecal MMP‐9: A new noninvasive differential diagnostic and activity marker in ulcerative colitis. *Inflammatory bowel diseases* (2012).
243. Adeyemi, E. & Hodgson, H. Faecal elastase reflects disease activity in active ulcerative colitis. *Scandinavian journal of gastroenterology* **27**, 139-142 (1992).
244. Romanos, J. et al. Improving coeliac disease risk prediction by testing non-HLA variants additional to HLA variants. *Gut* **63**, 415-22 (2014).
245. Pruteanu, M. & Shanahan, F. Digestion of epithelial tight junction proteins by the commensal Clostridium perfringens. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **305**, G740-G748 (2013).
246. Wu, S., Lim, K., Huang, J., Saidi, R. & Sears, C. Bacteroides fragilis enterotoxin cleaves the zonula adherens protein, E-cadherin. *PNAS* **95**, 14979–14984 (1998).

247. Steck, N. et al. Enterococcus faecalis Metalloprotease Compromises Epithelial Barrier and Contributes to Intestinal Inflammation. *Gastroenterology* **141**, 959-971 (2011).
248. Steck, N. et al. *Enterococcus faecalis* metalloprotease compromises epithelial barrier and contributes to intestinal inflammation. *Gastroenterology* **141**, 959-971 (2011).
249. Fouts, D.E., Torralba, M., Nelson, K.E., Brenner, D.A. & Schnabl, B. Bacterial translocation and changes in the intestinal microbiome in mouse models of liver disease. *Journal of hepatology* **56**, 1283-1292 (2012).
250. Clarke, L.L. A guide to Ussing chamber studies of mouse intestine. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **296**, G1151-G1166 (2009).
251. Karin, M., Lawrence, T. & Nizet, V. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell* **124**, 823-835 (2006).
252. Macpherson, A.J. & Smith, K. Mesenteric lymph nodes at the center of immune anatomy. *Journal of Experimental Medicine* **203**, 497-500 (2006).
253. Berg, R.D. & Garlington, A.W. Translocation of certain indigenous bacteria from the gastrointestinal tract to the mesenteric lymph nodes and other organs in a gnotobiotic mouse model. *Infection and immunity* **23**, 403-411 (1979).
254. Berg, R.D. Bacterial translocation from the gastrointestinal tract. *Trends in microbiology* **3**, 149-154 (1995).
255. Niess, J.H. et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* **307**, 254-258 (2005).
256. Medina-Contreras, O. et al. CX3CR1 regulates intestinal macrophage homeostasis, bacterial translocation, and colitogenic Th17 responses in mice. *The Journal of clinical investigation* **121**, 4787-4795 (2011).
257. Johansson, M.E., Larsson, J.M.H. & Hansson, G.C. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host–microbial interactions. *Proceedings of the national academy of sciences* **108**, 4659-4665 (2011).
258. Watanabe, O. et al. Confocal endomicroscopy in patients with ulcerative colitis. *Journal of Gastroenterology and Hepatology* **23**, S286-S290 (2008).
259. Gersemann, M. et al. Differences in goblet cell differentiation between Crohn's disease and ulcerative colitis. *Differentiation* **77**, 84-94 (2009).
260. Lindén, S.K., Florin, T.H. & McGuckin, M.A. Mucin dynamics in intestinal bacterial infection. *PloS one* **3**, e3952 (2008).
261. Bergstrom, K.S. et al. Modulation of intestinal goblet cell function during infection by an attaching and effacing bacterial pathogen. *Infection and Immunity* **76**, 796-811 (2008).
262. Voss, E. et al. NOD2/CARD15 mediates induction of the antimicrobial peptide human beta-defensin-2. *Journal of Biological Chemistry* **281**, 2005-2011 (2006).

263. Möndel, M. et al. Probiotic *E. coli* treatment mediates antimicrobial human β-defensin synthesis and fecal excretion in humans. *Mucosal immunology* **2**, 166-172 (2009).
264. Schlee, M. et al. Induction of human β-defensin 2 by the probiotic *Escherichia coli* Nissle 1917 is mediated through flagellin. *Infection and immunity* **75**, 2399-2407 (2007).
265. Wehkamp, J. et al. Human β-defensin 2 but not β-defensin 1 is expressed preferentially in colonic mucosa of inflammatory bowel disease. *European journal of gastroenterology & hepatology* **14**, 745-752 (2002).
266. Coretti, L. et al. The Interplay between Defensins and Microbiota in Crohn's Disease. *Mediators of Inflammation* **2017** (2017).
267. Langhorst, J. et al. Elevated human β-defensin-2 levels indicate an activation of the innate immune system in patients with irritable bowel syndrome. *The American journal of gastroenterology* **104**, 404-410 (2009).
268. De Palma, G. et al. Transplantation of fecal microbiota from patients with irritable bowel syndrome alters gut function and behavior in recipient mice. *Science Translational Medicine* **9**, eaaf6397 (2017).
269. Zigmond, E. & Jung, S. Intestinal macrophages: well educated exceptions from the rule. *Trends in immunology* **34**, 162-168 (2013).
270. Rothmeier, A.S. & Ruf, W. in Seminars in immunopathology 133-149 (Springer, 2012).
271. Brenchley, J.M. et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nature Medicine* **12**, 1365-1371 (2006).
272. Knutson, L., Ahrenstedt, Ö., Odlind, B. & Hallgren, R. The jejunal secretion of histamine is increased in active Crohn's disease. *Gastroenterology* **98**, 849-854 (1990).
273. Barbara, G. et al. Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. *Gastroenterology* **126**, 693-702 (2004).
274. Baum, C.A., Bhatia, P. & Miner, P.B. Increased colonic mucosal mast cells associated with severe watery diarrhea and microscopic colitis. *Digestive Diseases and Sciences* **34**, 1462-1465 (1989).
275. Weston, A.P., Biddle, W.L., Bhatia, P.S. & Miner, P.B. Terminal ileal mucosal mast cells in irritable bowel syndrome. *Digestive diseases and sciences* **38**, 1590-1595 (1993).
276. Guilarte, M. et al. Diarrhoea-predominant IBS patients show mast cell activation and hyperplasia in the jejunum. *Gut* **56**, 203-209 (2007).
277. Camilleri, M., Lasch, K. & Zhou, W. Irritable bowel syndrome: methods, mechanisms, and pathophysiology. The confluence of increased permeability, inflammation, and pain in irritable bowel syndrome. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **303**, G775-G785 (2012).
278. Spiller, R. Recent advances in understanding the role of serotonin in gastrointestinal motility in functional bowel disorders: alterations in 5‐HT

signalling and metabolism in human disease. *Neurogastroenterology & Motility* **19**, 25-31 (2007).
279. Sjöqvist, A., Cassuto, J., Jodal, M. & Lundgren, O. Actions of serotonin antagonists on cholera‐toxininduced intestinal fluid secretion. *Acta Physiologica* **145**, 229-237 (1992).
280. Lundgren, O. (Elsevier, 1998).
281. Kordasti, S., Sjövall, H., Lundgren, O. & Svensson, L. Serotonin and vasoactive intestinal peptide antagonists attenuate rotavirus diarrhoea. *Gut* **53**, 952-957 (2004).
282. Edgington-Mitchell, L.E. Pathophysiological roles of proteases in gastrointestinal disease. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **310**, G234-G239 (2016).
283. Raithel, M. et al. Release of mast cell tryptase from human colorectal mucosa in inflammatory bowel disease. *Scandinavian journal of gastroenterology* **36**, 174-179 (2001).
284. Liang, W.-J. et al. Tryptase and protease-activated receptor 2 expression levels in irritable bowel syndrome. *Gut and liver* **10**, 382-390 (2016).
285. Tooth, D. et al. Characterisation of faecal protease activity in irritable bowel syndrome with diarrhoea: origin and effect of gut transit. *Gut*, gutjnl-2012-304042 (2013).
286. Garsed, K.C. et al. Increased Fecal Serine Protease in Patients With Irritable Bowel Syndrome (IBS) and Diarrhoea. *Gastroenterology* **140**, S-525 (2011).
287. Tobisawa, Y., Imai, Y., Fukuda, M. & Kawashima, H. Sulfation of colonic mucins by N-acetylglucosamine 6-O-sulfotransferase-2 and its protective function in experimental colitis in mice. *Journal of Biological Chemistry* **285**, 6750-6760 (2010).
288. Bergstrom, K. et al. Core 1–and 3–derived O-glycans collectively maintain the colonic mucus barrier and protect against spontaneous colitis in mice. *Mucosal Immunology* **10**, 91-103 (2017).
289. Hasnain, S.Z. et al. Immune-driven alterations in mucin sulphation is an important mediator of *Trichuris muris* helminth expulsion. *PLoS pathogens* **13**, e1006218 (2017).
290. Kalle, M. et al. Host defense peptides of thrombin modulate inflammation and coagulation in endotoxin-mediated shock and Pseudomonas aeruginosa sepsis. *PLoS One* **7**, e51313 (2012).
291. Van Der Plas, M.J. et al. *Pseudomonas aeruginosa* elastase cleaves a C-terminal peptide from human thrombin that inhibits host inflammatory responses. *Nature communications* **7** (2016).
292. LaFayette, S.L. et al. Cystic fibrosis–adapted *Pseudomonas aeruginosa* quorum sensing *lasR* mutants cause hyperinflammatory responses. *Science advances* **1**, e1500199 (2015).