IMPACT OF MICROBIOTA ON INTESTINAL BARRIER FUNCTION

IMPACT OF GUT MICROBIOTA ON INTESTINAL BARRIER FUNCTION AND INTEGRITY

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy

McMaster University

DESCRIPTIVE NOTE

Doctor of Philosophy (2018) (Medical Sciences)	McMaster University, Hamilton, Ontario
TITLE	Impact of gut microbiota on intestinal barrier function and integrity
AUTHOR	Christina L. Hayes, BSc
SUPERVISOR	Dr. Elena F. Verdu
NUMBER OF PAGES	xxi, 276

LAY ABSTRACT

The epithelial lining of the intestines, called the intestinal barrier, is the first line of defence against the potentially harmful microorganisms that populate the gut, collectively termed the microbiota. The intestinal barrier must moderate microbiota-host interactions to prevent infection and inflammation, while facilitating digestion and absorption of nutrients. Improper intestinal barrier function and altered composition of the gut microbiota have both been associated with inflammation and gastrointestinal diseases. Thus, in this thesis I investigated how the microbiota affects intestinal barrier function using mouse models. I determined that acquisition of gut microbiota was vital for establishment of normal intestinal barrier function, and also found the microbiota differentially stimulated production of an antimicrobial protein called RegIII-γ, particularly the bacterium *Bifidobacterium breve* NCC2950, which was also able to reduce susceptibility to intestinal injury and promoted recovery. Such findings demonstrate that the gut microbiota shapes intestinal barrier function and integrity, and consequently, impacts health.

ABSTRACT

The intestinal barrier is responsible for digestion, absorption of nutrients and elimination of waste, while maintaining local tolerance towards the luminal microbiota and limiting antigen exposure. Immune and physiological characteristics are used to facilitate these functions and mediate intestinal immune responses in order to maintain homeostasis. Integrity and function of the intestinal barrier are critical to maintaining health, as dysfunction and aberrant barrier-microbiota interactions are evident in chronic inflammatory intestinal disorders. However, to elucidate the relative contributions of microbiota and barrier dysfunction in disease, first we need to better understand their interactions. Thus, the overall goal of my thesis is to determine whether and how the gut microbiota impacts the intestinal barrier.

In **Chapter 3** of this thesis I characterized the changes in intestinal barrier function and integrity that follow colonization of germ-free mice with human commensal microbiota. I determined that colon structure and paracellular permeability reach the physiological state within a week of colonization, resulting in resilience towards chemically induced inflammation and reduced systemic bacterial antigen exposure. In **Chapter 4**, germ-free and *in vitro* experiments were implemented to ascertain the effect of microbiota on innate immune barrier function. In collaboration with other lab members, I determined that bacteria differentially impact intestinal epithelial cell production of the antimicrobial peptide RegIII-γ, mediated by MyD88-Ticam1 signaling. Finally, in **Chapter 5** I

explored the therapeutic potential of *Bifidobacterium breve* NCC2950 and found that live *B. breve* promoted resilience towards chemically induced injury and inflammation, and recovery was promoted by live and heat inactivated formulations. Together these findings demonstrate the key role of the microbiota in establishing and modulating intestinal barrier function and integrity, providing a basis from which dysfunctional microbiota-barrier interactions can be better understood. Furthermore, these findings support the investigation of microbiota-targeted therapies to promote intestinal barrier integrity.

ACKNOWLEDGEMENTS

To my supervisor, Dr. Elena Verdu, I express great gratitude for the opportunities for learning and growth you have provided me the course of my graduate studies. I cannot thank you enough for your continuing faith and patience, your invaluable guidance and knowledge.

Jennifer Jury, your guidance, technical abilities and hard work have been integral to my achievements over the years. Heather Galipeau, you have always been willing to help and provide insightful perspectives. Beyond the lab, you both have been there through the highs and lows of research, as well as life. I am very grateful for our friendship, and will sincerely miss our morning coffe breaks.

To all the lab members and collaborators I have had the fortune of working with over the years, thank you for your advice, assistance, the laughs and memories; you have made the lab a positive, enjoyable environment.

To my supervisory committee Dr. John Wallace, Dr. Michael Surette, and Dr. David Armstrong: thank you for your invaluable counsel, constructive feedback and insights throughout my graduate studies, they are truly appreciated.

I would also like to express my profound gratitude to my friends and family for providing immeasurable love and laughter. Mom and Dad, from a young age you taught me to dream big but work hard to achieve my goals, and I am thankful for your unwaivering encouragement. Krist, I am so thankful for your understanding and compassion throughout this entire journey. Your reassurance and love have been instrumental in getting me through the insecurities, anxiety and frustrations, and I would not be where I am today without your support. Last but not least, I must thank my dearest Elsa Noelle. The fearlessness, enthusiasm and joy with which you take on the world are simply inspiring. Your beautiful, inquisitive brain dazzles me every single day, and I cannot wait to share my passion for science with you.

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CHAPTER 3

LIST OF ABBREVIATIONS AND SYMBOLS

3’	Three prime end
5’	Five prime end
^{51}Cr	Chromium-51 isotope
AB	Alcian blue
AEC	3-Amino-9-ethylcarbazole
AGU	Axenic Gnotobiotic Unit
AJ	Adherens junction
ANOVA	Analysis of variance
APC	Antigen presenting cell
ASBT	Apical sodium bile acid transporter
ASF	Altered Schaedler flora
ATCC	American Type Culture Collection
ATM	Antimicrobial
B	Bacteria
Bb	*Bifidobacterium breve*
BSA	Bovine serum albumin
CA	California
CBA	Cytometric bead assay
CCL	C-C motif ligand
CD	Cluster of differentiation
CD	Celiac disease (Appendix I only)
cDNA	Complementary deoxyribonucleic acid
CECED	Club D’Études des Cellules Epitheliales Digestives
CFU	Colony forming units
CFTR	Cystic fibrosis transmembrane conductance regulator
CIID	Chronic inflammatory intestinal disorder
cm	Centimetres
CNS	Central nervouse system
CO_2	Carbon dioxide
CT	Threshold cycle
Cu	Copper
CX_3CR1	C-X3-C motif receptor 1
Da	Daltons
DAPI	4’,6-Diamidine-2’-phenylindole dihydrochloride
DC	Dendritic cell
DL	Detection limit
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DRA	Downregulated in adenoma
DSS	Dextran sulphate sodium
DTT	Dithiothreitol
EC	Epithelial cell
EDTA	Ethylenediaminetetra-acetic acid
EM	Electron microscopy
EPLIN	Epithelium protein lost in neoplasm
FBS	Fetal bovine serum
FISH	Fluroescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3
fwd	Forward
g	Gram
GAP	Goblet cell-associated antigen passage

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GALT	Gut-associated lymphoid tissue
GF	Germ-free
GI	Gastrointestinal
h	Hour
H^+	Hydrogen ion
H+L	Heavy and light chains
HCl	Hydrochloric acid
HEPES	4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid
HI	Heat inactivated
HIP/PAP	Hepatocarcinoma-intestine-pancreas/pancreatic-associated protein
HLA	Human leukocyte antigen
HPA	Hypothalamic-pituitary-adrenal
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IEL	Intraepithelial lymphocyte
IF	Immunofluorescence
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
ILC	Innate lymphoid cell
ILF	Isolated lymphoid follicle
Ig	Immunoglobulin
Inc	Incorporated
iNK	Invariant natural killer
Isc	Short circuit current
JAM	Junctional adhesion molecule
kDa	Kilodalton
kV	Kilovolts
LB	Luria broth
LPS	Lipopolysaccharide
M	Molar (mol/L)
M cell	Microfold cell
MAMP	Microbe associated molecular pattern
MCP-1	Monocyte chemoattractant protein-1
MDP	Muramyl dipeptide
MDR-1	Multidrug resistance protein 1
MEM	Minimial essential media
mg	Milligram
MHC	Major histocompatibility complex
ml	Millilitre
MLN	Mesenteric lymph node
mm	Millimetre
mM	Millimolar
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MRS	de Man, Rogosa, and Sharpe
Mu	Mucus
Mv	Microvillus
MyD88	Myeloid differentiation primary response gene 88
Na^+	Sodium ion

NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NPC1L1	Niemann-Pick C1-like protein 1
NEMO	NFκB essential modulator
nFcR	Neonatal Fc receptor
NFκB	Nuclear factor κ B
ng	Nanogram
NHE3	Sodium-hydrogen antiporter 3
NIH	National Institutes of Health
NK	Natural killer
NLR	NOD-like receptor
nm	Nanometre
NOD	Nucleotide-binding oligomerization domain-containing protein
NOD	Non-obese diabetic (Appendix I only)
RPMI	Roswell Park Memorial Institute
PAS	Periodic acid-Schiff
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Potential difference
PEG	Polyethylene glycol
Pen-strep	Penicillin-streptomysin
pH	Potential of hydrogen
PLEK-HA7	Pleckstrin homology domain containing A7
PMN	Polymorphonuclear cell
Prof.	Professor
PRR	Pattern recognition receptor
PSA	Polysaccharide A
O_2	Dioxygen
OVA	Ovalbumin
QIIME	Quantitative Insights into Microbial Ecology
qPCR	Quantitative polymerase chain reaction
RAG1	Recombinant activating gene 1
RegIII	Regenerating islet-derived protein 3
RELMβ	Resistin-like molecule beta
rev	Reverse
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
RT-qPCR	Quantitative real-time polymerase chain reaction
SCFA	Short chain fatty acid
SCID	Severe combined immunodeficiency
SCM	Spent culture medium
SD	Standard deviation
SEAP	Secreted embryonic alkaline phosphatase
SEM	Standard error of the mean
SERT	Serotonin transporter
SFB	Segmented filamentous bacteria

sIgA	Secretory immunoglobulin A
SMCT-1	Sodium-coupled monocarboxylate transporter 1
SPF	Specific pathogen free
spp.	Species pluralis
SRA	Sequence Read Archive
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF	Transforming growth factor
T_H	Helper T cell
Ticam1	Toll interleukin-1 receptor-containing adaptor molecule 1
TJ	Tight junction
TLR	Toll-like receptor
TNBS	2,4,6-Trinitrobenzenesulfonic acid
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
Treg	Regulatory T cell
TRIF	Toll interleukin-1 receptor-containing adaptor-inducting interferon-β
UC	Ulcerative colitis
USA	United States of America
ZO	Zonula occludens
v/v	Volume per volume
w/v	Weight per volume
vs	Versus
X	Times (magnification)
α	Alpha
Å	Angstrom
β	Beta
γ	Gamma
Δ	Delta
δ	Delta
κ	Kappa
µCi	Microcurie
µg	microgram
µl	Microlitre
µm	Micrometer
µM	Micromolar
°C	Degrees Celsius
®	Registered trademark
=	Equals
<	Less than
>	Greater than
%	Percent
±	Plus/minus
$^{-/-}$	Gene knockout
~	Approximately

DECLARATION OF ACADEMIC ACHIEVEMENT

I, Christina L. Hayes, declare I am the sole author of this document. The work presented in Chapters 3 and 5 of this thesis was completed by myself, with technical support and guidance from my supervisor, co-authors and collaborators. Experiments described in Chapter 4 were performed by my colleague Jane M. M. Natividad; I was responsible for writing the manuscript.

CHAPTER 1

INTRODUCTION

1.1 The Gastrointestinal Tract

The gastrointestinal (GI) tract is the largest mucosal interface between the host and external environment. It forms the digestive system along with the accessory organs: liver, gall bladder, pancreas, salivary glands and tongue. The GI tract is uniquely designed to facilitate digestion, absorption of nutrients and elimination of the resulting waste. Following mechanical and gastric acid breakdown of food in the stomach, secretion of digestive enzymes and bile acids into the small intestine further aid in food degradation, releasing nutrients that are absorbed. Peristalsis propels luminal contents to the colon where resident microorganisms digest complex dietary fibers before remnants are expelled from the body. Digestion is a complex process requiring coordination of intestinal physiology, accessory organs, the enteric nervous system and endocrine signaling (Gribble and Reimann 2016).

The GI tract is colonized by a diverse collection of microorganisms, which are predominantly bacteria, but also includes viruses, fungi, protozoa and Archaea, collectively referred to as the *microbiota* (Hugon et al. 2017; Qin et al. 2010). Although the ratio of bacterial to host cells is estimated to be 1:1, the genomic content of the microbiota (*microbiome*) is estimated to be approximately 150 times more than the human genome (Qin et al. 2010; Sender, Fuchs, Milo 2016). Thus, the complex microbial ecosystem of the gut is diverse in terms of composition and function, and greatly influences host physiology and health. The gut microbiota with which we have coevolved a symbiotic relationship are

referred to as *commensal* microbiota, a term derived from Latin *com-* and *-mensa* meaning sharing a table. Although the term *commensal* is widely used to describe the microbiota, it implies one member benefits from the symbiotic relationship and the other is unaffected. In truth, we exist in a *mutualistic* symbiotic relationship where both members benefit: the host provides a suitable environment and nutrients for the microbiota, and in turn, the microbiota aid in extraction of nutrients and energy from our food, and produce metabolites used by the host. We exist in a relatively stable, or *homeostatic*, state with our commensal microbiota, though many microbes exhibit pathogenic potential and can invade host tissues, disrupting intestinal homeostasis. Therefore, protective immune responses must be stimulated when a threat is encountered, but a level of local immune tolerance must be exhibited towards the commensal microbiota. In order to maintain homeostatic host-microbiota interactions, the intestinal barrier of the GI tract mediates complex crosstalk between the microbiota and the immune system. Using immune and physiological properties, the intestinal barrier prevents luminal antigen invasion yet also permits absorption of nutrients and passage of fluids, electrolytes and molecules. Given the essential roles in digestion and protection from luminal antigens, integrity and function of the intestinal barrier is critical to maintaining host health.

1.2 The Intestinal Immune System

The intestinal immune system, referred to as the *gut-associated lymphoid tissue* (GALT), is vital to maintaining intestinal homeostasis and overall health.

With such a high level and range of antigen exposure encountered by the GALT, a number of protective measures are in place to limit antigen exposure and inflammation. The intestinal barrier is considered part of the GALT, acting as a physical and chemical barrier to the microbiota, as well as a key mediator between the luminal antigens and intestinal immune cells (detailed below). Based on interactions with the microbiota, the barrier can relay signals to immune cells to either promote local tolerance or stimulate inflammation. Termed *mucosally induced immune tolerance*, local intestinal immune cells can become unresponsive to bacterial antigens encountered in the colon during homeostasis (Diehl et al. 2013; Pabst and Mowat 2012). This differs from *oral tolerance*, where antigens encountered in the small intestine can generate both gut and systemic immune unresponsiveness. Reference to tolerance in this thesis will henceforth infer mucosally induced immune tolerance/unresponsiveness towards microbial antigens. Lamina propria immune cells must rapidly respond to bacterial invasion and limit dissemination beyond the GALT since the systemic immune system does not exhibit tolerance towards commensal bacteria (Konrad et al. 2006; Macpherson and Harris 2004). Therefore, the GALT must restrict systemic antigen exposure in order to maintain homeostasis.

The GALT is comprised of effector sites as well as organized lymphoid structures where antigen-specific responses are generated, called inductive sites. The epithelium and lamina propria are effector sites; mesenteric lymph nodes (MLNs), Peyer's patches, isolated lymphoid follicles and cryptopatches are

inductive sites. Isolated lymphoid follicles and cryptopatches are dispersed throughout the lamina propria, and in the small intestine, Peyer's patches are situated in the submucosa. The follicle-associated epithelium contains microfold (M) cells specialized in delivering luminal antigens to underlying antigen presenting cells, specifically, dendritic cells (DCs) and macrophages (Mabbott et al. 2013). Antigen presenting cells activate naïve T cells, generating memory cells specific to that antigen. Activated T cells can interact with B cells in the germinal centers of Peyer's patches and isolated lymphoid follicles, resulting in differentiation into antibody producing plasma cells (Macpherson et al. 2000). Lymphatic vessels convey activated T cells, B cells and migratory DCs to effector sites and MLNs where further activation and expansion of memory T cells and B cells can occur. The MLNs act as the final line of GALT defense against antigens; dissemination beyond the MLNs can result in systemic immune responses (Macpherson and Uhr 2004).

The lamina propria is seeded with a variety of innate and adaptive immune cells, including antigen presenting cells, granulocytes, macrophages, lymphocytes and innate lymphoid cells. Effector T cells, plasma cells, and intraepithelial lymphocytes (IELs) are the predominant lymphocytes in the lamina propria. Innate immune cells kill invading microbes and secrete immune mediators to inhibit or stimulate further inflammation and induce adaptive responses. In addition to innate-like functions, DCs and macrophages uptake antigens and present them to T cells and B cells. The cytokine milieu during antigen

presentation influences T cell differentiation into either effector T helper (T_H) cells, functionally classified as T_H1, T_H2 or T_H17, or regulatory cells. $Foxp3^+$ regulatory T cells, Type 1 regulatory cells and regulatory macrophages suppress effector T cells functions through IL-10 production (Coombes et al. 2007; Kayama et al. 2012; Vieira et al. 2004). Regional distribution of immune cells is evident; compared to the colon, the small intestine has a higher abundance of IELs, eosinophils, plasma cells, lamina propria DCs and T cells, specifically type 1 regulatory cells and T_H17 cells (Bowcutt et al. 2014; Suzuki 2009). Overall, the structure and cellular composition of the GALT enables regulation and compartmentalization of responses to the microbiota in order to maintain homeostasis (Figure 1).

1.3 The Intestinal Barrier

The intestinal barrier is the all-encompassing term describing the epithelium and associated elements that act as the interface between luminal contents and the host. The intestinal barrier performs a variety of essential functions, namely: digestion of disaccharides by brush border enzymes, nutrient and fluid absorption; monitoring of luminal microbiota; limiting antigen exposure; and inducing immune responses, when appropriate. Such functions are mediated through regional innate immune, structural, and permeability aspects of the intestinal barrier that reflect differing environmental challenges, roles in digestion, and immune surveillance.

1.3.1 Intestinal Barrier Structure

The intestinal barrier is comprised of a polarized, semipermeable, continuous monolayer of epithelial cells that form the physical barrier between luminal contents and the internal milieu. Intestinal epithelial cells (IECs) are organized into crypts, and in the small intestine, villi project into the lumen to increase the absorptive surface area. Multipotent stem cells at the crypt base proliferate and give rise to absorptive enterocytes or specialized cells, which migrate up the villus/crypt axis and, upon death, are expelled into the lumen. Polarity of the epithelium is maintained by apical cellular junction complexes. Cellular adhesion complexes include desmosomes and gap junctions, which respectively, provide cell-cell adhesion and passage of ions, small molecules and electrical signals between adjacent cells.

Differentiation into diverse IEC lineages enables the barrier to perform its absorptive, microbial surveillance, antigen exclusion and immune stimulatory functions. Enterocytes comprise approximately 80% of IECs, and contribute to the numerous barrier functions by expressing enzymes to aid with digestion, uptaking fluid and nutrients, secreting antimicrobial factors, and producing immune mediators. In addition to antigen sampling by M cells as described above, a number of secretory IEC lineages perform specific barrier functions: enteroendocrine cells produce hormones; goblet cells secrete mucins and barrier promoting factors; Paneth cells produce antimicrobial peptides and growth factors in the small intestine; and tuft cells stimulate immune responses towards parasites

(Gerbe et al. 2016). In the steady state, epithelial turnover is completed every 3-5 days in the small intestine, and within 5-7 days in the colon. Paneth cells, however, are an exception, found only at the crypt base in the small intestine and are renewed every 3-6 weeks. Enteroendocrine cells in distal intestinal regions also have slow turnover, with evidence suggesting a lifespan over 60 days (Bohorquez et al. 2015). IELs and intraepithelial innate lymphoid cells are able to incorporate themselves into the epithelium and perform important functions for maintenance of barrier integrity, and therefore are considered a part of the intestinal barrier. Thus, it is through diverse cell lineages that the barrier facilitates its many functions.

In addition to the epithelium, the mucus layer aids in exclusion of luminal contents, acting as a physical barrier as well as a medium for secreted antimicrobial factors. In the small intestine, the mucus layer is loose and discontinuous, whereas in the colon it is more uniform and organized into distinct layers (Ermund et al. 2013). Such differential mucus structure is believed to ensure bioavailability of nutrients in the small intestine while still obstructing bacteria. In the colon, transmembrane mucins -3, -12 and -17 expressed on the apical surface of enterocytes form a dense coating approximately 1μm thick, called the *glycocalyx*. Secreted mucins form the dense inner mucus layer of the colon, which extends 50μm from the apical surface in mice, whereas the outer layer is looser and approximately 100μm thick (Johansson et al. 2008). While both mucus layers are primarily composed of mucin-2 secreted by goblet cells, the

mucin-2 of the inner layer is cleaved by proteases, expanding the volume, forming the looser outer layer (Johansson et al. 2008). In the steady state, the inner mucus layer of the colon is devoid of bacteria, aided by secretory IgA (sIgA) and antimicrobial peptides (discussed below), as well as a fast turnover rate of 1-2 hours, but bacteria are able to colonize the outer layer (Johansson et al. 2008; Johansson 2012). Goblet cells also secrete trefoil factors and resistin-like molecule-β (RELMβ), which exert a number of barrier promoting effects. Specifically, trefoil factors have been shown to inhibit IEC apoptosis, protect IECs from damage and increased permeability, as well as promote barrier restitution (Mashimo et al. 1996; Sun et al. 2014; Taupin, Kinoshita, Podolsky 2000). RELMβ aids with exclusion of nematodes and Gram-negative bacteria, and can induce T_H2 responses (Artis et al. 2004; Propheter et al. 2017).

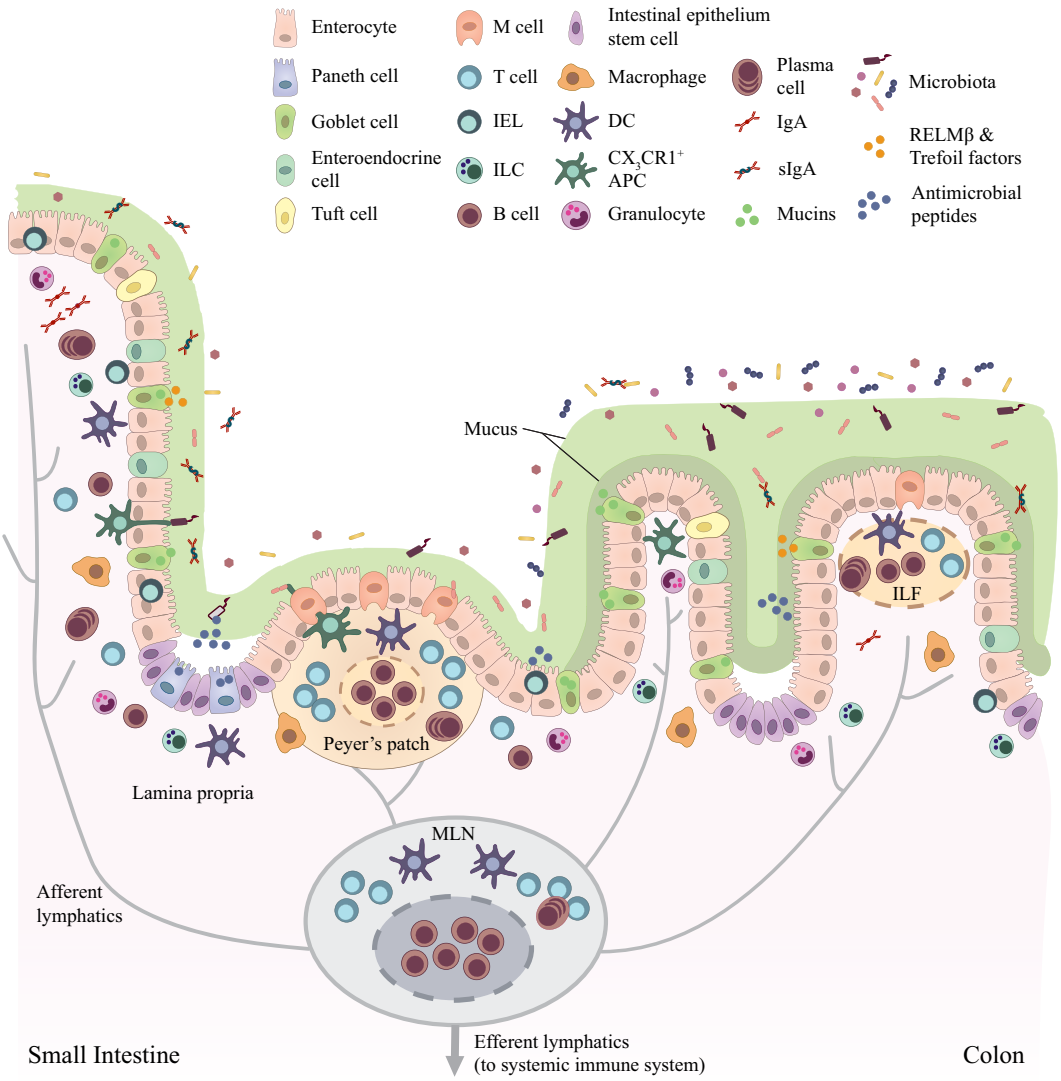

Figure 1. Overview of the mucosal immune system and barrier structure.
The intestinal immune system consists of effector sites (intestinal barrier and lamina propria) and inductive sites (Peyer's patches in the small intestine, isolated lymphoid follicles (ILF), mesenteric lymph nodes (MLN)). The intestinal barrier is comprised of absorptive enterocytes, specialized secretory cells and intraepithelial lymphocytes (IELs) that create a physical and chemical barrier to luminal antigens. The barrier interacts with diverse populations of innate and adaptive immune cells in the lamina propria. Migratory immune cells can reach MLNs and systemic immunity using afferent and efferent lymphatics, respectively. APC, antigen presenting cell; DC, dendritic cell; ILC, innate lymphoid cell; sIgA, secretory IgA; RELMβ, resistin-like molecule β.

Enteroendocrine cells release hormones and peptides that act as neurotransmitters, paracrine factors and circulating hormones to orchestrate intestinal motility, secretion of digestive juices, glucose and lipid metabolism, as well as appetite in response to food-related stimuli. Apical expression of transporters, channels and G-protein coupled receptors facilitates surveillance of local luminal metabolites, stimulating hormone release only when appropriate (Gribble and Reimann 2016). Regional localization of enteroendocrine cell subtypes further helps coordinate digestion across the GI tract. Hormone products of enteroendocrine cells can also alter intestinal permeability and immune responses; for example, serotonin decreases intestinal permeability in healthy individuals, but can promote inflammation in experimental colitis (Ghia et al. 2009; Keszthelyi et al. 2014).

1.3.2 Innate Immune Functions of the Intestinal Barrier

In addition to using physical barriers to exclude luminal contents from the epithelium, IECs also secrete antimicrobial and immune factors into the intestinal lumen. Intrinsic innate immune functions of the intestinal barrier are also utilized to monitor the luminal environment and induce appropriate immune and physiological responses in order to maintain homeostasis.

1.3.2.1 Pattern Recognition Receptors

Surveillance and immune induction rely on pattern recognition receptors (PRRs) that detect molecular motifs called microbe-associated molecular patterns

(MAMPS) conserved across a variety of microorganisms. PRRs expressed by IECs include nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), toll-like receptors (TLRs), DNA cytosolic sensors, C-type lectins, and RIG-I-like receptors that detect microbial nucleic acids, cell membrane and wall components. Upon activation of PRRs, intracellular signaling pathways are activated, stimulating production of immune mediators and, ultimately, induction of immune responses and changes in intestinal barrier function. Differential expression of PRRs ensures inflammation is stimulated only when a MAMP is encountered where it threatens the host; thus, PRRs have differing regional and IEC lineage expression, as well as specific intracellular or membrane localization (Abreu 2010). As an example, TLR9 can be expressed on IEC apical, basolateral and endosome membranes in the colon, but in the small intestine is only expressed on the apical surface of follicle-associated epithelial cells (Abreu 2010). In addition to differential expression, PRR localization affects the response upon activation. For instance, activation of basolaterally expressed TLR9 leads to an inflammatory response, but apical activation does not, and can even inhibit subsequent responses to activation of basolateral TLR9 and other TLRs (Lee et al. 2006). Furthermore, PRR redundancy enhances MAMP detection and prevents microbe subversion; a single PRR can recognize MAMPs from multiple sources, and multiple PRRs can to detect the same MAMP (Sellge and Kufer 2015). For instance, TLR5 and NLRC4 both recognize bacterial flagellin, but detect distinct

epitopes, and NLRC4 is also activated by a structural protein of the bacterial type III secretion system (Miao et al. 2007).

1.3.2.2 Secretion of Antimicrobial Immune Factors

In addition to physically excluding luminal contents, the intestinal barrier also forms a biochemical barrier through secretion of antimicrobial peptides and sIgA into the mucus. Antimicrobial peptides expressed in the intestines include defensins, cathelicidins, C-type lectins, lysozyme, phospholipase A2, resistins, as well as a chemokine and other select peptides (Table 1). MAMP activation of PRRs in IECs and IELs stimulates the expression of antimicrobial peptides in the steady state, which prevents infiltration of the mucus (Ismail et al. 2011; Muniz, Knosp, Yeretssian 2012). Antimicrobial peptides target conserved outer structural components essential to microbial survival, allowing for broad specificity and limited microbial subversion. The cationic, hydrophobic characteristics of many antimicrobial peptides facilitates binding to negatively charged bacterial membranes, neutralizing their ability to activate PRRs, and in many cases, cause membrane disruption resulting in bacterial lysis (Zhang and Gallo 2016). Similar to PRRs, antimicrobial peptide production can be specific to the cell lineage, intestinal region, and localization within the villus-crypt axis (Jager, Stange, Wehkamp 2010; Muniz et al. 2012).

Table 1. Intestinal antimicrobial peptides.

Antimicrobial Family	Intestinal Antimicrobials	Expression	Mechanism of Action
Defensins	α-defensin -5 & -6 (humans) cryptdins (mice)	Paneth cells (C)	Electrostatic membrane disruption
	β-defensin 1	Enterocytes (C) Paneth cells (C)	
	β-defensin -2, -3 & -4	Colonocytes (I) Paneth cells (C)	
Cathelicidins	LL37 (humans) Cathelicidin-related antimicrobial peptide (mice)	Upper crypt coloncytes (C)	
C-type lectins	RegIII-α, -β, -γ,	Colonocytes (C) Paneth cells (C) IELs (C)	Membrane disruption
Lysozyme	Lysozyme C	Paneth cells (C)	Enzymatic membrane disruption
Phospholipase A2	Secretory phospholipase A2	Paneth cells (C)	
RNase	Angiogenin 4	Paneth cells (C)	Membrane disruption*
Resistins	Resistin (humans) RELM-β (mice)	Goblet cells (C)	Bacterial membrane disruption; bind nematode chemosensory apparatus
Chemokine	CCL20	Enterocytes (I)	Electrostatic membrane disruption
Other Peptides	Bactericidal/ permeability-increasing protein	Enterocytes (C)	Bind & neutralize LPS
	Elafin	Enterocytes (C)	Membrane disruption*
	Secreted leukocyte protease inhibitor	Enterocytes (C)	Membrane disruption*

*mechanism inferred based on structure
(C) constitutive expression; (I) induced expression during inflammation; RELM, resistin-like molecule; LPS, lipopolysaccharide.

In addition to antimicrobial peptide secretion, IECs also facilitate the secretion of immunoglobulins into the intestinal lumen, an essential function for limiting bacterial translocation to the GALT (Macpherson and Uhr 2004). Dimeric IgA, and to a lesser extent, pentameric IgM produced by plasma cells in the lamina propria are secreted (Magri et al. 2017). IECs express polymeric immunoglobulin receptors on the basolateral membrane where it binds to IgA. The receptor-IgA complex passes through the IEC and IgA is released into the lumen with the secretory component attached to inhibit degradation. Secretory IgA inhibits immune induction by MAMPs, called *immune exclusion*, by binding to microbes in the lumen, mucus, lamina propria, or during intracellular passage. Moreover, the secretory component alone can neutralize bacterial antigens (Perrier, Sprenger, Corthesy 2006).

1.3.2.3 Intraepithelial Lymphocytes

IELs play an integral role in maintaining barrier integrity and homeostasis through interactions with IECs and immune cells, in addition to antimicrobial activities. The population of IELs is heterogeneous, with differing T cell receptor (TCR) and co-receptor expression, cellular origins, and regional localization. Natural/unconventional/type b IELs are present at birth, decrease with age, can express TCRαβ or TCRγδ, and are either $CD8\alpha\alpha^-$ or $CD8\alpha\alpha^+$ (Cheroutre, Lambolez, Mucida 2011). TCR^- subsets of natural IELs that can express CD8αα or intracellular CD3 have been described, as well as TCR^- IELs with innate

lymphoid cell characteristics (Olivares-Villagomez and Van Kaer 2017). Natural IELs are believed to acquire an activated phenotype through self-antigen exposure during thymic development, and directly migrate to the intestinal epithelium. Upon activation by antigen, conventional $CD4^+$ or $CD8\alpha\beta^+$ $TCR\alpha\beta^+$ T cells migrate to the GALT and differentiate to induced/conventional/type a IELs, the abundance of which increases with age. IEL abundance is higher in the small intestine compared to the colon, with an estimated ratio of 1 IEL per 10 IECs, and a predominance of $TCR\gamma\delta^+$ cells; in the colon, $TCR\alpha\beta^+CD4^+$ IELs are the prevalent subset (Beagley et al. 1995). It should be noted that differences in IEL populations have also been identified between mice and humans. Humans harbour less $TCR\gamma\delta^+$ IELs and more $TCR\alpha\beta^+CD8\alpha\beta^+$ IELs than mice, and $TCR\alpha\beta^+CD8\alpha\alpha^+$ IELs are not commonly found (Olivares-Villagomez and Van Kaer 2017). In mice, innate lymphoid cell type 3-like and intracellular $CD3^+$ IELs have not been described, but mice harbour innate lymphoid cell type 1-like IELs co-expressing natural killer cell-associated marker NK1.1 that have not been identified in humans (Olivares-Villagomez and Van Kaer 2017).

High mobility of IELs allows for extensive barrier monitoring and rapid responses towards antigens. Expression of junctional proteins allows IELs to insert themselves between adjacent IECs without disrupting epithelium continuity (cellular junctions are described below). Although classified as innate cells, the functional characteristics of IELs are diverse, as they exhibit rapid innate and adaptive, effector and regulatory immune functions that do not require priming.

IELs can limit pathogen infiltration by secreting antimicrobial peptides and effector cytokines, such as RegIII-γ and IFNγ, and cytotoxic activities eliminate infected IECs (Ismail, Behrendt, Hooper 2009; Nielsen, Witherden, Havran 2017). IELs can also limit barrier damage by suppressing T cell effector responses and secreting keratinocyte growth factor and IL-6 to promote barrier integrity (Chen et al. 2002; Kober et al. 2014; Kuhn et al. 2017). Therefore, IELs play a vital role in maintaining barrier function and regulating immune responses.

1.3.2.4 IEC Crosstalk with Lamina Propria Immune Cells

Production of immune mediators by IECs influences local tolerance towards the commensal bacteria and immune responses. Secretion of thymic stromal lymphopoietin and retinoic acid by IECs inhibits mast cell activation, and conditions lamina propria DCs to favour regulatory T cell differentiation (Rimoldi et al. 2005). Production of B-cell-activating factor and a proliferation-inducing ligand by IECs promotes class switching and B cell IgA production. IECs can also process antigens and release exosomes containing MHC II-antigen complexes that also promote tolerogenic DC-mediated T cell activation (Karlsson et al. 2001; Ostman, Taube, Telemo 2005). The intestinal barrier also alters its function in response to signals from immune cells. For instance, IL-22 and IL-17 produced by type 3 innate lymphoid cells and T_H17 cells promotes integrity of cellular junction complexes, antimicrobial peptide and mucin production, and inhibits bacterial translocation in the steady state (Duffin et al. 2016; Liang et al. 2006).

1.3.3 Intestinal Permeability

The intestinal barrier must prevent invasion by microbes and harmful antigens, yet still permit passage of fluids, electrolytes and nutrients, as well as monitor the microbiota to maintain tolerance (Pabst and Mowat 2012). Thus, the barrier must carefully regulate permeability in response to environmental and physiological signals. Intestinal permeability is mediated through a number of pathways: paracellular passage between adjacent IECs, the transcellular pathway through IECs, and direct sampling of the lumen by immune cells.

1.3.3.1 Paracellular Permeability

The paracellular pathway facilitates passive flow of ions, water and hydrophilic molecules across the epithelium according to their concentration gradient. Paracellular permeability is mediated by cellular junction complexes that connect adjacent cells. Tight junctions (TJs) are the most apically localized cell junction and are composed of membrane-spanning proteins and cytosolic adaptor proteins. Transmembrane occludin, tricellulin, claudin and junctional adhesion molecule proteins of adjacent cells bind together to form pores with their extracellular loops through which passage occurs, and are anchored to actin filaments of the cytoskeleton by zonula occludens (ZO) proteins (Figure 2). Adherens junctions (AJ) are also important cellular adhesion complexes. Located below TJs, AJ maintain physical connections between adjacent IECs; thus, diminished AJ integrity leads to separation of cells and increased paracellular

permeability. Transcellular E-cadherin proteins link adjacent cells and are bound to cytosolic α- and β-catenin proteins (Figure 2). The entire E-cadherin-α-catenin-β-catenin complex is anchored to the cytoskeleton by epithelial protein lost in neoplasm (EPLIN) (Abe and Takeichi 2008). It has also been suggested that AJ also the microtubules network; the cytoplasmic domain of E-cadherin also binds p120 catenin, which interacts with microtubules through Pleckstrin homology domain containing A7 (PLEKHA7) and Nezha proteins (Meng and Takeichi 2009).

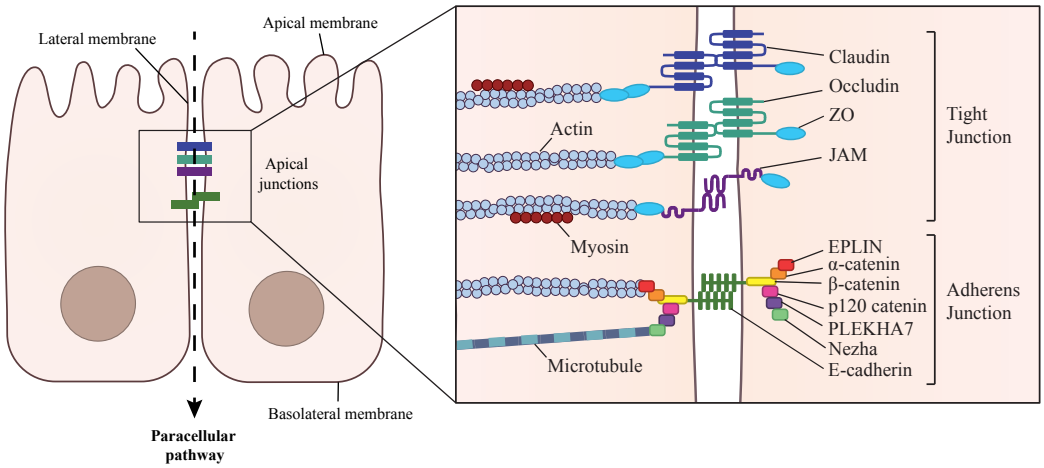

Figure 2. Apical cell junctions of the intestinal epithelium.
Tight junctions (TJ) and adherens junctions (AJ) connect adjacent intestinal epithelial cells and regulate paracellular permeability. TJs consist of transmembrane claudin, occludin and junctional adhesion molecule (JAM) proteins that are anchored to the actinomyosin cytoskeleton by adaptor ZO proteins. AJs consist of E-cadherin transmembrane proteins anchored to the cytoskeleton by the α-cadherin-β-cadherin-EPLIN complex, or the microtubule network by the p120 catenin-PLEKHA7-Nezha complex.

Paracellular permeability is regulated through changes in TJ protein expression and localization in response to a variety of host and environmental stimuli (summarized in Table 2). Size selectivity of paracellular passage can be mediated by the *pore* or *leak* pathways (Figure 3). Claudin expression selectively determines passage of ions and small solutes via the pore pathway, where certain claudins are associated with limiting permeation, called *barrier-forming* claudins, while *pore-forming* claudins permit passage of cations or anions. Passage by the pore pathway occurs abundantly at the villus tip, and diminishes down the villus-crypt axis (Fihn, Sjoqvist, Jodal 2000). The leak pathway mediates non-specific passage of macromolecules, and is associated with increased claudin-2 expression, and decreased tricellulin expression at tricellular junctions. Passage of macromolecules in the crypts suggests preference of the leak pathway, though under homeostatic conditions, crypts are not exposed to luminal contents. Diminished TJ integrity can also increase paracellular flux through intracellular sequestration of TJ proteins in endosomes, pore dilation or ZO dissociation by cytoskeletal retraction. Regional expression of claudins along the GI tract and cell membrane also contributes to differing permeability characteristics along all axes of the intestines (Figure 4).

Table 2. Summary of factors that alter intestinal paracellular permeability.

Permeability	Factor	Mechanism
Decrease	Amino acids (glutamine, tryptophan)	Promote apical junction assembly
	Peptides (casein, cheese)	Promote TJ assembly
	Short chain fatty acids	Promote TJ assembly
	Polyphenols (quercitin, epicatechin)	Increase TJ protein levels
	Vitamin D	Promote TJ assembly
	TGF-β	Increase claudin-1 protein
	Epidermal growth factor	Promote apical junction assembly
	Serotonin	Promote ZO-1 TJ localization
	Probiotics	Promote TJ assembly & protein expression
Increase	Glucose	Decrease ZO-1 TJ localization
	Leptin	Decreased occludin protein
	High fat diet	TJ disassembly
	Acetaldehyde/ethanol	TJ disassembly
	Pro-inflammatory cytokines (IFNγ, IL-1β, IL-6, TNFα)	TJ disassembly, increase claudin-2 protein
	Proteinase-activated receptor 2	Decrease ZO-1 TJ localization
	Hydrogen peroxide	Decrease ZO-1 TJ localization
	Keratinocyte growth factor	Increase claudin-2 protein
	Pathogens & Toxins	TJ disassembly

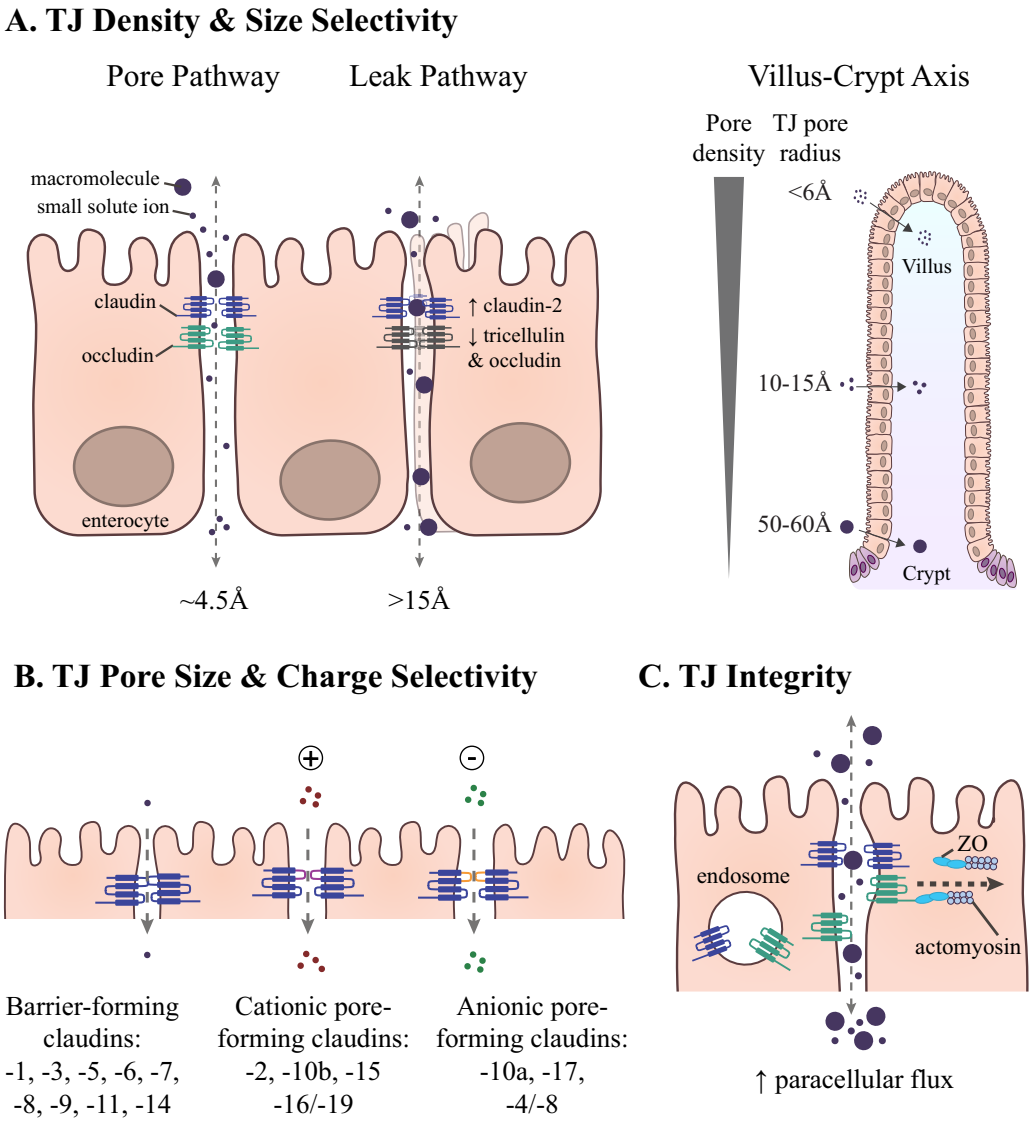

Figure 3. Mechanisms of paracellular permeability modulation.

(A) The tight junction (TJ) pore and leak pathways regulate paracellular flux of small solutes, such as ions, and macromolecules, respectively, along with changes in the density of TJ pores along the villus-crypt axis. Increased expression of claudin-2 and diminished tricellulin expression at tricellular junctions is associated with increased macromolecular flux via the leak pathway. (B) Expression of barrier-forming, cationic- and anionic-pore forming claudins further regulates size and charge selectivity of the pore pathway. (C) TJ integrity can be diminished by TJ dilation or disassembly through ZO dissociation and endosomal sequestering of TJ proteins, resulting in increased paracellular permeability.

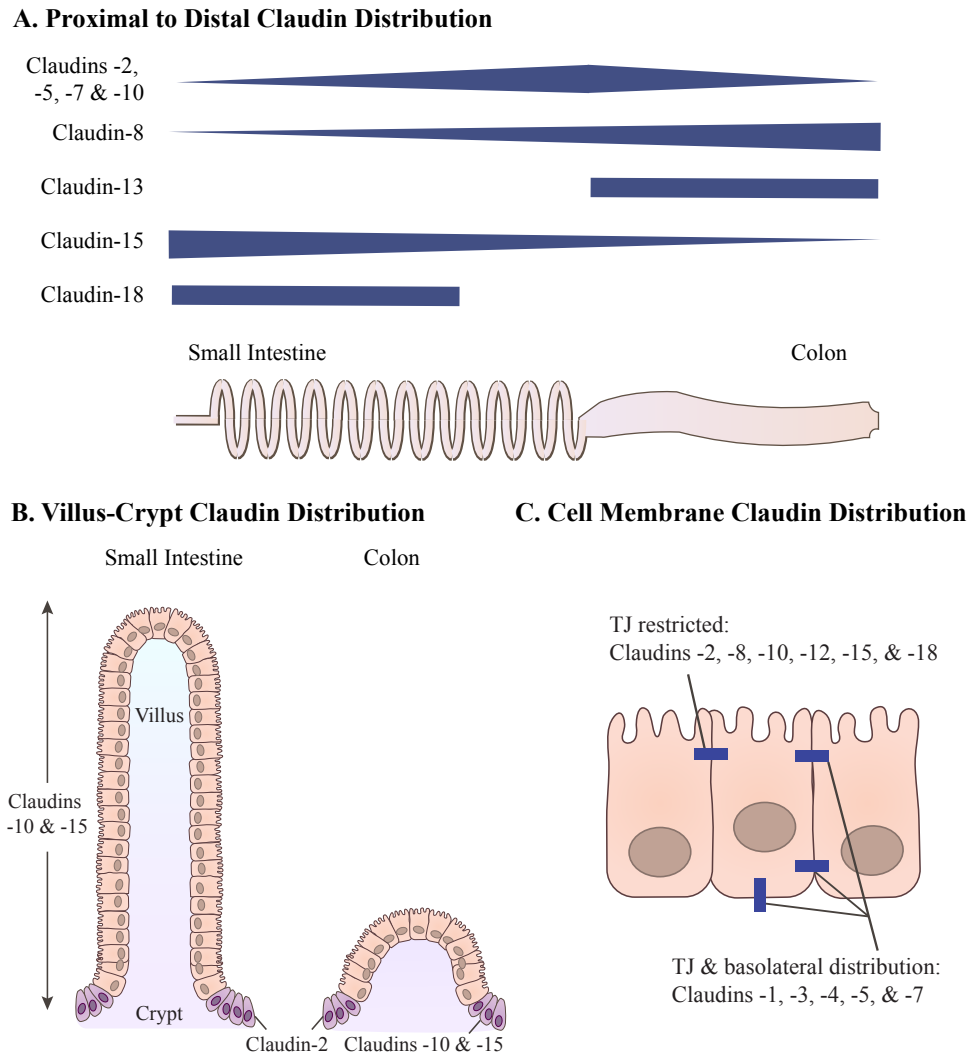

Figure 4. Intestinal distribution & localization of claudin proteins.
(A) Claudins are regionally expressed along the GI tract, (B) villus-crypt axis and (C) cell membrane.

The paracellular pathway also plays a role in immune surveillance. It is postulated the leak pathway facilitates antigen sampling for immune surveillance. Although it is debated whether these cells are DCs or macrophages, CX_3CR1^+

cells interact with TJs to extend transepithelial dendrites into the lumen and uptake antigens (Medina-Contreras et al. 2011; Niess et al. 2005; Rescigno et al. 2001). Expression of TJ proteins along the dendrite allows for barrier integrity to be maintained (Farache et al. 2013). CX_3CR1^+ cells also use the paracellular pathway for transepithelial migration in order to uptake pathogens in the lumen (Nicoletti, Arques, Bertelli 2010). Antigen-laden DCs migrate to the MLNs and stimulate IgA production (Macpherson and Uhr 2004; Niess et al. 2005). It is speculated pathogens may utilize CX_3CR1^+ cells to gain entry to host tissues, but bacterial translocation is unaffected in CX_3CR1 deficient mice, indicating alternative modes of entry are predominantly used (Nicoletti et al. 2010). However, transepithelial dendrite formation has been reported to be a rare occurrence, and antigen uptake and delivery to the MLN may be predominantly mediated by Peyer's patch and isolated lymphoid follicle derived antigen presenting cells (Macpherson and Uhr 2004; McDole et al. 2012).

1.3.3.2 Transcellular Permeability

Under steady-state conditions, the paracellular pathway of intestinal permeability is limited to small, hydrophilic molecules moving according to their concentration gradient; thus, uptake of nutrients, fluids, as well as antigens for surveillance, also occurs through the transcellular pathway. Nutrients that move transcellularly include sugars, short chain fatty acids, amino acids, vitamins, and small peptides. Transcellular passage can be facilitated through pinocytosis,

passive or facilitated diffusion, as well as active transport or receptor-mediated endocytosis (Figure 5). Water and small, hydrophobic molecules such as fatty acids and monoglycerides, can passively diffuse through the cell membrane of enterocytes. Transporters, receptors and channels are expressed in the apical membrane for facilitated diffusion and active transport of macromolecules, many of which exhibit regional specificity (Kiela and Ghishan 2016). For example, the Na^+/H^+ exchanger NHE3 is expressed throughout the small intestine and colon to facilitate water and Na^+ absorption. Conversely, expression of monocarboxylate transporter 1 is localized to the colon where it uptakes short chain fatty acids, the primary energy source of colonocytes. Basolaterally expressed transporters and channels allow nutrients to exit the cell, but most large endocytosed proteins are degraded in endosomes before being released to lamina propria antigen presenting cells (Heyman et al. 1986; Menard, Cerf-Bensussan, Heyman 2010).

Transcellular permeability is also an important mechanism of antigen delivery for surveillance of luminal contents. As discussed above, M cells in the epithelium of Peyer's patches and isolated lymphoid follicles uptake luminal antigens and release them to underlying immune cells. Such uptake can be through non-selective pinocytosis, receptor-mediated endocytosis, or transcytosis of sIgA-antigen complexes (Mabbott et al. 2013). M cells can also create transceullar pores through which DCs extend trans-M-cell dendrites to acquire luminal antigens (Lelouard et al. 2012).

Goblet cells have also been implicated in transcellular passage of antigens. In a phenomenon termed goblet cell-associated antigen passages (GAPs), goblet cells uptake and preferentially deliver luminal antigens to lamina propria CX_3CR1^- $CD103^+$ DCs in the small intestine during the steady state (McDole et al. 2012). Evidence suggests luminal antigens enter following the release of large quantities of mucin granules (Hirose et al. 2016). Uptake of beads 0.2-1.0μm by GAPs does not occur, and although 70 kDa dextran was detectable in goblet cells, it was not delivered to DCs; together, these findings suggest GAPs are restricted to passing small molecules (McDole et al. 2012). GAPs are not observed in the steady state colon unless TLR-mediated microbial sensing is disrupted, indicating regional specificity (Knoop et al. 2016). Collectively, these mechanisms of transcellular permeability allow for uptake and systemic dissemination of nutrients, delivery of antigens to immune cells, as well as maintenance of electrolyte and fluid balances.

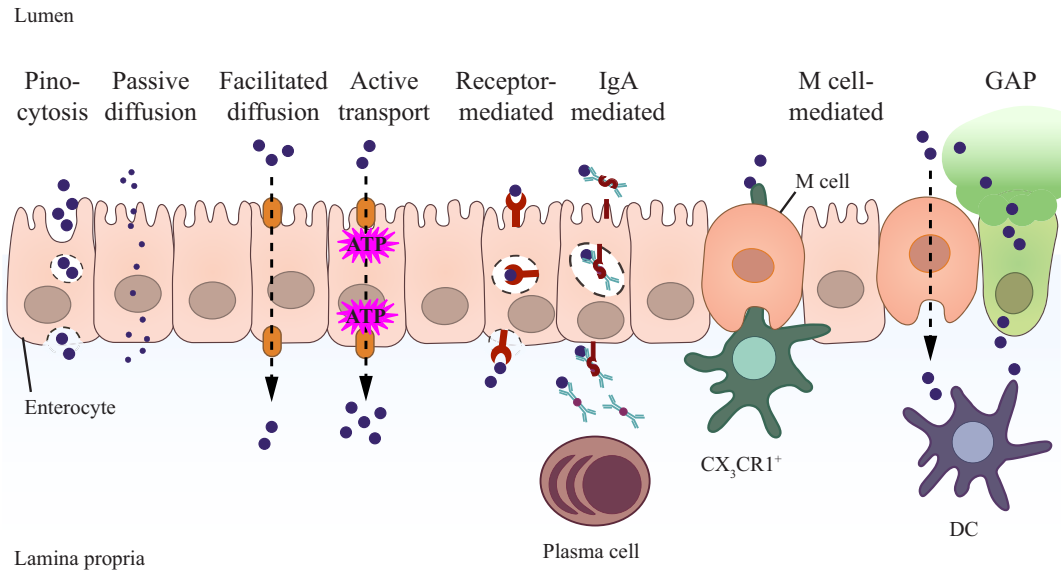

Figure 5. Transcellular permeability.
Intestinal transcellular transport can be mediated by: pinocytosis of luminal contents; passive diffusion of small, lipid soluble molecules; facilitated diffusion through channels or transporters; active transport through transporters, which requires energy (adenosine triphosphate, ATP); receptor or IgA-mediated binding; sampling of luminal contents by M cells or CX_3CR1^+ cells through M cell pores; or delivery of antigens to lamina propria dendritic cells (DC) through goblet cell-associated antigen passages (GAPs).

1.3.3.3 Techniques for Evaluation of Intestinal Permeability

Given the vital role of intestinal permeability in regulating antigen exposure, changes in permeability can be an indicator of overall barrier integrity and host health. A number of techniques are available to study intestinal permeability to probes *in vivo*, *ex vivo* using fresh tissues, or *in vitro* using cell lines (Table 3). Fundamentally, these techniques involve measuring flux of one or more probes across the intestinal barrier. Measurement of permeability *in vivo* allows for evaluation with inclusion of the complex microbial and host

physiological dynamics; however, intestinal motility and fluid retention can impact probe retention time, bacterial overgrowth can lead to probe degradation, and inflammation can alter villus surface area and TJ-mediated paracellular size exclusion properties. Thus, since *in vivo* evaluation of intestinal permeability can present a number of challenges, alternative assessment methods can be preferable. The *ex vivo* Ussing chambers technique has proven to be a valuable and versatile tool in evaluation of intestinal permeability (Figure 6) (Clarke 2009).

Table 3. Common experimental techniques to evaluate intestinal permeability.

Test	Site Assessed	Probe(s) & Measurement	Advantages (•) & Limitations (o)
in vivo Permeability			
Administration of sugars or PEGs of differing molecular weights	Small intestine, stomach	Lactulose/mannitol with or without sucrose in urine/blood	• Non-invasive o Physiological confounding factors o Not site-specific
	Colon	Lactulose/mannitol with sucralose in urine/blood	
Administration of a single, inert probe	Small intestine & colon	^{51}Cr-EDTA, OVA, FITC-dextran in urine or blood	• Non-invasive • OVA models protein antigen permeability o Not site-specific o ^{51}Cr-EDTA is radioactive
ex vivo/in situ Permeability			
Ussing chambers	Site-specific	Ions, PEG, sugars, inulin, ^{51}Cr-EDTA, OVA, FITC-dextran, HRP, bacteria, drugs in tissue	• Mechanistic studies • Differentiation of permeability pathways & ion transport o Invasive o Limited tissue viability

Intestinal loop	Site-specific	Ions, PEG, sugars, ^{51}Cr-EDTA, OVA, FITC-dextran, HRP, bacteria, drugs in blood or extraintestinal sites	• Mechanistic studies • Differentiation of permeability pathways • Complete host physiological systems o Invasive
in vitro Permeability			
Cell culture	Intestinal epithelial cell monolayers	Transepithelial resistance, PEG, sugars, inulin, ^{51}Cr-EDTA, OVA, FITC-dextran, HRP, bacteria, drugs in media	• Controlled environment o Lacks host complexity
Barrier Integrity Biomarkers			
Bacterial translocation	Intestine	Live bacteria in lymph nodes, liver, spleen, and/or blood	• Complete host physiological systems o Invasive o Culture conditions will determine amount and type of bacteria detected o Not site-specific
Bacterial antigen(s) or specific antibodies	Intestine	Lipopolysaccharide, muramyl dipeptide in blood	• Non-invasive o Indirect evidence of altered permeability or acute damage o Not site-specific
Morphology	Site-specific	Apical junction protein expression & localization, electron microscopy of tissue	• Integrity of barrier and apical junction structure can be evaluated o Invasive

EDTA, Ethylenediaminetetraacetic acid; EM, electron microscopy; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; OVA, ovalbumin; PEG, polyethylene glycol.

Adapted from (Galipeau and Verdu 2016)

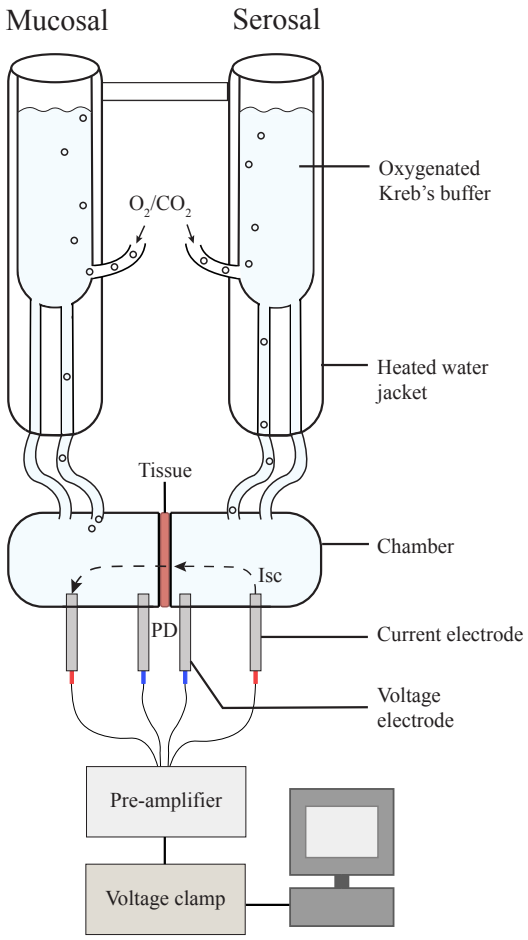

Figure 6. Ussing chambers.

The two halves of the Ussing chamber are separated by the tissue sample, where the mucosal surface is exposed to one side of the chamber and the serosal side to the other half. Water jackets warm separate reservoirs of oxygenated, isotonic physiological buffer. Voltage electrodes measure the potential difference (PD) across the tissue. Using the preamplifier and voltage clamp, current electrodes apply a short circuit current (Isc), a current equal and opposite to that generated by net ion transport across the tissue, to clamp the voltage (PD=0). Paracellular and transcellular permeability can be determined by administering inert probes, such as ^{51}Cr-EDTA and horseradish peroxidase, respectively, to the mucosal side of the chamber and measuring the amount permeated to the serosal side.

1.4 Intestinal Microbiota

Humans have coevolved a complex, mutualistic relationship with the intestinal microbiota, where the host provides a nutrient rich environment for the microbiota, and in turn, they aid with digestion, produce essential metabolites, protect from pathogens, enhance barrier function and stimulate immune maturation. Development of cost effective, high-throughput sequencing methods has allowed for comprehensive characterization of the microbiota composition, structure, and elucidation of effects on host physiology. Sequencing the highly variable regions of the 16S rRNA gene allows for identification of bacterial and Archaea taxa. Due to ease of sample collection and analysis, many studies focus on the fecal bacterial community; however, density and diversity are different between regions of the GI tract, a reflection of the differing environmental and host conditions (Stearns et al. 2011). The taxonomic structure of the microbiota is further influenced by: initial and successive colonizers, developmental stage, diet, intestinal barrier and immune function, host lifestyle, as well as geographic location (Rodriguez et al. 2015).

1.4.1 Microbiota Acquisition and Succession

The long-held belief that microbiota is acquired at birth has been contested; viruses can be transmitted through placental blood exchange, and growing evidence suggests controlled bacterial exposure begins *in utero* prior to delivery through placental transfer of maternal microbiota (Middelkamp, Reed,

Patrizi 1967; R. W. Walker et al. 2017). However, this exposure may relate to bacterial products, rather than live cells. Older studies report few cases of bacteria cultured from meconium, and many contemporary studies rely solely on molecular techniques for detection (Perez-Munoz et al. 2017). Thus, currently it is unclear when initial colonization occurs, and the impact of *in utero* microbial exposure on fetal development remains largely unknown. During birth, the maternal vaginal microbiota is vertically transferred to infants, resulting in dominance of *Lactobacillus* and *Prevotella* in feces (Dominguez-Bello et al. 2010). Within a week, these initial colonizers then give way to anaerobes such as *Bacteroides*, *Clostridium* and dominant *Bifidobacterium* spp. (C. J. Hill et al. 2017). Over the first year of life, fecal microbiota diversity increases, and by 2-3 years of age, resembles the adult microbiota in terms of composition, structure, diversity and function (Koenig et al. 2011; Yatsunenko et al. 2012). Microbiota acquisition and succession can be affected, however, by birth mode, diet, antibiotic exposure and other environmental factors, which may have long-term consequences on immune development and health (Gensollen et al. 2016). The adult fecal microbiota is primarily composed of Firmicutes and Bacteroidetes phyla, with Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia detectable; Clostridiaceae, Ruminococcaceae, Lachnospiraceae and Bacteroidaceae are the predominant families (Eckburg et al. 2005; Hugon et al. 2015). In the absence of significant diet, lifestyle and health changes, the adult core microbiota remains relatively stable. Although inter-individual microbiota

composition is variable, it is proposed that function of the microbiota may be comparable between communities (Qin et al. 2010). The elderly exhibit shifts in microbiota structure, with increased *Bacteroides* spp., changes in Clostridiaceae distribution, and an overall decrease in diversity (Claesson et al. 2011). Such elderly microbiota changes are associated with altered diet, fecal metabolic profiles, increased inflammatory markers and declining health, suggesting microbiota-diet interactions affect health status of the elderly (Claesson et al. 2012).

1.4.2 Regional Differences in Microbiota Composition and Density

Differing functions and physiology between regions of the GI tract impacts microbiota composition. Bacterial diversity and density both increase from the proximal to distal gut as well as along the tissue-lumen axis (Figure 7). The high levels of oxygen, antimicrobials and nutrients, low pH, and rapid transit time of the small intestine provides a habitat that restricts colonization to facultative anaerobes that favour simple sugar and amino acid metabolism and can grow rapidly on the mucus or epithelium. As such, Lactobacilliales, Proteobacteria, Bacteroidia and Clostridia have been reported as the predominant taxa in the small intestine of humans and mice, with Clostridia being enriched in the mucus (Gu et al. 2013; Jakobsson et al. 2015). In the colon, antimicrobial peptides are less abundant, transit time is slower, complex carbohydrates resistant to host digestion are available, and an oxygen gradient diffusing from the barrier

into the lumen occurs, all of which contribute to shaping the microbiota. The colon and fecal microbiota is predominantly made up of anaerobes capable of metabolizing complex carbohydrates, particularly Lachnospiraceae, Ruminoccocaceae, Bacteroidaceae, Prevotellaceae, and Rikenellaceae (Gu et al. 2013). However, taxonomic differences exist between areas of colonic structure. Enrichment of Ruminoccocaceae and Lachnospiraceae in the transverse folds of the proximal colon has been described in mice (Nava, Friedrichsen, Stappenbeck 2011). Select bacteria are able to colonize the outer mucus layer, such as *Akkermansia muciniphila*, and evidence also suggests *Acinetobacter* spp. colonize the crypts in the steady state (Pedron et al. 2012; Png et al. 2010).

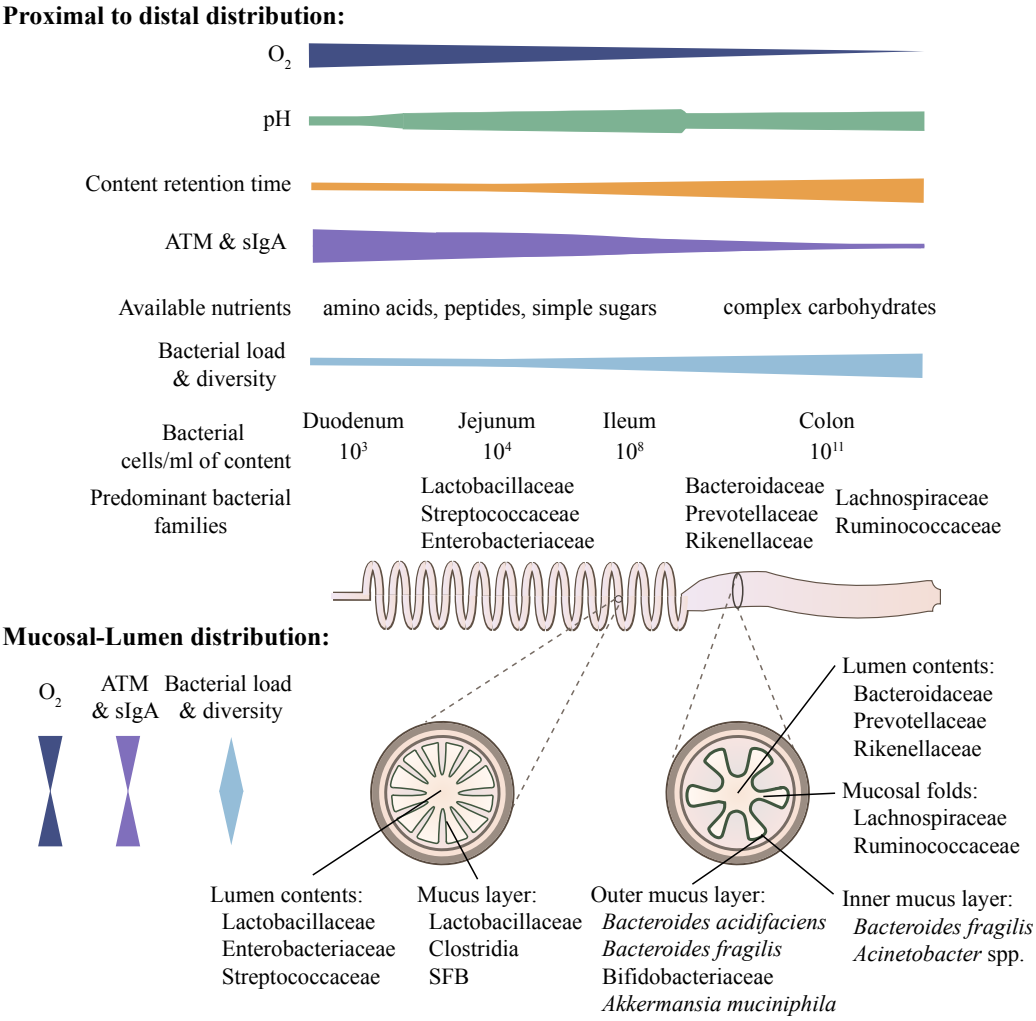

Figure 7. Intestinal regional environment characteristics and bacterial distribution.

Luminal oxygen levels, pH, antimicrobial (ATM) peptides, secretory IgA (sIgA), type of nutrients available, as well as bacterial load, diversity and composition along the intestinal tract and mucosal-lumen axis.

1.5 Microbiota-Host Interactions

The microbiota and intestinal barrier have coevolved complementary functions to maintain a mutualistic relationship. Indeed, the gut microbiota is a significant mediator of host health, influencing a number of host physiological systems, including the intestinal barrier, gut-brain-axis, as well as immune and metabolic functions. The fundamental role of the microbiota in intestinal barrier and immune development is detailed in **Appendix I**. A key tool in deciphering the impact of the microbiota on host physiology is the gnotobiotic, or germ-free, mouse model. Germ-free mice do not harbour live bacteria and are raised under strict sterile conditions to prevent contamination. Comparison to conventional counterparts can reveal physiological features affected by the microbiota. For instance, germ-free mice have altered intestinal morphology, motility and an immature immune system compared to conventionally raised specific pathogen free mice (Smith, McCoy, Macpherson 2007). The effect of specific microbes on host physiology can be elucidated by colonization with select bacteria or microbial communities, and can be used to gain insight into the establishment and mechanisms behind microbiota-driven changes. Thus, the germ-free mouse model is a valuable tool for studying host-microbiota interactions.

1.5.1 Aberrant Microbiota-Barrier Interactions

The homeostatic state of the gut relies on complex interactions between the intestinal barrier, immune system, microbiota and the luminal environment.

Thus, host factors such as genetic predisposition, as well as environmental changes, including infection, anxiety, drugs and dietary elements, can disrupt intestinal homeostasis. Failure to preserve the symbiotic balance of environmental-host interactions can lead to barrier dysfunction, microbiota imbalances and intestinal inflammation, which if unresolved, can contribute to chronic intestinal inflammatory disorders.

Such is the case with inflammatory bowel disease (IBD). IBD is a collection of chronically relapsing inflammatory intestinal disorders, the most common of which are ulcerative colitis and Crohn's disease. Though the etiology of IBD is not fully understood, genetic, immunological, microbial and other environmental factors are believed to be involved. Genome-wide association studies have identified over 200 IBD risk loci, many of which are involved in regulating immune responses and barrier function (Jostins et al. 2012). In particular, loss of function mutation of the *NOD2* gene is associated with a high risk of Crohn's disease. In association with risk loci, IBD patients exhibit many dysregulated barrier and immune characteristics, including increased intestinal permeability, diminished mucus integrity and elevated T_H17 responses, to name a few (Johansson et al. 2014; Kevans et al. 2015; Kobayashi et al. 2008). Although genetic factors certainly contribute to disease pathogenesis, they are insufficient to induce disease. For instance, evaluation of first-degree relatives of Crohn's disease patients that also harbour *NOD2* risk alleles did not exhibit elevated intestinal permeability, suggesting permeability alterations may not be heritable

(Kevans et al. 2015). Imbalances in microbiota taxonomic structure have been described in IBD patients in comparison to healthy controls; commonly reported changes include reduced Firmicutes, particularly members of Clostridium clusters XIVa and IV, enrichment of Proteobacteria, and decreased diversity (Frank et al. 2007; A. W. Walker et al. 2011). Differences between the bacterial communities of inflamed and non-inflamed regions have also been reported (A. W. Walker et al. 2011). It has been difficult to prove a causal role of the microbiota in IBD pathogenesis, as no clear microbial signature has been identified. Furthermore, most studies compare patients with active inflammation to those with drug-induced remission or healthy controls, but given these conditions are multifactorial and patient populations are highly heterogenious, this provides limited pathophysiological insight. It is therefore possible that microbiota changes are a consequence of inflammation. Regardless of causation, depletion of bacteria known to promote barrier function, such as *Faecalibacterium prausnitzii*, in IBD patients may contribute to the perpetuation or exacerbation of inflammation (Sokol et al. 2008). IBD patients also exhibit changes in viral and fungal communities, although the pathophysiological consequences of these changes are not well characterized or understood (Chu et al. 2017). Other environmental factors have also been associated with IBD, such as smoking, diet, infection, and hygiene (Molodecky et al. 2011).

Altogether, it is generally accepted that IBD results from aberrant immune responses towards the microbiota in genetically susceptible hosts. Given the

intricate nature of the microbiota-host relationship, elucidating causal roles of microbiota and barrier dysfunction in established disease has proven difficult; thus, a number of animal models have been developed to mimic some of the inflammation and barrier dysfunction characteristics of IBD (Table 4). The dextran sulfate sodium (DSS) chemical model of intestinal injury and inflammation is widely used. DSS causes damage to the intestinal epithelium, leading to microbial invasion that drives acute inflammation (Wirtz et al. 2007). Overall, before pathophysiological microbiota-barrier interactions can be deciphered, how the microbiota affects barrier function in the non-inflamed, steady state needs to be characterized.

Table 4. Select murine models of IBD-like intestinal inflammation.

Type of Model	Model	Type of Inflammation
Chemical	Dextran sulphate sodium	Acute/chronic, driven by microbiota
	2,4-di- or 2,4,6-trinitrobenzene sulfonic acid	Acute/chronic, localized to colon, T_H1 response
	Oxazolone	Acute/chronic, localized to colon, NK T cell response
Barrier Defect	Mucin or glycan deficiencies	Spontaneous/chronic, driven by microbiota
	IEC-specific NEMO deficiency	Spontaneous/chronic, throughout GI tract
	IEC-specific IKK-β deficiency	Increased colitis susceptibility
	SAMP1/Yit mouse	Spontaneous/chronic, localized to terminal ileum-cecum, T_H1 response, possibly T_H2 also
	IEC-specific STAT5 deficiency	Increased colitis susceptibility, microbiota driven

Innate Immune Defect	Macrophage/neutrophil STAT3 deficiency	Spontaneous/chronic, T_H1 response
Adaptive Immune Defect	$CD45^+CD45RB^{hi}$ T cell adoptive transfer into SCID or *Rag1*$^{-/-}$ mice	Chronic, T_H1 responses
	IL-10 deficiency	Spontaneous/chronic, initially T_H1 driven, later T_H2
	$TNF^{\Delta ARE}$ mice	Spontaneous/chronic, localized to ileum, T_H1 response
Infection	*Citrobacter rodentium*	Acute/lethal, infects cecum & colon, translocation to other organs
	Salmonella enterica serovar Typhimurium	Acute/lethal, infects colon, translocation to other organs

1.5.2 Targeting Barrier Function through Microbiota Modulating Therapies

Given the immense impact of the microbiota on barrier function and inflammation, a number of therapeutic strategies aimed at modulating barrier function through microbiota-barrier interactions have been developed (Table 5). In the last 15 years, ingestion of specific "probiotic" microbes to confer health benefits has gained popularity. The term probiotic derives from Latin *pro-* to be in favour of, and Greek *-biotic* meaning life, and was first used to describe protozoan-derived substances that promote survival of other microorganisms (Morelli and Capurso 2012). In 1974, the definition was changed to designate beneficial host health effects mediated through the microbiota, specifically to describe animal feed supplements. The definition has since gone through a number of revisions, and in 2002 was described by the World Health Organization

as, "live organisms through which, when administered in adequate amounts, confer a health benefit on the host." Probiotics can include bacteria and yeast, and although the current definition of probiotic stipulates the microbe must be live, beneficial effects can also be elicited by inactivated bacteria and probiotic-derived bioactive molecules (Plaza-Diaz et al. 2017). Probiotics belonging to the *Bifidobacterium*, *Lactobacillus*, and *Saccharomyces* genera are commonly used. A broad range of barrier promoting and anti-inflammatory effects can be elicited by probiotics, including: inhibition of IEC apoptosis, IEC and immune cell proinflammatory responses, as well as promotion of anti-inflammatory signaling, TJ protein expression, mucus fortification, restitution, ultimately improving resilience to damage (Plaza-Diaz et al. 2017). However, the efficacy of probiotics has been demonstrated to be strain-specific. For instance, *Faecalibacterium prausnitzii* strains A2-165 and HTF-F reduce clinical symptoms of acute DSS-induced injury, but only *F. prausnitzii* HTF-F attenuates colon mucosal damage (Rossi et al. 2015). Additionally, probiotics can be given in combination with prebiotics, called synbiotics, in order to improve the survival and fermentation activity of the probiotic. Indeed, a small trial of synbiotics in ulcerative colitis patients demonstrated reduced clinical disease scores and inflammation, as well as improved endoscopic scores (Ishikawa et al. 2011). However, deciphering whether the prebiotic, probiotic, interactions together or with other bacteria are responsible for beneficial outcomes has proven difficult. Overall, heterogeneity of clinical trial design and quality, chronic inflammatory intestinal disorder

investigated, probiotic, concomitant therapies, as well as the state of intestinal inflammation when administered makes it difficult to make recommendations for clinical trial and therapeutic use. Moreover, the effects of probiotics vary based on dose, formulation and strain(s) administered. Therefore, probiotics need to be thoroughly characterized prior to clinical investigation.

Table 5. Microbiota-targeting therapies for GI disorders.

Therapy	Strategy	Advantages	Limitations
Antibiotic	Drug that eliminates or inhibits replication of bacteria	• Treat infections • Bacterial diminishment can be beneficial in chronic inflammatory intestinal disorders • Non-invasive	• Broad specificity can alter bacterial community structure • Risk of resistance development with prolonged use
Fecal Microbiota Transfer	Transfer of fecal material from a donor to a patient in order to alter microbiota composition and confer health benefits	• Entire microbial community administered • Very effective *Clostridium difficile* treatment	• Invasive • Safety risks in patients with poor barrier integrity • Benefit in IBD patients unclear; some patient populations may respond better than others • Lack of guidelines for donor selection & screening

Low fermentable oligo-, di- and mono-saccharide and polyol diet	Restrict consumption of poorly absorbed sugars, sugar alcohols and fibre to reduce fermentation in the colon	• Can help diminish symptoms of irritable bowel syndrome • Non-invasive	• May provide limited benefit in non-functional GI disorders • Inadequate nutrition a concern with long-term use
Prebiotics	Consumption of specific dietary fibres fosters particular bacteria & alters the metabolic profile in the colon	• Barrier-enhancing & anti-inflammatory effects have been demonstrated • Non-invasive	• Difficult to decipher exact mechanism(s) of beneficial effects
Probiotics	Ingestion of live bacteria to confer health benefits	• A broad range of barrier enhancing and anti-inflammatory effects have been demonstrated • Non-invasive	• Difficult to decipher exact mechanism(s) of beneficial effects • Effects may be dependent on state of barrier integrity & inflammation • Safety risks in patients with poor barrier integrity
Synbiotics	Ingestion of pre- and probiotics together	• May improve probiotic efficacy • Non-invasive	

CHAPTER 2

THESIS OBJECTIVES

2.1 Thesis Scope

Evidence from patients with chronic gastrointestinal disorders indicates interactions between the microbiota and intestinal barrier can greatly impact host health; however, the fundamental role of commensal microbiota in determining intestinal barrier function and integrity remains poorly understood. Furthermore, characterization of how specific members of the microbiota impact function and integrity of the intestinal barrier is essential for development of microbiota-targeted strategies to promote a resilient intestinal barrier.

Therefore, the goal of my thesis was to investigate how the gut microbiota affects intestinal barrier integrity and function. I assessed how commensal microbiota contributes to the establishment of intestinal barrier function and integrity through colonization of germ-free mice and induction of acute chemical injury (**Chapter 3**). I also investigated how the microbiota can promote barrier function and integrity during the steady state; specifically, differential induction of the antimicrobial peptide REGIII by different bacteria, including *Bifidobacterium breve* NCC2950, was evaluated (**Chapter 4**). The ability of *B. breve* NCC2950 to alter susceptibility to DSS-induced intestinal injury and inflammation was assessed in **Chapter 5**.

I have also co-authored a textbook chapter titled *Importance of the Microbiota in Early Life and Influence on Future Health* in which the impact of colonization on the gut-brain axis, immune and intestinal development are

described, as well as the implications altered colonization patterns may have on future health (**Appendix I**).

2.2 Thesis Aims

The goal of this thesis is examined through three aims, each of which is investigated in a separate chapter. The specific aims are as follows:

Aim 1: To determine how the microbiota shapes intestinal barrier function and integrity.

CHAPTER 3: Commensal microbiota determines colonic barrier structure and permeability. The manuscript pertaining to this objective is currently under review at *Scientific Reports*:

> Hayes CL, Dong J, Galipeau H, Jury J, McCarville JL, Huang X, Naidoo A, Anbazhagan AN, Libertucci J, Sheridan C, Dudeja PK, Bowdish DME, Surette MG, Verdu EF. Commensal microbiota induces colonic barrier structure and permeability functions that contribute to homeostasis.

Aim 2: To examine induction of antimicrobial REGIII by specific microbiota components.

CHAPTER 4: Differential induction of antimicrobial peptide REGIII by intestinal microbiota and *Bifidobacterium breve* NCC2950. These findings have been published in the following manuscript:

> Natividad JM*, Hayes CL*, Motta JP, Jury J, Galipeau HJ, Philip V, Garcia-Rodenas CL, Bercik P, Verdu EF. Differential induction of

antimicrobial peptide REGIII by intestinal microbiota and *Bifidobacterium breve* NCC2950. *Appl Environ Microbiol.* 2013 Dec; 79(24):7745-54. doi: 10.1128/AEM.02470-13.
*Natividad JM and Hayes CL contributed equally to this manuscript.

Aim 3: To evaluate the effects of *Bifidobacterium breve* NCC2950 on susceptibility to intestinal injury and inflammation.

CHAPTER 5: Efficacy of *Bifidobacterium breve* NCC2950 against DSS-induced colitis is dependent on bacterial preparation and timing of administration. These findings were published in the following manuscript:

Hayes CL, Natividad JM, Jury J, Martin R, Langella P, Verdu EF. Efficacy of *Bifidobacterium breve* NCC2950 against DSS-induced colitis is dependent on bacterial preparation and timing of administration. *Benef Microbes*. 2014 Mar;5(1):79-88. doi: 10.3920/BM2013.0039.

The work presented in this thesis was completed with collaboration and support from colleagues. Specific author contributions for each manuscript are outlined in the preface of each chapter.

CHAPTER 3

COMMENSAL MICROBIOTA DETERMINES COLONIC BARRIER STRUCTURE AND PERMEABILITY

Commensal microbiota determines colonic barrier structure and permeability

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The material in this chapter is in preparation for submission to *Scientific Reports*.

Preface: The work I performed on this manuscript was carried out from Sept. 2013 to the present. I am the primary author of the paper. I planned and conducted the majority of the experiments and analyzed the data with my supervisor E. Verdu. I wrote the manuscript with input from E. Verdu and H. Galipeau. A. Anabazhagan performed RT-qPCR on protein transporters. I received technical assistance from J. Dong, J. Jury, H. Galipeau, J. McCarville, X. Huang, XY. Wang, A. Naidoo, J. Libertucci and C. Sheridan. Scientific input and critical assessment of the manuscript was given by P. Dudeja, D. Bowdish, and M. Surette.

SUMMARY AND SIGNIFICANCE

In this manuscript, I assess the role of the microbiota in determining intestinal barrier structure, function and integrity through colonization of germ-free mice with human fecal microbiota. Within a week of colonization, physiological structure and permeability of the colonic barrier is reached, and is associated with diminished claudin-1 expression, transient IL-18 production and serotonin transporter expression, as well as diminished systemic exposure to bacterial antigens and enhanced resilience towards DSS-induced inflammation. Together, these results demonstrate the microbiota plays a key role in establishment of physiological colonic barrier structure and function, resulting in increased resilience and integrity.

Commensal microbiota determines colonic barrier structure and permeability

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ABSTRACT

The intestinal barrier encompasses structural, permeability and immune aspects of the gut mucosa that, when disrupted, may contribute to chronic inflammation. Although the effects of microbiota on immunity have been extensively studied, its impact on barrier function remains unclear. We compared germ-free and conventional mice, as well as mice colonized with human fecal microbiota that were followed for 21 days post-colonization. Colonic barrier structure was investigated by immunohistochemistry, molecular and electron microscopy techniques. Permeability was assessed in colon tissue by Ussing chambers, and by serum LPS and MDP detection using TLR4- and NOD2-NFκB reporter assays. Microbiota profile was determined by Illumina 16S rRNA gene sequencing. Low dose dextran sodium sulfate was administered to assess microbiota-induced barrier changes on resistance to colonic injury. Permeability to paracellular probes and mucus layer structure resembled that of conventional mice by day 7 post-colonization, coinciding with reduced claudin-1 expression and transient IL-18 production by intestinal epithelial cells. These post-colonization adaptations were associated with decreased systemic bacterial antigen exposure and reduced susceptibility to intestinal injury. In conclusion, commensal colonization promotes physiological barrier structural and functional adaptations that contribute to intestinal homeostasis.

INTRODUCTION

Host-microbe interactions are key determinants of health and disease through their influence on immune development, which has been demonstrated in gnotobiotic studies [1-5]. The intestinal barrier is the all-encompassing term given to the physical and chemical barrier that makes up the first line of defense against luminal antigens and pathogens. Influenced by interactions with mucosal immune cells and the intestinal microbiota, this contiguous layer of epithelial cells (ECs) determines intestinal permeability, mucus production, and secretion of immune factors, such as antimicrobial peptides. Intestinal barrier dysfunction and altered microbiota composition have been associated with chronic inflammatory gastrointestinal conditions, such as inflammatory bowel disease [6-8]. However, deciphering the role of dysfunctional microbiota-barrier interactions during the course of disease has proven difficult, particularly since there is little understanding of how the microbiota impacts intestinal barrier in the non-inflamed, physiological state.

We therefore studied intestinal structure and permeability in germ-free (GF) and in conventionally raised, specific pathogen-free (SPF) mice. The dynamics of structural and permeability adaptations induced were assessed over a 21-day period following *de novo* colonization of GF mice with human fecal microbiota from a healthy donor previously used in a successful fecal microbiota transfer clinical trial [9]. In order to evaluate the pathophysiological consequences of colonization-induced barrier changes, resistance towards dextran sulfate

sodium (DSS)-induced injury was assessed. We show that microbial colonization with commensal microbiota induced rapid colonic barrier structural and permeability functions important for maintenance of homeostasis.

RESULTS

The microbiota is a key determinant of a physiological colonic barrier.

We first compared colonic barrier permeability and structure between GF and conventional SPF mice, whereby characteristics observed in SPF mice were considered the standard, physiological state. Paracellular permeability to the probe ^{51}Cr-EDTA was lower in the proximal colon of GF mice compared with SPF mice (Figure 1A). No statistically significant changes in transcellular permeability to horseradish peroxidase (HRP) was observed (Figure 1B). Semi-quantification of TJ protein expression by immunofluorescence revealed higher expression of claudin-1 and occludin in GF mice compared to SPF mice (Figure 1C,D). No significant change in ZO-1 protein was observed (Supplementary Figure S1). Tight junction (TJ) mRNA expression by real-time (RT)-qPCR showed GF mice had lower claudin-1 and higher occludin mRNA expression compared with SPF mice (Supplementary Figure S1). A non-statistically significant trend for higher ZO-1 mRNA expression was also found in GF mice. To further assess the impact of microbiota on barrier function, expression apical membrane transporters were evaluated.

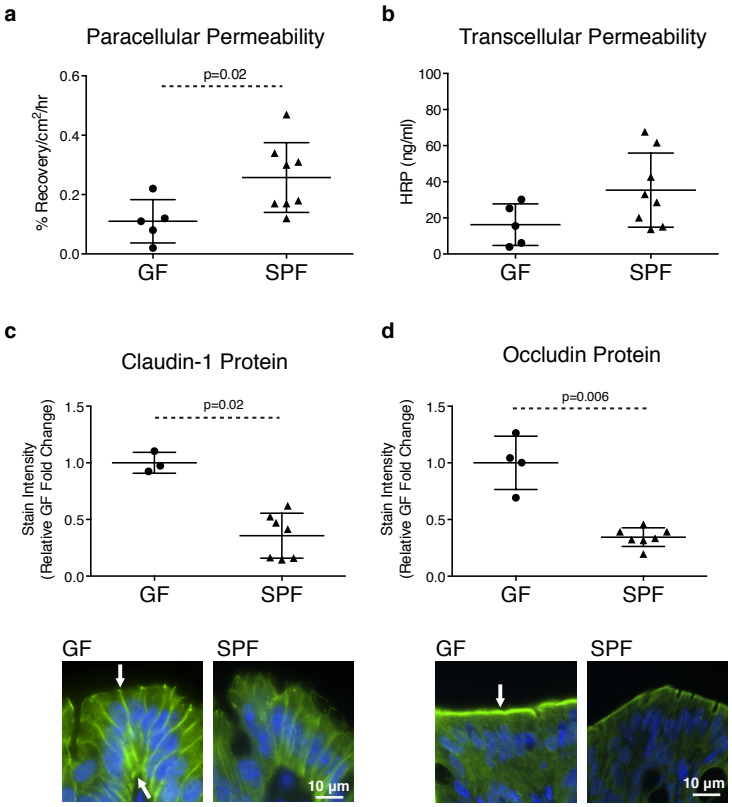

Figure 1. Microbiota is required for establishment of physiological colonic barrier permeability.

(a) Paracellular permeability to ^{51}Cr-EDTA and (b) transcellular permeability to HRP in the proximal colon. Evaluation of (c) claudin-1 and (d) occludin protein expression by IF and (e) representative images. Target proteins are shown in green, nuclei were stained with DAPI (blue). Arrows indicate areas of high expression. Each data point represents one mouse; the horizontal line and whiskers depict the mean±standard deviation (SD). Data was collected over 2-5 independently executed experiments. Significance was determined using an unpaired, two-tailed Student's t test.

No significant differences in serotonin transporter (SERT), apical sodium bile acid transporter (ASBT), cystic fibrosis transmembrane conductance regulator (CFTR), downregulated-in-adenoma (DRA), monocarboylate transporter 1 (MCT-1), Niemann-Pick C1-like protein 1 (NPC1L1), sodium-hydrogen

exchanger 3 (NHE3), or sodium-coupled monocarboxylate transporter (SMCT-1) expression were observed between GF and SPF mice (Supplementary Figure S2). Electron microscopy (EM) assessment demonstrated the mucus layer of SPF mice excluded bacteria from the epithelial surface, while no bacteria were observed in EM of GF mice. GF mice also had higher microvilli length compared with SPF mice (Figure 2). These findings indicate that the presence of commensal microbiota impacts colonic ultrastructure, TJ proteins and paracellular permeability.

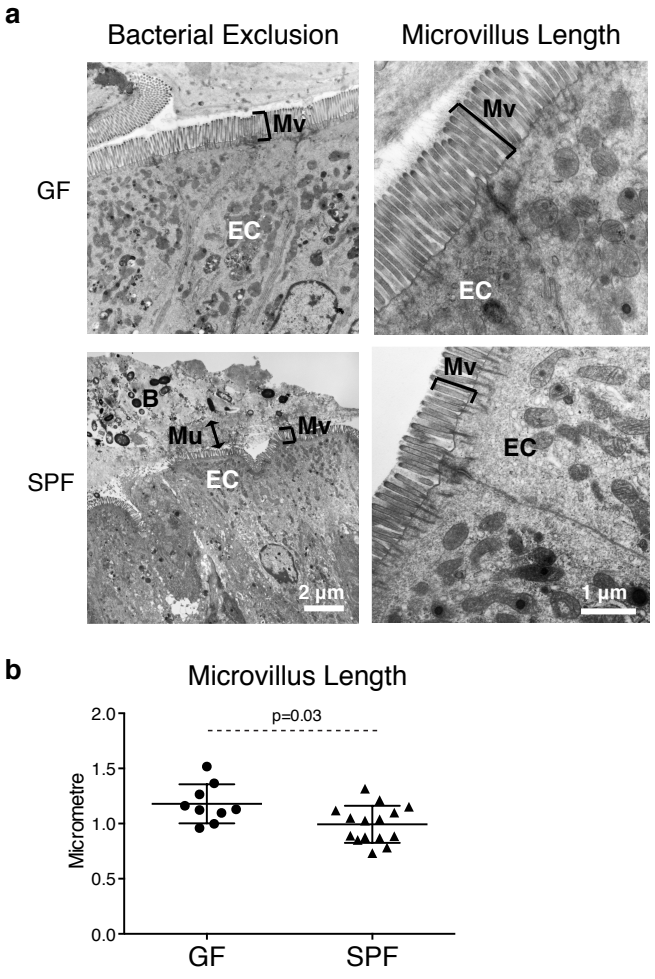

Figure 2. Microbiota is required for establishment of physiological colonic barrier mucus and microvillus structure.

Mucus structure and microvillus length were evaluated in GF and SPF mice. (a) Representative EM pictures demonstrating bacterial exclusion and microvillus length. Markings indicate microvilli (Mv), epithelial cells (EC), and the black arrow indicates separation of bacteria (B) from the epithelium by the mucus (Mu) layer. (b) Microvillus length. Each data point represents analysis of one image; the horizontal line and whiskers depict the mean±SD. Data was collected over 2-5 independently executed experiments. Significance was determined using an unpaired, two-tailed Student's t test.

Colonic microbiota taxonomic structure changes over the first week of colonization.

In order to evaluate the dynamics of intestinal barrier changes after colonization, GF mice were colonized with human microbiota from one healthy donor previously shown to be free of common pathogens (Supplementary Figure S3)[9]. The fecal bacterial profile of mice colonized with this donor was monitored over the first 21 days of colonization by 16S rRNA gene sequencing. Fecal bacterial composition shifted over the first week post-colonization (Figure 3A,B); compared to days 7 and 21 post-colonization, a high abundance of Firmicutes with significantly more Turicibacteraceae, Clostridiaceae, other Clostridia, and other Firmicutes was observed at day 1 post-colonization ($p<0.05$ days 7 and 21 post-colonization vs day 1). By day 7 post-colonization, expansion of Verrucomicrobia and Bacteroidetes was observed, with other Bacteroidales, Porphyromonadaceae, Rikenellaceae, and Verrucomicrobiaceae significantly increased compared to day 1 ($p<0.05$ days 7 and 21 post-colonization vs day 1).

Overall, the Firmicutes:Bacteroidetes ratio was significantly higher at day 1 post-colonization compared to days 7 and 21 post-colonization (Supplemental Figure S4). Principal component analysis with weighted UniFrac and Bray Curtis dissimilarity ordinations showed the microbiota at day 1 post-colonization clustered separately from days 7 and 21 (Figure 3C,D). Although no statistically significant changes in taxonomic composition were observed between days 7 and 21 post-colonization, Bray Curtis dissimilarity values between mice at day 21 post-colonization were higher than at days 1 and 7 post-colonization, indicating higher interindividual variation (Figure 3E). When comparing Bray Curtis dissimilarity values of each individual mouse post-colonization, the values were lower between days 7 and 21 post-colonization compared to day 1 vs 7 and day 1 vs 21 (Figure 3F). No significant changes in Shannon diversity, PD whole tree, Chao1 or observed species were found (Supplementary Figure S5). Together, these findings indicate changes in the taxonomic composition of the fecal microbiota occur over the first 21 days post-colonization.

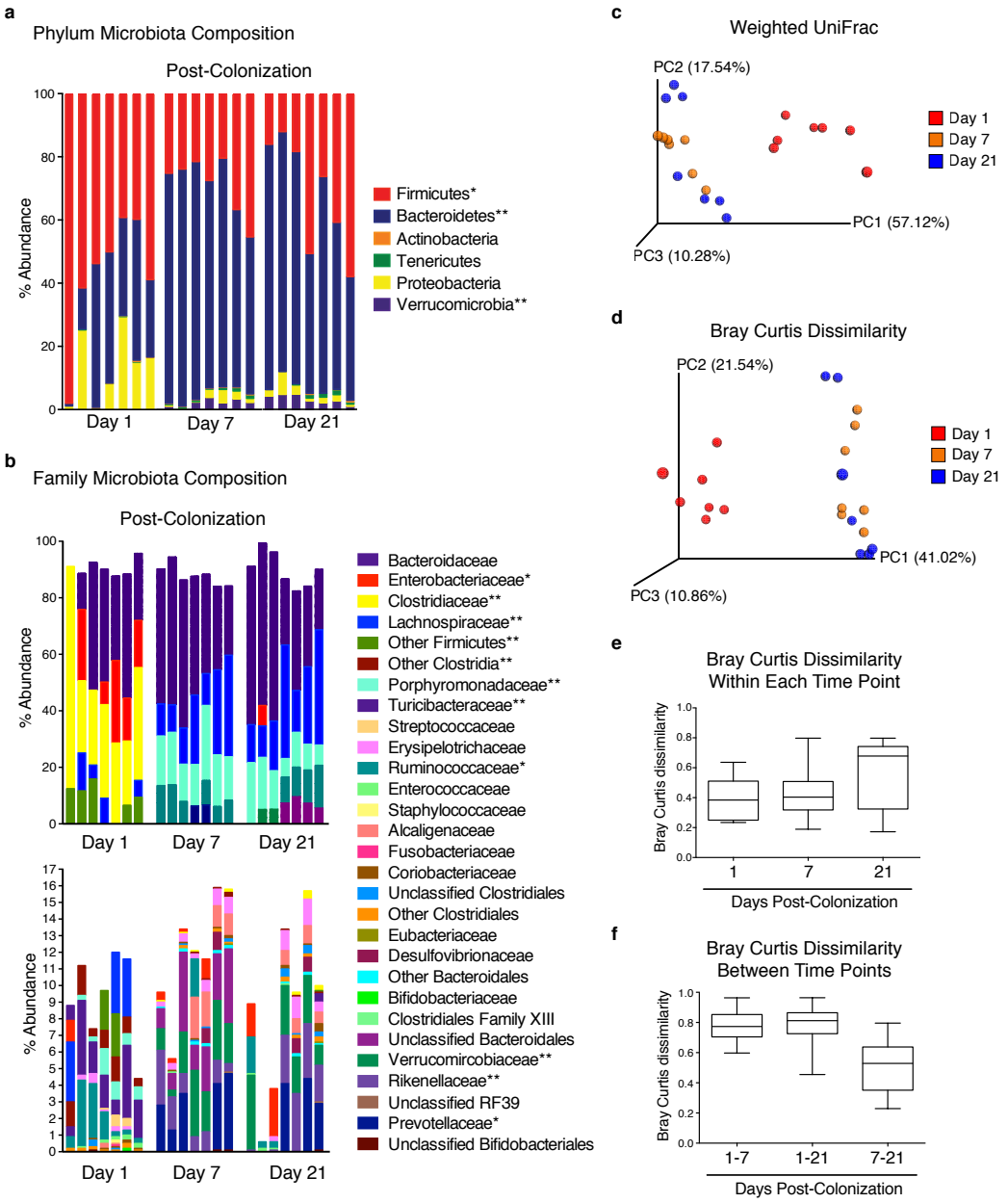

Figure 3. Microbiota taxonomic composition shifts post-colonization.

Fecal microbiota was assessed by 16S rRNA gene sequencing of the same 7 mice at days 1, 7 and 21 post-colonization. Fecal microbiota taxonomic structure at the (a) phylum and (b) family levels. *$p<0.05$; **$p<0.01$ as determined by Kruskal-Wallis test with Bonferroni correction. (c) Principle component (PC) analysis using weighted UniFrac and (d) Bray Curtis dissimilarity ordination. Each data point represents one mouse. (e) Bray Curtis dissimilarity values between the 7 mice at each time point and (f) between time points. Data was collected in a single experiment.

Colonization induces physiological levels of colonic paracellular permeability.

By day 7 post-colonization, uptake of the paracellular probe ^{51}Cr-EDTA increased to a level comparable to that observed in SPF mice, and this was maintained up to day 21 post-colonization (Figure 4A). As with SPF mice, no changes in transcellular permeability were observed post-colonization (Figure 4B). The increase in paracellular probe uptake coincided with lower expression of claudin-1 protein at days 7 and 21 post-colonization (Figure 4C,D). Occludin protein expression tended to be lower by day 21 post-colonization, but did not reach statistically significance (Figure 4E). Only ZO-1 mRNA expression was diminished at day 1 post-colonization (Supplementary Figure S6). Histological evaluation did not reveal damage to the mucosa (Figure 4G). The increase in paracellular permeability at day 7 post-colonization was also observed in a supplemental experiment in which mice were colonized by an alternative mode with the same donor instead of intragastric gavage, whereby the microbial inoculum was applied to the face and paws (Supplementary Figure S7). As with SPF mice, no significant differences in SERT, ASBT, CFTR, DRA, MCT-1, NPC1L1, NHE3, or SMCT-1 expression were observed (Supplementary Figure S8). Overall, these findings suggest that colonic paracellular permeability and some TJ protein characteristics are dynamically and rapidly affected by colonization, in a manner that resembles the steady state observed in conventional mice.

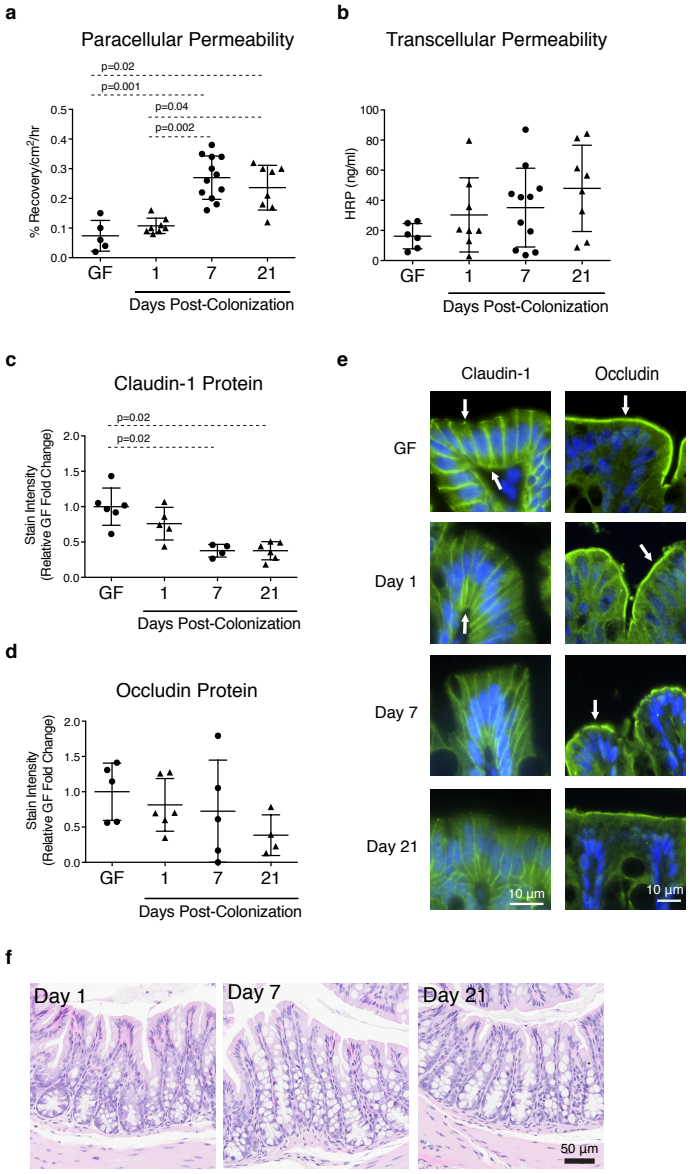

Figure 4. Microbiota induces maturation of colonic permeability.

Colonic permeability as well as TJ mRNA and protein expression were assessed at days 1, 7 and 21 post-colonization. (a) Paracellular permeability to ^{51}Cr-EDTA and (b) transcellular permeability to HRP. (c) Evaluation of claudin-1 and (d) occludin protein expression by IF. (e) Representative IF images of TJ proteins. Target proteins are shown in green, nuclei were stained with DAPI (blue). Arrows indicate areas of high expression. (f) Representative images of colon mucosal structure. Each data point represents one mouse; the horizontal line and whiskers depict the mean±SD. Data was collected over 2-5 independently executed experiments. Significance was determined by Kruskal-Wallis test with Dunn's post-hoc test.

Colonization induces maturation of colonic barrier structure.

We focused our structural immunohistochemistry (IHC) and EM analysis between days 1 and 7 post-colonization, when paracellular permeability, microbiota and TJ protein expression changes were more marked. At day 1 post-colonization, a thin, patchy mucus layer composed primarily of alcian blue-stained mucins was observed, as well as direct contact between microbes and ECs (Figure 5A,B). By day 7 post-colonization, a mixture of alcian blue- and periodic acid/Schiff-stained mucins were produced, and the mucus layer physically segregated luminal bacteria from the epithelial surface (Figure 5A,B). Bacterial exclusion from the epithelium was maintained up to day 21 post-colonization (Figure 5B). A 20% reduction in microvillus length was observed by day 7 post-colonization, resembling the structure of SPF mice (Figure 5B,C). Bacterial exclusion and microvillus shortening were also observed in mice colonized for 7 days using the aforementioned alternative mode of colonization (Supplementary Figure S9). These results indicate mucus and microvillus structure reach the physiological state by day 7 post-colonization.

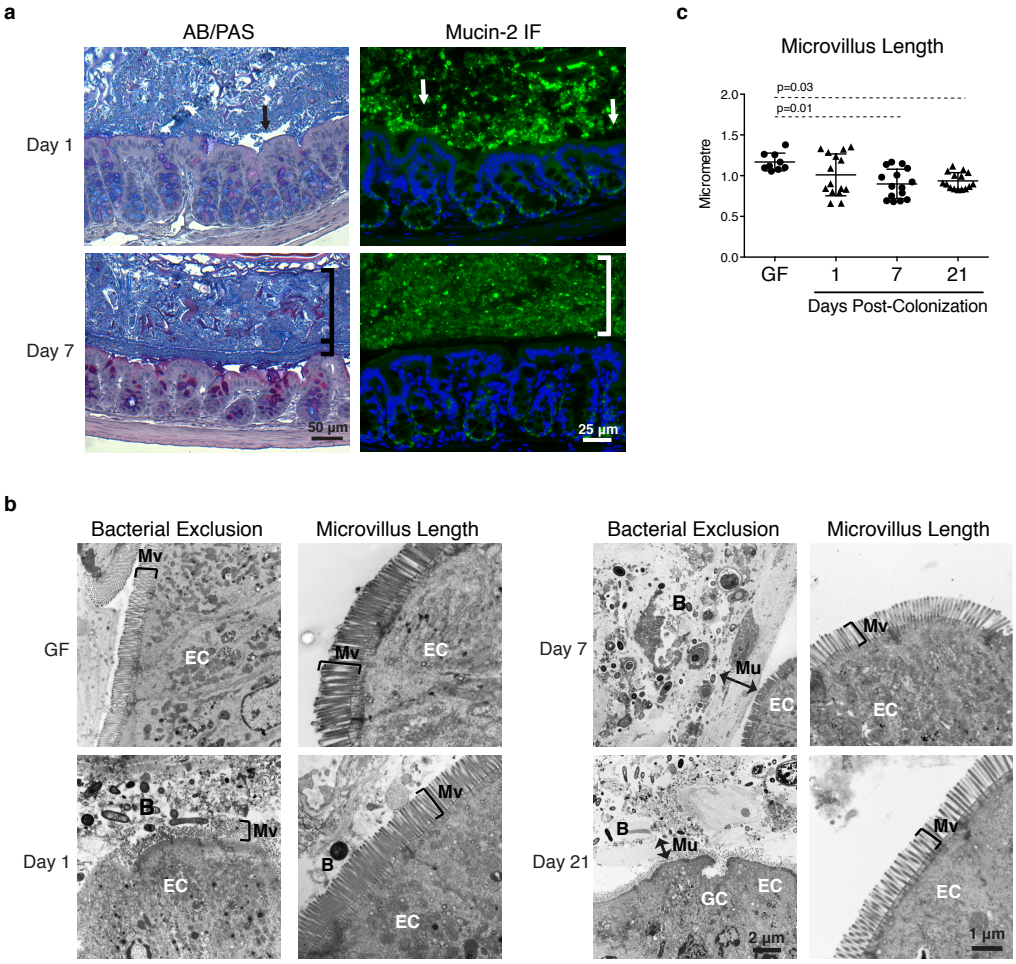

Figure 5. Microbiota induces maturation of colonic barrier structure.

Mucus structure and microvillus length were assessed at days 1, 7 and 21 post-colonization. (a) Representative pictures of mucus structure evaluated by alcian blue/periodic acid-Schiff (AB/PAS) staining (left panel) and mucin-2 IF staining (right panel). Mucin-2 is shown in green, nuclei were stained with DAPI (blue). Arrows designate areas of inconsistent mucus, brackets indicate areas of uniform, thick mucus. (b) Representative EM images demonstrating bacterial exclusion (left images) and microvillus length (right images). Markings indicate microvilli (Mv), epithelial cells (EC), and the black arrows indicate separation of bacteria (B) from the epithelium by the mucus (Mu) layer. (c) Microvillus length. Each data point represents analysis of one image; the horizontal line and whiskers depict the mean±SD. Data was collected over 2-5 independently executed experiments. Significance was determined by Kruskal-Wallis test with Dunn's post-hoc test.

Colonization induces a transient increase in IL-18 production by colonic epithelial cells.

A number of cytokines produced by cells in the epithelial compartment, such as intraepithelial lymphocytes (IELs) and ECs, regulate intestinal permeability [10-14]. In order to gain insight into mechanisms that may influence colonic barrier changes post-colonization, expression of proinflammatory genes in the IEL-enriched compartment of the colon were evaluated. Out of a panel of 254 genes, 43 were found to be significantly different between days 1 and 7 post-colonization (Figure 6A; Supplementary Figure S10). No significant changes in *Tnf*, *Ifng*, *Il6*, or *Il1b* were found (Supplementary Figure S11), but *Il18* was increased at day 7 post-colonization. IL-18 is a regulator of paracellular permeability that is produced by ECs [15-18]. Therefore, IL-18 localization and expression was assessed by IHC. At day 7 post-colonization, an increase in IL-18 protein expression was detected in surface ECs compared to GF mice (Figure 6B). By day 21 post-colonization, the IL-18 expression level had diminished, resembling that observed in SPF mice (Figure 6B,C). These findings suggest transient epithelial IL-18 production at day 7 post-colonization may drive physiological changes in the expression of TJ proteins and paracellular permeability.

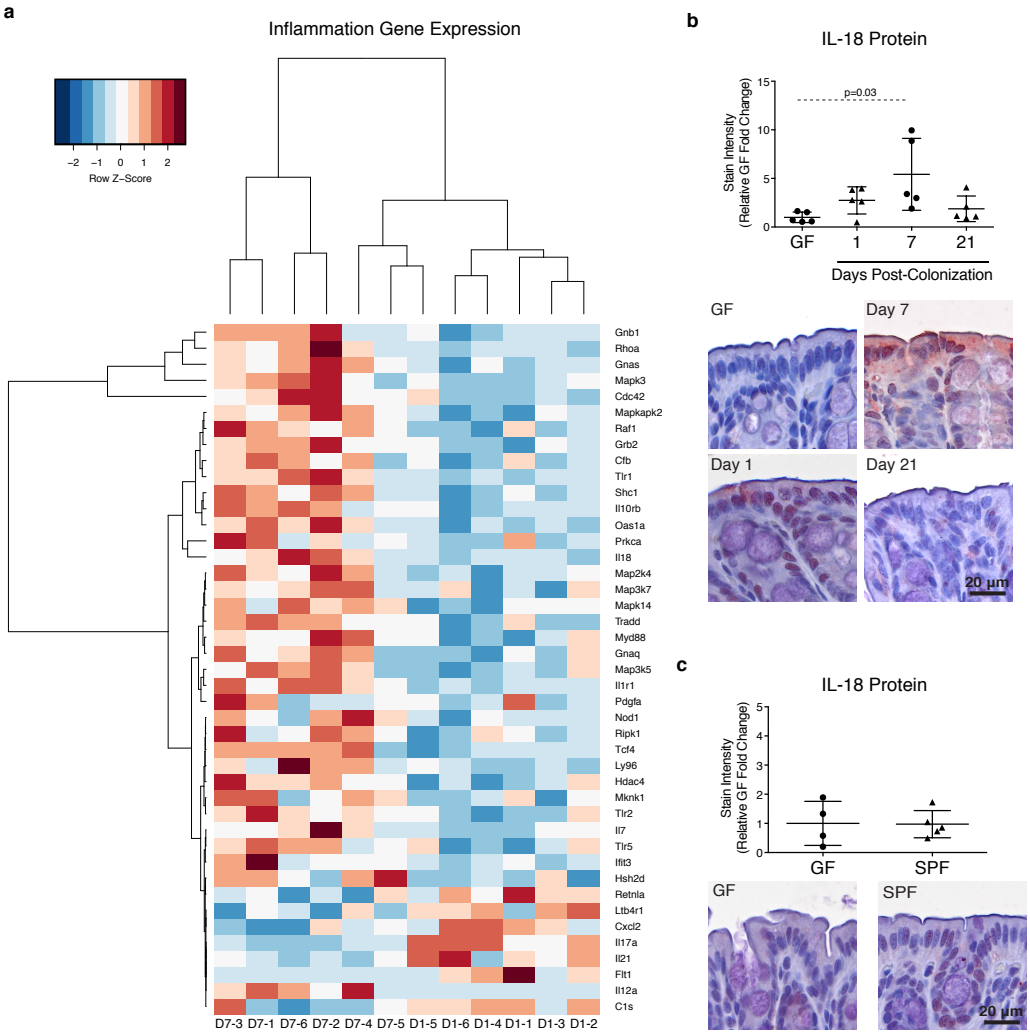

Figure 6. Microbiota induces a transient increase in colonic epithelial cell IL-18 at day 7 post-colonization.

(a) Heat map of significantly altered Log2 transformed inflammation-associated genes in the colonic IEL-enriched compartment as assessed by NanoString at days 1 and 7 post-colonization (D1 and D7, respectively, samples 1-6). Evaluation of IL-18 protein expression by IHC and representative images (b) post-colonization and (c) in SPF mice. Each data point represents one mouse; the horizontal line and whiskers depict the mean±SD. Data was collected over 2-5 independently executed experiments. Significance was determined by Kruskal-Wallis test with Dunn's post-hoc test or an unpaired, two-tailed Student's t test.

Barrier adaptations post-colonization reduce systemic microbial antigen exposure.

To gain insight into the functional significance of structural and permeability changes post-colonization, immunostimulatory bacterial components lipopolysaccharide (LPS) and muramyl dipeptide (MDP) were semi-quantified in serum at days 1 and 7 post-colonization. Both LPS and MDP were increased at day 1 post-colonization, while no significant changes were observed at day 7 post-colonization (Figure 7). These findings indicate colonization-induced barrier adaptations are associated with a transient increase in circulating bacterial products that resolves as barrier function reaches the physiological state.

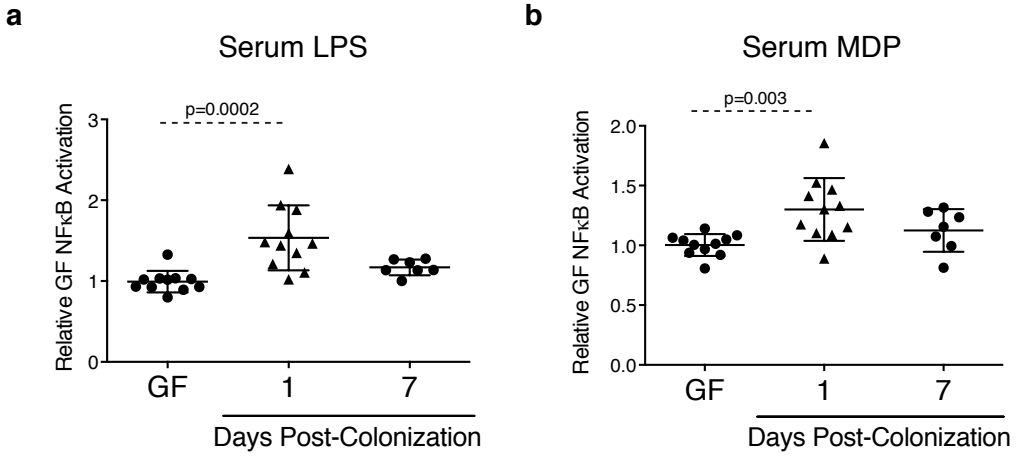

Figure 7. Barrier adaptations post-colonization limit systemic microbial antigen exposure.

Bacterial components (a) LPS and (b) MDP were assessed in serum at days 1 and 7 post-colonization. Each data point represents one mouse; the horizontal line and whiskers depict the mean±SD. Data was collected over 2-5 independently executed experiments. Significance was determined by Kruskal-Wallis test with Dunn's post-hoc test.

Resistance to DSS-induced intestinal inflammation increases within a week of colonization.

To determine the pathophysiological significance of structural and permeability changes post-colonization, intestinal injury and subsequent microbiota-driven inflammation was induced using the DSS model. SPF mice and mice colonized for 1 and 7 days were exposed to low dose (2%) DSS for 5 days. SPF mice developed moderate microscopic damage (Figure 8A,B). Compared to SPF mice, mice colonized for 1 day prior to DSS administration had significantly higher microscopic damage and weight loss, severe diarrhea, colon shortening, blood detectable in stool, translocation of live bacteria to the liver, as well as a non-statistically significant trend of translocation to the spleen (Figure 8A-J). Mice colonized for 7 days prior to DSS exhibited diarrhea and colon shortening, but weight loss was less marked, and no significant bacterial translocation was observed. Fluid consumption was monitored throughout the experiment, and mice colonized for 7 days consumed significantly more DSS than SPF and 1-day colonized mice (average DSS/water consumption: 19±0.93ml SPF mice, 28±3.9ml day 1 colonized, 40±4.4ml day 7 colonized ($p<0.0001$ vs SPF), 25±5.9ml water control (mean±SD; n=5-14)). These findings suggest a longer colonization period is associated with higher resistance to DSS-induced inflammation.

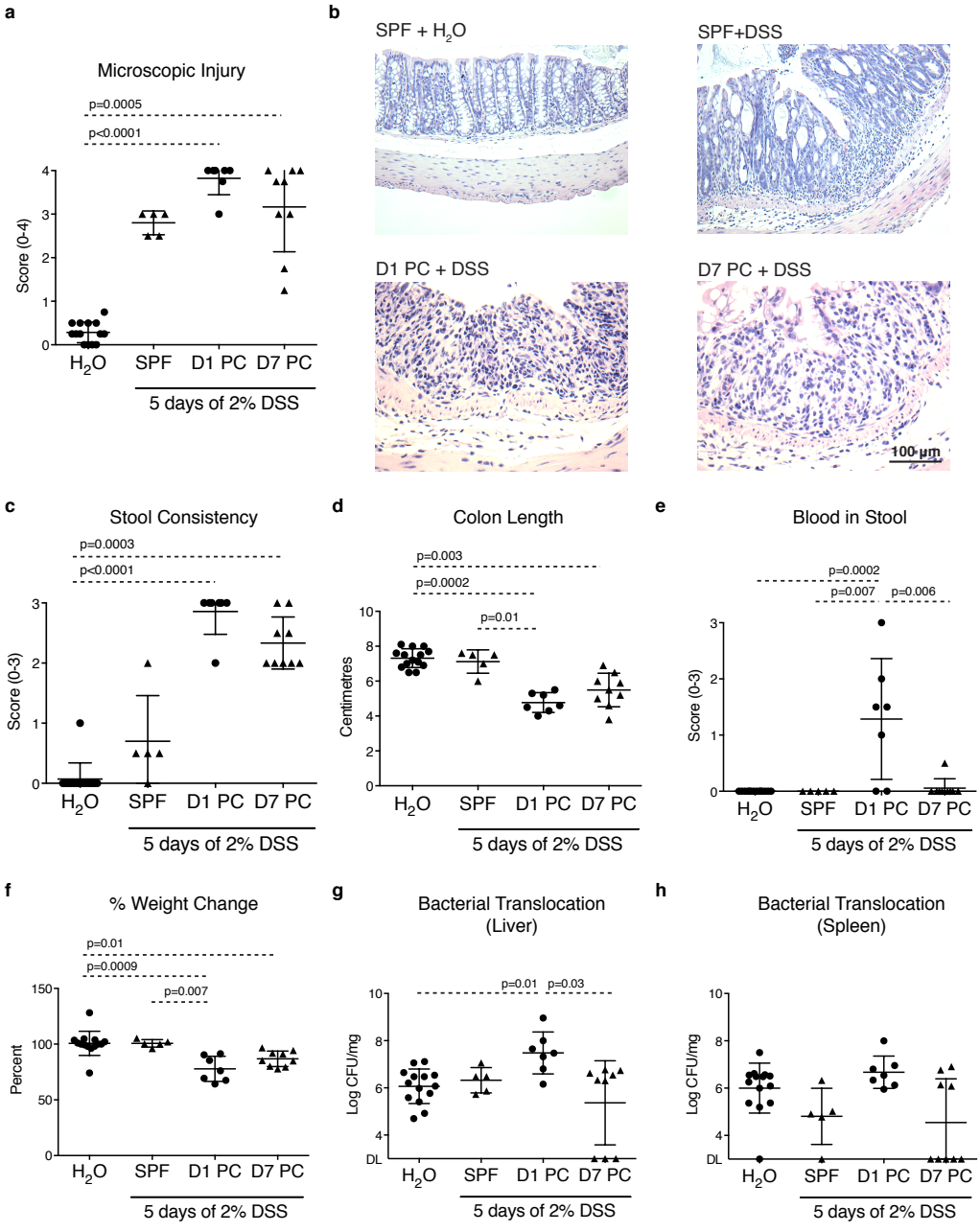
a
Microscopic Injury
p=0.0005
p<0.0001
Score (0-4)
H2O
SPF
D1 PC
D7 PC
5 days of 2% DSS
b
SPF + H2O
SPF+DSS
D1 PC + DSS
D7 PC + DSS
100 µm
c
Stool Consistency
p=0.0003
p<0.0001
Score (0-3)
d
Colon Length
p=0.003
p=0.0002
p=0.01
Centimetres
e
Blood in Stool
p=0.0002
p=0.007
p=0.006
Score (0-3)
f
% Weight Change
p=0.01
p=0.0009
p=0.007
Percent
g
Bacterial Translocation (Liver)
p=0.01
p=0.03
Log CFU/mg
DL
h
Bacterial Translocation (Spleen)
Log CFU/mg
DL

Figure 8. Barrier adaptations post-colonization increase resistance to DSS-induced injury.
SPF mice and mice at days 1 and 7 post-colonization (PC) were given 2% DSS in drinking water for 5 days and sacrificed 2 days thereafter to assess mucosal injury, inflammation, and bacterial translocation. Mice given water were used as controls for each group and pooled together. (a) Microscopic injury score and (b) representative haematoxylin and eosin stained images. (c) Stool consistency score. (d) Colon length. (e) Stool blood score. (f) Percent weight change. (g) Quantification of anaerobic bacteria in the liver and (h) spleen. DL = detection limit (3.33 log CFU/mg of tissue). Each data point represents one mouse; the horizontal line and whiskers depict the mean±SD. Data was collected over 2 independently executed experiments. Significance was determined by Kruskal-Wallis test with Dunn's post-hoc test.

DISCUSSION

Commensal colonization induces mucosal and systemic immune maturation important for the maintenance of homeostasis [1-4, 19, 20]; however, the role of microbiota in shaping intestinal barrier structure and permeability is poorly defined. Here we determined that within a week of commensal colonization, dynamic changes in several aspects of the colonic barrier occur that favor development of a steady state between the microbiota and host.

We first determined that, compared to conventional SPF mice, GF mice had lower paracellular uptake of an inert probe in the proximal colon, suggesting the microbiota is necessary for establishment of some semi-permeable paracellular features of the colon. To study the dynamics of how these barrier changes are established, adult GF mice were colonized with fecal microbiota from one healthy human donor screened for the absence of common pathogens [9]. The fecal bacterial taxonomic composition of colonized mice changed significantly over the

first week of colonization but was less marked between days 7 and 21 post-colonization, in agreement with previous findings [3, 21, 22]. Concurrently, paracellular permeability of the colon reached the physiological state within a week of colonization. This was not associated with mucosal damage, and was independent of the colonization mode, suggesting it was not due to an overt inflammatory response towards the microbial inoculum or the intragastric gavage procedure. Paracellular permeability changes post-colonization were paralleled by decreased intercellular claudin-1 expression and a transient increase in epithelial IL-18. In support of our findings in this colonization model, IL-18 has previously been associated with changes in claudin-1 expression and increased paracellular permeability [23]. IL-18 has been postulated to play a dichotomous role in barrier homeostasis depending on the cellular source and stage of inflammation; on one hand, epithelial IL-18 promotes barrier integrity during initiation of inflammation, while immune cell-derived IL-18 enhances inflammation at later stages [14, 15, 17, 18, 24-26]. The modest, transient increase in epithelial IL-18 observed in this study occurred in the absence of tissue damage or inflammation, consistent with a homeostatic response. Furthermore, increased IL-18 was concurrent with lower systemic exposure to bacterial immunostimulatory components, as well as decreased bacterial translocation and susceptibility to inflammation following DSS-induced injury. Together, these findings indicate that commensal colonization stimulates induction of physiological paracellular permeability

within a week, in association with transient IL-18 production and diminished claudin-1 expression, without compromising overall barrier integrity.

Structural integrity of the colonic barrier is essential for limiting host-microbe interactions and maintaining proper barrier function. Our results show that the structure of the mucus layer, comparable to that observed in SPF mice, was induced within the first week of colonization. This led to effective segregation of bacteria from the epithelial surface and coincided with reduced systemic microbial antigen exposure. In support of this, fortification of the mucus layer and increased diversity of mucin glycosylation was observed within 48 hours of human intestinal organoid colonization with human-derived, non-pathogenic *E. coli* [27]. Johansson *et. al.* [28] demonstrated exclusion of bacteria-sized beads by the mucus layer at 2 weeks post-colonization of GF mice with conventional murine cecal microbiota, although the area of exclusion was smaller than that of conventional mice and was not observed in all samples. In contrast to our EM findings, fluorescence *in situ* hybridization of bacterial DNA also suggested bacteria were in contact with colonic epithelium at 2 weeks post-colonization [28]. It is possible differing techniques of assessment and microbiota background account for some of these differences, as host and microbiota-specific effects on mucus structure have been described [29-31]. Nevertheless, our finding that mucus fortification is associated with increased resistance against intestinal injury and bacterial translocation is in accordance with previous studies [32-36]. Overall, our data demonstrates mucus fortification occurs rapidly following

colonization, coinciding with TJ protein expression changes and physiological paracellular permeability induction, which may play a role in controlling microbial antigen uptake.

In summary, features of the intestinal epithelial colonic barrier characteristic of conventional mice were reached within a week of commensal human microbiota colonization. This coincided with shifts in bacterial taxonomic structure and decreased claudin-1 expression, as well as transiently increased epithelial IL-18. Since colonization was performed beyond the postnatal and weaning periods to avoid developmental and diet related confounders, the results indicate the microbiota-mediated effects on some aspects of colonic barrier maturation are not restricted to a window of perinatal exposure. The changes resulted in a more resilient barrier capable of limiting systemic microbial exposure and increasing resistance to chemically induced intestinal injury. We believe this constitutes a useful and practical model to investigate the therapeutic potential of barrier-modulating microbiota therapies and the role of disease-associated microbial communities, or individual strains, on intestinal barrier dysfunction.

METHODS

Mice

Male and female adult GF C57BL/6 mice (2-3 months old) were obtained from the Axenic Gnotobiotic Unit at McMaster University. Conventionally raised, adult, male and female SPF C57BL/6 mice were obtained from Taconic (Taconic

Biosciences Inc, Germantown, New York; from rooms IBU04 and IBU14; 2-3 months old) and used for experiments after a week of acclimatization to the McMaster Animal Facility. Animals were kept on a twelve-hour light and dark cycle, and housed in sterile vent/rack cages with *ad libitum* access to food (Teklad S-2335 Mouse Breeder Sterilizable Diet) and water at the McMaster Animal Facility. All experiments were performed in accordance with McMaster University animal utilization protocols and conducted under the Canadian Council on Animal Care Guidelines.

Overall design

Comparisons between GF and SPF mice as well longitudinal studies in colonized mice at days 1, 7 and 21 post-colonization were performed. For the latter, GF mice were colonized by intragastric gavage with 0.2ml of fecal slurry (1:10 w/v) from one healthy adult human donor previously shown to be free of known pathogens (Supplementary Figure S3) [9]. Fecal slurry was prepared as described previously [37]. In a supplemental experiment, fecal slurry from the same donor was applied to the face and front paws of GF mice instead of by intragastric gavage, as an alternative colonization mode.

Intestinal permeability measurements

Intestinal permeability was evaluated *ex vivo* using Ussing chambers as previously described [38]. Paracellular and transcellular permeability were assessed

using 6μCi/ml of ^{51}Cr-EDTA and 5x10^{5} M HRP (type II; Sigma Aldrich) probes, respectively. Serosal ^{51}Cr-EDTA was quantified using a liquid scintillation counter and reported as percent recovery/cm^{2}/hour. A modified Worthington assay was used to quantify serosal HRP [39].

RNA extraction

Fresh colon tissue or the IEL-enriched compartment were incubated overnight at 4°C in RNAlater (Life Technologies). Colon tissues were stored at -20°C. RNA was extracted using the RNeasy Mini Kit (Qiagen) with DNase treatment. RNA concentration was determined using a NanoDrop spectrophotometer (Qiagen) and quality assessed by agarose gel electrophoresis.

Real-time quantitative PCR of tight junction and apical barrier transport proteins

To evaluate tight junction protein gene expression, reverse transcription of 1μg of RNA template was performed using iScript Reverse Transcriptase (Bio-Rad). Quantitative real-time PCR was performed using cDNA (50 ng/μl), primers (0.5 μM) and SsoFast EvaGreen Supermix (Bio-Rad), (Supplementary Table S1), and amplified using a Mastercycler ep realplex^{4} (Eppendorf). Enzyme activation was induced at 95°C for 30 seconds, denaturation at 95°C for 5 seconds and annealing/extension at 60°C for 20 seconds was cycled 45 times.

For assessment of apical membrane transport protein gene expression, 50ng of cDNA with 0.15 μM primers (Supplementary Table S1) was reverse

transcribed and amplified in one step reaction using Brillliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies) in Stratagene Mx3005P (Agilent Technologies). PCR proceeded 40 cycles of denaturation at 95°C for 20 seconds and annealing/extension at 60°C for 22 seconds. Gene expression of tight junction proteins and apical membrane transport proteins was normalized to *Gapdh*, and the fold expression changes relative to the GF mouse *Gapdh* mean determined using the $2^{-\Delta\Delta CT}$ method.

Mucin-2 and TJ protein expression by immunofluorescence

Mucin-2 and TJ protein expression in the proximal colon were assessed by immunofluorescent staining of formalin-fixed, paraffin-embedded sections. Sections were cut to 4μm, deparaffinized and rehydrated. For claudin-1 antigen retrieval, sections were steamed in sodium citrate buffer (pH 6.0) for 30 minutes. For occludin antigen retrieval, sections were subjected to proteinase K (20μg/ml) for 10 minutes at room temperature. For claudin-1 and occludin staining, tissue sections were blocked with 2% BSA/PBS/0.05% Tween 20 for 1 hour. For ZO-1 staining, sections were treated 0.4% pepsin in 0.01N HCl for 30 minutes at 37°C, and blocked with 10% goat serum for 1 hour at room temperature. Tissue sections were incubated overnight at 4°C with one of the following anti-rabbit primary antibodies in 1% BSA/PBS/0.05% Tween 20: claudin-1 (1:100; Abcam ab15098), occludin (1:200; Bioss Antibodies bs-1495R), or ZO-1 (1:200; Invitrogen 40-2200). Slides were incubated with goat anti-rabbit Alexa Fluor 488 (1:250;

Thermo Fisher Scientific A11070) secondary antibody for 2 hours at room temperature, and mounted with Prolong Gold Antifade with DAPI (Thermo Fisher Scientific). Controls included slides stained in the absence of primary antibody as well as absorptive controls, where primary antibody was applied with excess peptide. Carnoy's-fixed (60% methanol, 30% chloroform, 10% glacial acetic acid), paraffin-embedded slides were used for mucin-2 (1:100; Abcam ab76774) staining, as well as with the aforementioned secondary antibody. Images were acquired using ImagePro Plus (Media Cybernetics). Staining was blindly assessed using ImageJ v.1.49 (NIH), and shown as the fold increase of signal intensity relative to the GF controls, which was arbitrarily set to 1.

Evaluation of intestinal structure by EM

Cross sections of colon no more than 0.4cm thick were fixed overnight with 2% glutaraldehyde (v/v) in 0.1M sodium cacodylate buffer (pH 7.4) at 4°C. Sections were post-fixed with 1% osmium tetroxide in 0.1M sodium cacodylate buffer, dehydrated, rinsed with propylene oxide, subjected to a graded series of Spurr's resin, polymerized overnight in embedding moulds filled with 100% Spurr's resin at 60°C, cut using a Leica UCT ultramicrotome, and picked up onto Cu grids. Sections were post-stained with uranyl acetate and lead citrate, viewed using a JOEL JEM 1200 EX TEMSCAN transmission electron microscope at an accelerating voltage of 80kV. Images were obtained using an AMT 4-megapixel digital camera (Advanced Microscopy Techniques).

Microvillus length was measured from EM images using ImageJ (NIH). Colon sections from three mice were evaluated per group, 5-6 images evaluated per section, and 10 longitudinally cut microvilli measured per image. Each data point depicts the average microvillus length (μm) per image.

Microbiota Analysis

Fecal samples from the same 7 mice were collected at days 1, 7 and 21 post-colonization, flash frozen, and stored at -80°C. DNA was extracted using the MagMAX Express 96 (Life Technologies), the hypervariable V3 region of the bacterial 16S rRNA gene was sequenced using the Illumina MiSeq platform, and analysis was performed as described previously [40, 41]. Briefly, sequences were edited using Cutadapt version 1.2.1 [42] aligned using PANDAseq v.2.8 [43]. Abundant OTU was used to select operational taxonomic units [44]. Greengenes reference database was used to assign taxonomy, and QIIME was used to filter out operational taxonomic units below 0.01% abundance [45]. The fecal microbiota was sampled at a mean depth of 98 779 reads per sample (median = 83 515). Operational taxonomic unit abundance and Firmicutes:Bacteroidetes graphs were created in GraphPad Prism 6.0 (GraphPad Software, Inc). Principal component analysis using unweighted UniFrac and Bray Curtis dissimilarity ordinations was performed in QIIME.

Mucus layer staining

Proximal colon mucus layer structure was assessed using alcian blue and periodic acid Schiff staining of Carnoy's-fixed (60% methanol, 30% chloroform, 10% glacial acetic acid), paraffin-embedded sections. Deparaffinized, rehydrated tissue sections were treated with 3% acetic acid for 3 minutes, alcian blue for 15 minutes, periodic acid for 5 minutes, Schiff's reagent for 15 minutes, and counterstained with haematoxylin.

Expression of inflammation-associated genes in the IEL-enriched compartment

The IEL-enriched compartment of the colon was isolated using a modified protocol [46]. Briefly, colons were collected, cleared of contents and cut into 3-5mm pieces. Tissue pieces were incubated in DTT/HEPES/Hank's for 15 minutes at 37°C on a shaker, and 5 times in EDTA/HEPES/Dulbecco's PBS, with vortexing and straining between incubations. Supernatant was passed through 70μm strainers and the IEL-enriched compartment isolated by Percoll gradient. Cells were resuspended in RNAlater and kept at 4°C overnight prior to RNA extraction. Quality of RNA was first checked using an RNA Nano Chip and Agilent 2100 Bioanalyzer (Agilent Technologies). Gene expression was determined using the nCounter® Mouse Inflammation v2 XT CodeSet (NanoString Technologies Inc; Supplementary Table S2), analysed with nSolver Analysis Software v2.5.34 (NanoString Technologies Inc), and normalized, Log2 transformed gene values

depicted in heat maps with Euclidean clustering created in RStudio v1.0.143 (RStudio Inc).

IL-18 expression by immunohistochemistry

Formalin-fixed, paraffin-embedded colon sections were cut to 4μm, deparaffinized, rehydrated, and blocked with dual endogenous enzyme blocking reagent (Dako S2003) for 10 minutes. Slides were steamed in sodium citrate buffer (pH 6.0) for 30 minutes, blocked with 2% BSA/PBS/0.05% Tween 20 for 2 hours, as well as Peroxidase Block (Dako K4004) for 5 minutes. Samples were stained with rabbit anti-mouse IL-18 (1:100; Abcam ab71495) overnight at 4°C in 1% BSA/PBS/0.05% Tween 20, treated with Envision System-HRP Labeled Polymer Anti-Rabbit (Dako K4003) for 30 minutes, AEC substrate chromogen (Dako K3464), counterstained with haematoxylin and mounted with Aqua-Mount (Thermo Fisher Scientific). Controls included slides stained in the absence of primary antibody as well as an absorptive control, where primary antibody was applied with excess peptide. Images were acquired using ImagePro Plus (Media Cybernetics). Staining was blindly assessed using the IHC Toolbox in ImageJ (NIH) and shown as the fold increase of signal intensity relative to the GF controls, which was arbitrarily set to 1.

TLR4 and NOD2 NFκB-SEAP Reporter Assays

To detect LPS and MDP in serum, colorimetric NFκB-SEAP reporter assays were performed as described previously [47]. Briefly, heat inactivated serum diluted 1:5 in PBS, 1:1 in water, and finally 10ul into 190ul in HEK Blue Detection Media (Invitrogen) was incubated with HEK293 cell line expressing TLR4, MD2 and CD14 transfected with pNifty2-SEAP for LPS detection, or HEK293T cells transfected with NOD2 and pNifty2-SEAP for MDP detection. After a 24-hour incubation at 37°C, readings were performed at 630nm, and NFκB activation relative to the GF controls calculated.

Dextran Sodium Sulfate-Induced Injury

Intestinal injury and inflammation were induced by administration of 2% DSS (36,000-50,000 MW; MP Biomedicals LLC) in drinking water for 5 days, followed by 2 days on normal drinking water prior to sacrifice. Negative control mice for each group were given normal drinking water, and were pooled into one group as no significant differences were observed. Colon length, percent weight change, stool consistency and presence of blood were evaluated at sacrifice. Stool consistency and blood were each scored on a scale of 0-3 as outlined in Supplementary Table S3. Haematoxylin and eosin stained colon sections were blindly evaluated for microscopic damage on a scale of 0-4 as previously described [48].

Bacterial Translocation

Translocation of live bacteria was evaluated by homogenizing liver and spleen (1:10 w/v) in pre-reduced PBS, plating duplicate serial dilutions on brain heart infusion media (BD) supplemented with 0.5% sheep's blood (Cedarlane), and incubating at 37°C under anaerobic conditions for 48 hours. Data is shown as the log CFU/mg of tissue.

Statistics

Significance was determined by Kruskal-Wallis test with Dunn's post-hoc test or an unpaired, two-tailed Mann-Whitney test, when appropriate. For microbiota analysis, Kruskal-Wallis tests with Bonferroni correction were performed in QIIME. $P<0.05$ was considered significant.

Data Availability

The 16S gene sequencing dataset generated during this study is available in NCBI's SRA repository with BioProject ID PRJNA430097; http://www.ncbi.nlm.nih.gov/bioproject/430097.

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SUPPLEMENTARY MATERIAL

Supplemental Table S1. RT-qPCR primers.

	Protein	Gene	Forward	Reverse
Apical Junction Proteins	Claudin-1	*Cldn1*	5'-GGACTGTGGATGTCCTGCGTTT-3'	5'-GCCAATTACCATCAAGGCTCGG-3'
	Claudin-3	*Cldn3*	5'-TCATCGTGGTGTCCATCCTGCT-3'	5'-AGAGCCGCCAACAGGAAAAGCA-3'
	Occludin	*Ocln*	5'-TGGCAAGCGATCATACCCAGAG-3'	5'-CTGCCTGAAGTCATCCACACTC-3'
	ZO-1	*Tjp1*	5'-GTTGGTACGGTGCCCTGAAAGA-3'	5'-GCTGACAGGTAGGACAGACGAT-3'
	GAPDH	*Gapdh*	5'-CCATGGAGAAGGCTGGGG-3'	5'-CAAAGTTGTCATGGATGACC-3'
Protein Transporters	ASBT	*Slc10a2*	5'-TGGGTTTCTTCCTGGCTAGACT-3'	5'-TGTTCTGCATTCCAGTTTCCAA-3'
	CFTR	*Cftr*	5'-CTGGACCACACCAATTTTGAGG-3'	5'-GCGTGGATAAGCTGGGGAT-3'
	DRA	*Slc26a3*	5'-TGGTGGGAGTTGTCGTTACA-3'	5'-CCCAGGAGCAACTGAATGAT-3'
	MCT-1	*Slc16a1*	5'-TTGGACCCCAGAGGTTCTCC-3'	5'-AGGCGGCCTAAAAGTGGTG-3'
	NPC1L1	*Npc1l1*	5'-TGGACTGGAAGGACCATTTCC-3'	5'-GACAGGTGCCCCGTAGTCA-3'
	NHE-3	*Slc9a3*	5'-GGCCTTCATTCGCTCCCCAAG-3'	5'-ATGCTTGTACTCCTGCCGAGG-3'
	SMCT-1	*Slc5a8*	5'-TGCCATTTCCTTATGGGTAGG-3'	5'-AGTGGAGTCCTTTCCGCATTA-3'

	SERT	*Slc6a4*	5'-GGCTGAGATGAGGAACGAAG-3'	5'-CTGCAAACGTGCTATCCAAA-3'
	GAPDH	*Gapdh*	5'- TGTGTCCGTCGTGGATCTGA-3'	5'-CCTGCTTCACCACCTCTTGAT-3'

Supplementary Table S2. Inflammation-associated genes in the nCounter® Mouse Inflammation v2 XT NanoString CodeSet.

Gene Name	Accession #	Gene Name	Accession #
Ager	NM_007425.2	Ccl21a	NM_011124.4
Alox12	NM_007440.4	Ccl22	NM_009137.2
Alox15	NM_009660.3	Ccl24	NM_019577.4
Alox5	NM_009662.2	Ccl3	NM_011337.1
Areg	NM_009704.3	Ccl4	NM_013652.1
Arg1	NM_007482.3	Ccl5	NM_013653.1
Atf2	NM_001025093.1	Ccl7	NM_013654.2
Bcl2l1	NM_009743.4	Ccl8	NM_021443.2
Bcl6	NM_009744.3	Ccr1	NM_009912.4
Birc2	NM_007465.2	Ccr2	NM_009915.2
C1qa	NM_007572.2	Ccr3	NM_009914.4
C1qb	NM_009777.2	Ccr4	NM_009916.2
C1ra	NM_023143.3	Ccr7	NM_007719.2
C1s	NM_144938.2	Cd163	NM_053094.2
C2	NM_013484.2	Cd4	NM_013488.2
C3	NM_009778.2	Cd40	NM_011611.2
C3ar1	NM_009779.2	Cd40lg	NM_011616.2
C4a	NM_011413.2	Cd55	NM_010016.2
C6	NM_016704.2	Cd86	NM_019388.3
C7	XM_356827.6	Cdc42	NM_009861.1
C8a	NM_146148.1	Cebpb	NM_009883.3
C8b	NM_133882.2	Cfb	NM_008198.2
C9	NM_013485.1	Cfd	NM_013459.1
Ccl11	NM_011330.3	Cfl1	NM_007687.5
Ccl17	NM_011332.2	Chi3l3	NM_009892.1
Ccl19	NM_011888.2	Creb1	NM_133828.2
Ccl2	NM_011333.3	Crp	NM_007768.4
Ccl20	NM_016960.1	Csf1	NM_001113530.1

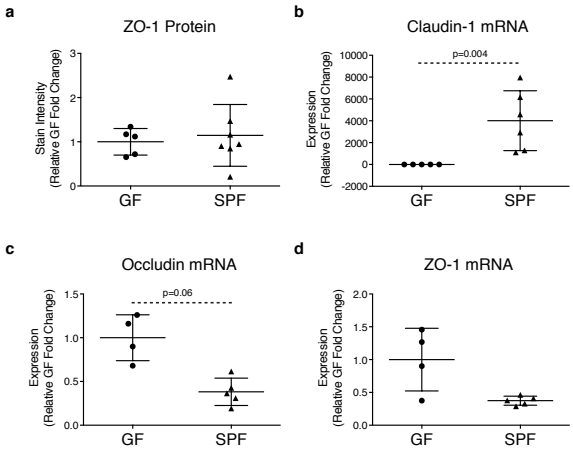
Csf2	NM_009969.4	Ifi27l2a	NM_029803.1
Csf3	NM_009971.1	Ifi44	NM_133871.2
Cxcl1	NM_008176.1	Ifit1	NM_008331.2
Cxcl10	NM_021274.1	Ifit2	NM_008332.2
Cxcl2	NM_009140.2	Ifit3	NM_010501.1
Cxcl3	NM_203320.2	Ifna1	NM_010502.2
Cxcl5	NM_009141.2	Ifnb1	NM_010510.1
Cxcl9	NM_008599.2	Ifng	NM_008337.1
Cxcr1	NM_178241.4	Iigp1	NM_021792.3
Cxcr2	NM_009909.3	Il10	NM_010548.1
Cxcr4	NM_009911.3	Il10rb	NM_008349.5
Cysltr1	NM_021476.4	Il11	NM_008350.2
Cysltr2	NM_001162412.1	Il12a	NM_008351.1
Daxx	NM_007829.3	Il12b	NM_008352.1
Ddit3	NM_007837.3	Il13	NM_008355.2
Defa-rs1	NM_007844.2	Il15	NM_008357.1
Elk1	NM_007922.4	Il17a	NM_010552.3
Fasl	NM_010177.3	Il18	NM_008360.1
Flt1	NM_010228.3	Il18rap	NM_010553.2
Fos	NM_010234.2	Il1a	NM_010554.4
Fxyd2	NM_052823.2	Il1b	NM_008361.3
Gnaq	NM_008139.5	Il1r1	NM_001123382.1
Gnas	NM_010309.3	Il1rap	NM_008364.2
Gnb1	NM_008142.3	Il1rn	NM_031167.4
Gngt1	NM_010314.2	Il2	NM_008366.3
Gpr44	NM_009962.2	Il21	NM_021782.2
Grb2	NM_008163.3	Il22	NM_016971.1
H2-Ea-ps	NM_010381.2	Il22ra2	NM_178258.5
H2-Eb1	NM_010382.2	Il23a	NM_031252.1
Hc	NM_010406.1	Il23r	NM_144548.1
Hdac4	NM_207225.1	Il3	NM_010556.4
Hif1a	NM_010431.2	Il4	NM_021283.1
Hmgb1	NM_010439.3	Il5	NM_010558.1
Hmgb2	NM_008252.3	Il6	NM_031168.1
Hmgn1	NM_008251.3	Il6ra	NM_010559.2
Hras1	NM_008284.2	Il7	NM_008371.2
Hsh2d	NM_197944.1	Il9	NM_008373.1
Hspb1	NM_013560.2	Irf1	NM_008390.1
Hspb2	NM_024441.3	Irf3	NM_016849.3

Irf5	NM_012057.3	Mrc1	NM_008625.1
Irf7	NM_016850.2	Mx1	NM_010846.1
Itgb2	NM_008404.4	Mx2	NM_013606.1
Jun	NM_010591.2	Myc	NM_010849.4
Keap1	NM_016679.4	Myd88	NM_010851.2
Kng1	NM_023125.3	Myl2	NM_010861.3
Limk1	NM_010717.2	Nfatc3	NM_010901.2
Lta	NM_010735.1	Nfe2l2	NM_010902.3
Ltb	NM_008518.2	Nfkb1	NM_008689.2
Ltb4r1	NM_008519.2	Nlrp3	NM_145827.3
Ltb4r2	NM_020490.2	Nod1	NM_172729.2
Ly96	NM_016923.1	Nod2	NM_145857.2
Maff	NM_010755.3	Nos2	NM_010927.3
Mafg	XM_001002362.1	Nox1	NM_172203.1
Mafk	NM_010757.2	Nr3c1	NM_008173.3
Map2k1	NM_008927.3	Oas1a	NM_145211.2
Map2k4	NM_009157.4	Oas2	NM_145227.2
Map2k6	NM_011943.2	Oasl1	NM_145209.2
Map3k1	NM_011945.2	Pdgfa	NM_008808.3
Map3k5	NM_008580.4	Pik3c2g	NM_011084.2
Map3k7	NM_172688.2	Pla2g4a	NM_008869.2
Map3k9	NM_177395.4	Plcb1	NM_019677.1
Mapk1	NM_001038663.1	Ppp1r12b	NM_001081307.1
Mapk14	NM_011951.2	Prkca	NM_011101.3
Mapk3	NM_011952.2	Prkcb	NM_008855.2
Mapk8	NM_016700.3	Ptger1	NM_013641.2
Mapkapk2	NM_008551.1	Ptger2	NM_008964.4
Mapkapk5	XM_990515.1	Ptger3	NM_011196.2
Masp1	NM_008555.2	Ptger4	NM_008965.1
Masp2	NM_010767.3	Ptgfr	NM_008966.3
Max	NM_008558.1	Ptgir	NM_008967.3
Mbl2	NM_010776.1	Ptgs1	NM_008969.3
Mef2a	XM_976032.1	Ptgs2	NM_011198.3
Mef2b	NM_001045484.1	Ptk2	NM_007982.2
Mef2c_Mm	NM_025282.2	Rac1	NM_009007.2
Mef2d	NM_133665.3	Raf1	NM_029780.3
Mknk1	NM_021461.4	Rapgef2	NM_001099624.2
Mmp3	NM_010809.1	Rela	NM_009045.4
Mmp9	NM_013599.2	Relb	NM_009046.2

Retnla	NM_020509.3	Tlr5	NM_016928.2
Rhoa	NM_016802.4	Tlr6	NM_011604.3
Ripk1	NM_009068.3	Tlr7	NM_133211.3
Ripk2	NM_138952.3	Tlr8	NM_133212.2
Rock2	NM_009072.2	Tlr9	NM_031178.2
Rps6ka5	NM_153587.2	Tnf	NM_013693.1
Shc1	NM_011368.4	Tnfaip3	NM_009397.2
Smad7	NM_001042660.1	Tnfsf14	NM_019418.2
Stat1	NM_009283.3	Tollip	NM_023764.3
Stat2	NM_019963.1	Tradd	NM_001033161.2
Stat3	NM_213659.2	Traf2	NM_009422.2
Tbxa2r	NM_001277265.1	Trem2	NM_031254.2
Tcf4	NM_013685.1	Tslp	NM_021367.1
Tgfb1	NM_011577.1	Twist2	NM_007855.2
Tgfb2	NM_009367.1	Tyrobp	NM_011662.2
Tgfb3	NM_009368.2	Cltc	NM_001003908.1
Tgfbr1	NM_009370.2	Gapdh	NM_008084.1
Tlr1	NM_030682.1	Gusb	NM_010368.1
Tlr2	NM_011905.2	Hprt	NM_013556.2
Tlr3	NM_126166.2	Pgk1	NM_008828.2
Tlr4	NM_021297.2	Tubb5	NM_011655.4

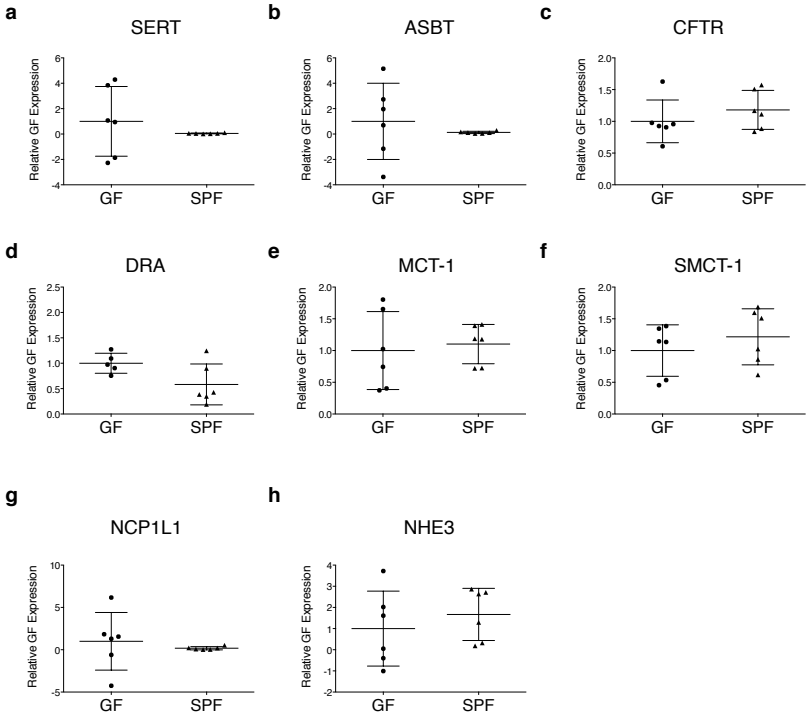
Supplemental Table S3. Scoring systems for stool consistency and presence of blood.

Score	Stool Consistency	Blood in Stool
0	Firm stool, distinctly formed pellets	Negative Hemoccult test
1	Soft stool, elongated pellets	No visible blood, positive Hemoccult test
2	Very soft stool, no distinct pellets	Some blood visible in stool
3	Completely liquid stool	Blood visible throughout stool



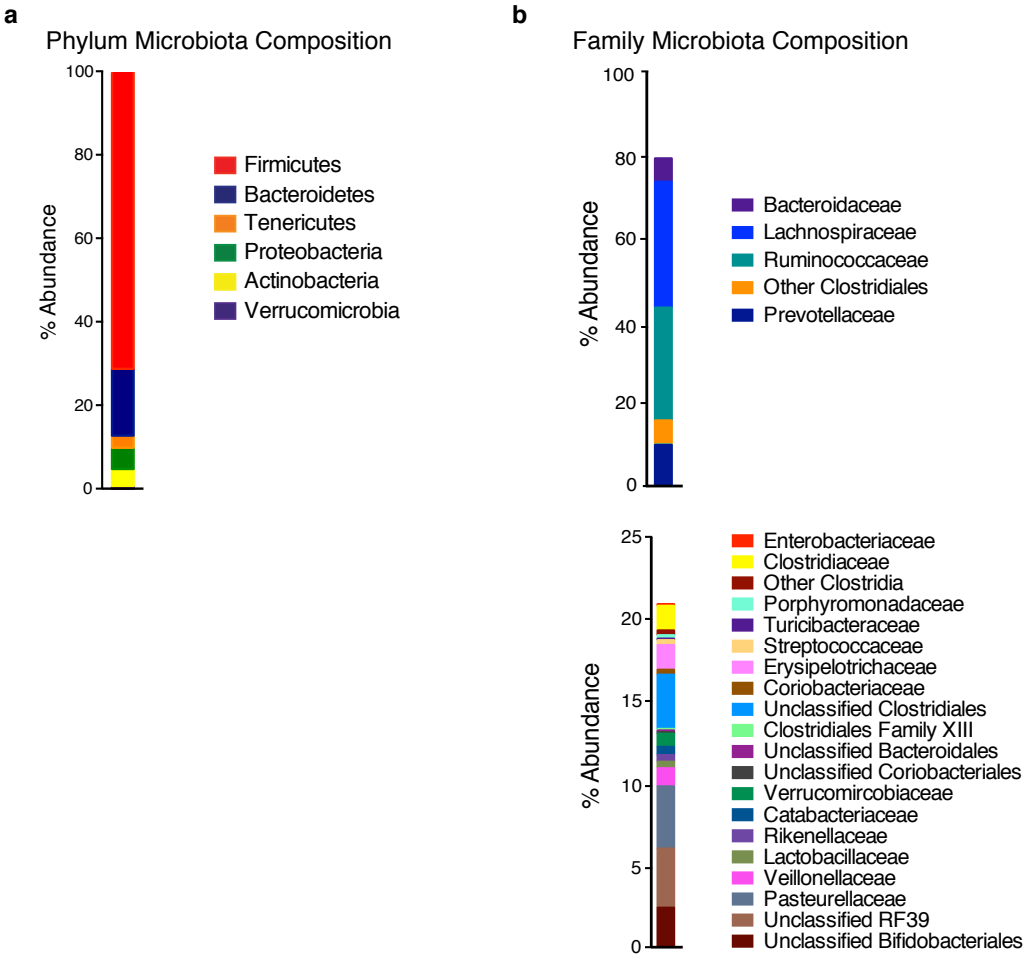
Supplementary Figure S1. Tight junction gene and protein expression in germ-free compared to conventional mice.

Tight junctions were evaluated in germ-free (GF) and conventionally raised specific pathogen free (SPF) mice. (a) Immunofluorescent staining was used to assess (d) ZO-1 protein expression. Real-time qPCR was used to evaluate mRNA expression of (b) claudin-1, (c) occludin, and (d) ZO-1 mRNA. Each data point represents one mouse; the horizontal line and whiskers depict the mean±standard deviation. Significance was assessed using by unpaired, two-tailed Mann-Whitney test.



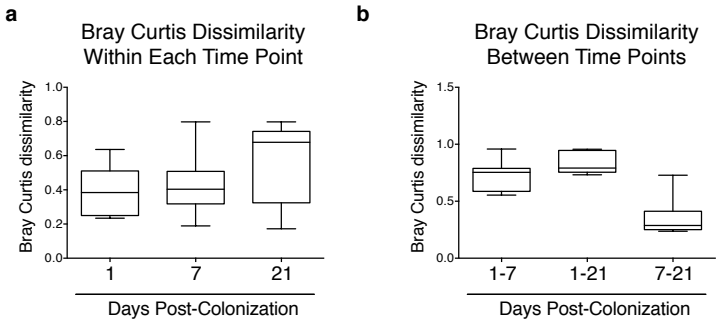
Supplementary Figure S2. Colonic apical membrane protein transporter expression.

Real-time qPCR was used to evaluate colon expression of apical membrane protein transporters in germ-free (GF) and specific pathogen free (SPF) mice. (a) SERT (b) ASBT (c) CFTR (d) DRA (e) MCT-1 (f) SMCT-1 (g) NCP1L1 (h) NHE3. Each data point represents one mouse; the horizontal line and whiskers depict the mean±standard deviation. Significance was assessed by unpaired, two-tailed Mann-Whitney test.

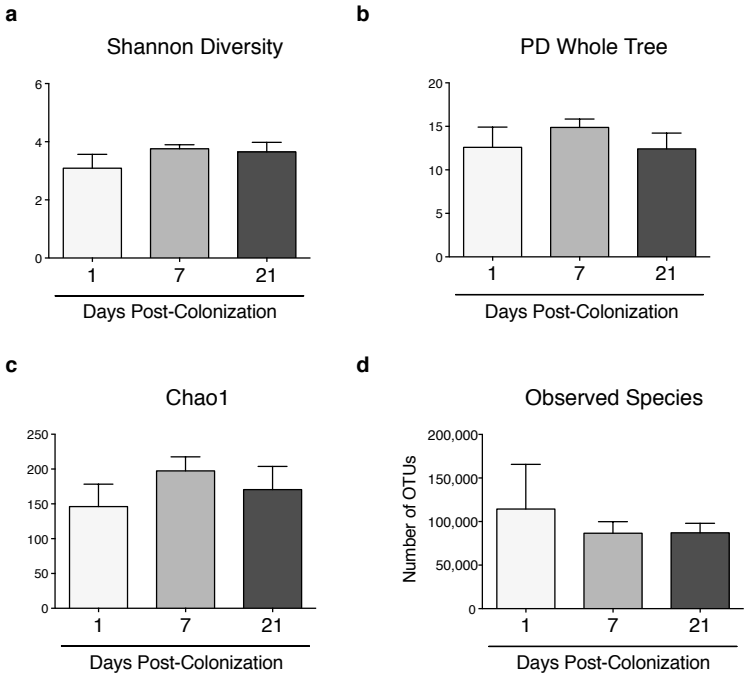


Supplementary Figure S3. Donor microbiota taxonomic composition and structure.

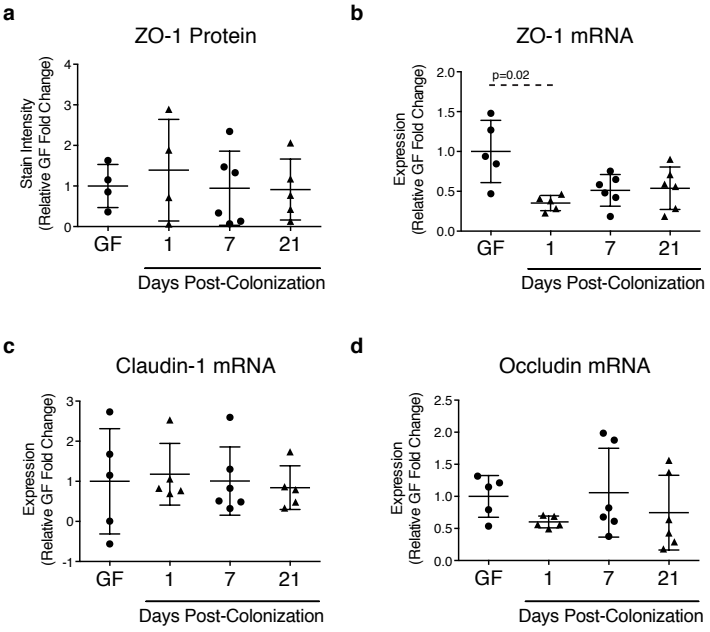
Donor fecal microbiota inoculum was assessed by 16S rRNA gene sequencing at the (a) phylum and (b) family levels.



Supplementary Figure S4. Bray Curtis dissimilarity post-colonization.
16S rRNA gene sequencing was performed on fecal samples collected in a single experiment from the same mice (n=7) at days 1, 7 and 21 post-colonization with human fecal microbiota. (a) Bray Curtis dissimilarity between mice within each time point. (b) Bray Curtis dissimilarity of the individual mice between time points. Whiskers depict the minimum and maximum.

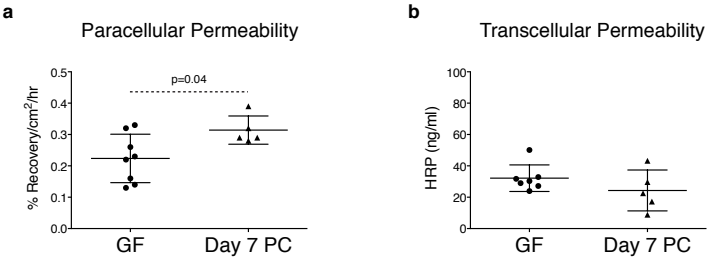


Supplementary Figure S5. Alpha diversity is not significantly changed post-colonization.
16S rRNA gene sequencing from fecal samples collected in a single experiment from the same mice (n=7) at days 1, 7 and 21 post-colonization with human fecal microbiota does not indicate significant changes in (a) Shannon diversity, (b) PD whole tree, (c) Chao1 or (d) observed species were found. Significance was evaluated using a Kruskal-Wallis test with Dunn's post-hoc test.



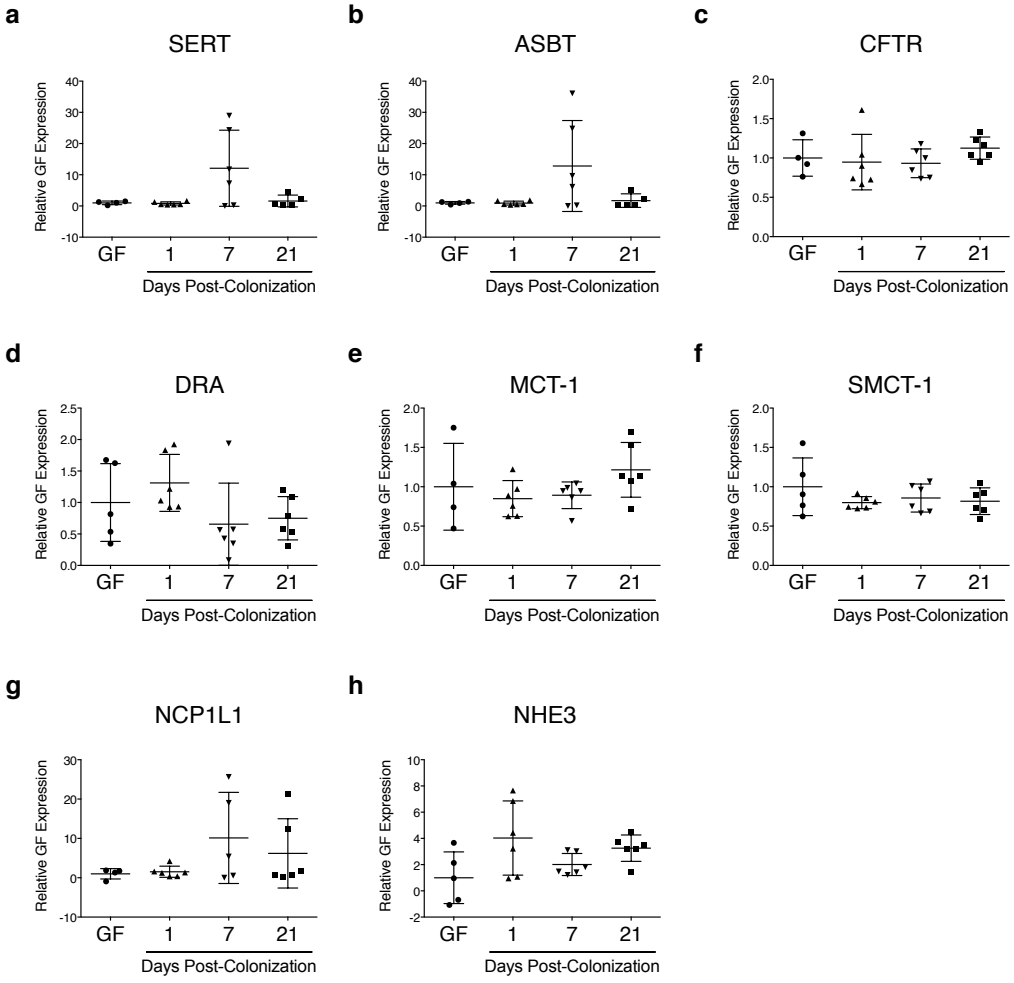
Supplementary Figure S6. Tight junction gene and protein expression post-colonization.

Tight junctions were evaluated in germ-free (GF) mice and at days 1, 7, and 21 post-colonization with human fecal microbiota. (a) Immunofluorescent staining was used to assess ZO-1 protein expression. Real-time qPCR was used to evaluate expression of (b) ZO-1 (c) claudin-1 and (d) occludin mRNA. Each data point represents one mouse; the horizontal line and whiskers depict the mean±standard deviation. Significance was assessed using by Kruskal-Wallis test with Dunn's post-hoc test.



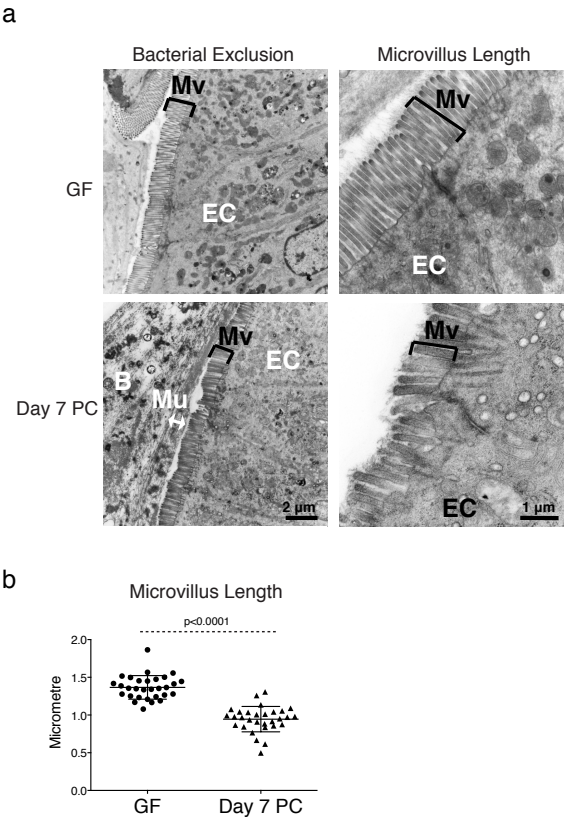
Supplementary Figure S7. Paracellular permeability changes by day 7 post-colonization are independent of colonization mode.

(a) Paracellular and (b) transcellular permeability to ^{51}Cr-EDTA and horseradish peroxidase, respectively, were evaluated by Ussing chambers in germ-free (GF) mice and at day 7 post-colonization by application of human fecal microbiota to face and paws. Each data point represents one mouse; the horizontal line and whiskers depict the mean±standard deviation. Significance was determined using an unpaired, one-tailed Mann-Whitney test.



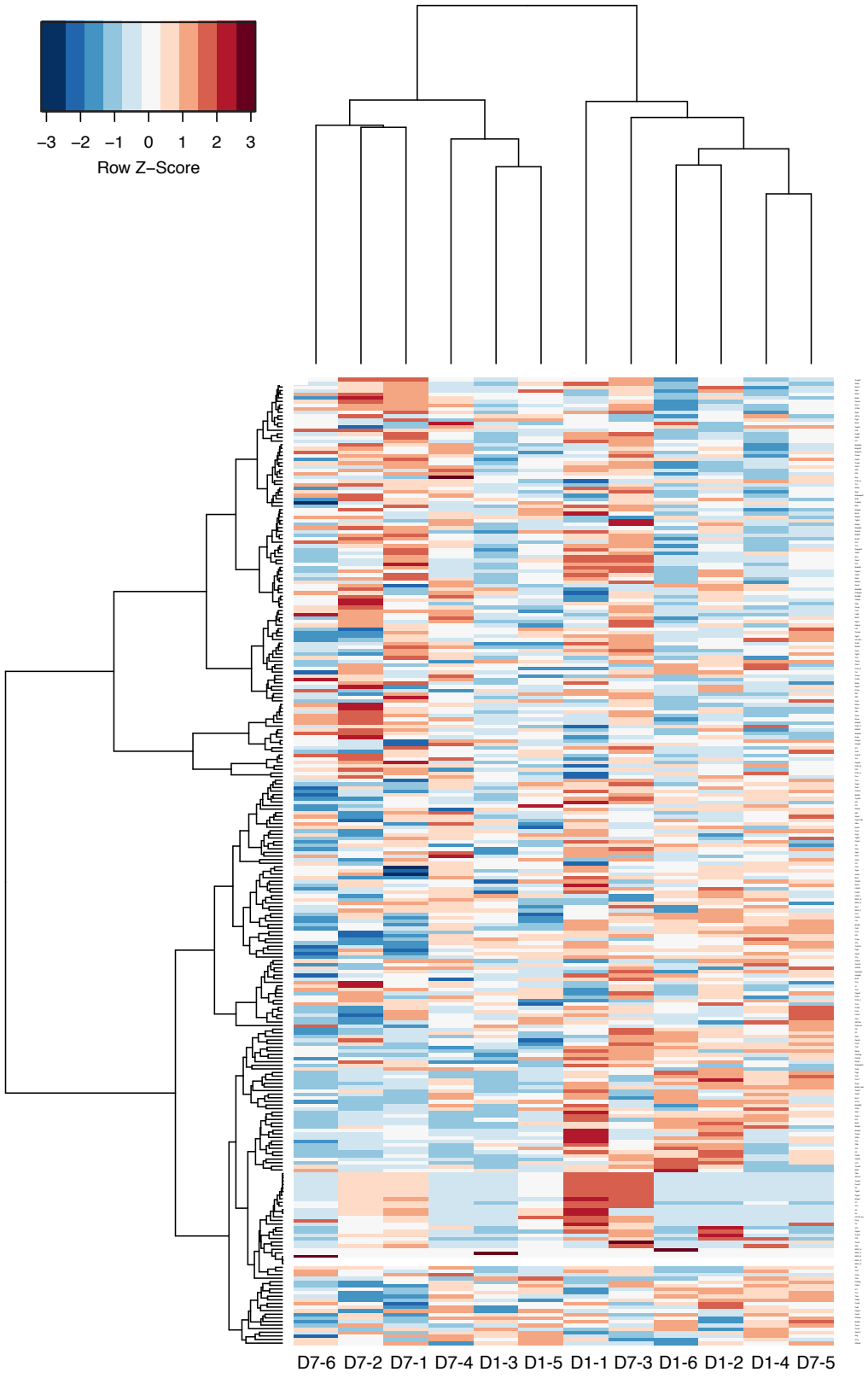
Supplementary Figure S8. Colonic mRNA expression of apical membrane protein transporters are not significantly changed post-colonization.

Real-time qPCR was used to evaluate expression of apical membrane protein transporters in the colon at day 1, 7 and 21 post-colonization with human fecal microbiota of germ-free (GF) mice. (a) SERT (b) ASBT (c) CFTR (d) DRA (e) MCT-1 (f) SMCT-1 (g) NCP1L1 (h) NHE3. Each data point represents one mouse; the horizontal line and whiskers depict the mean±standard deviation. Significance was assessed by Kruskal-Wallis test with Dunn's post-hoc test.



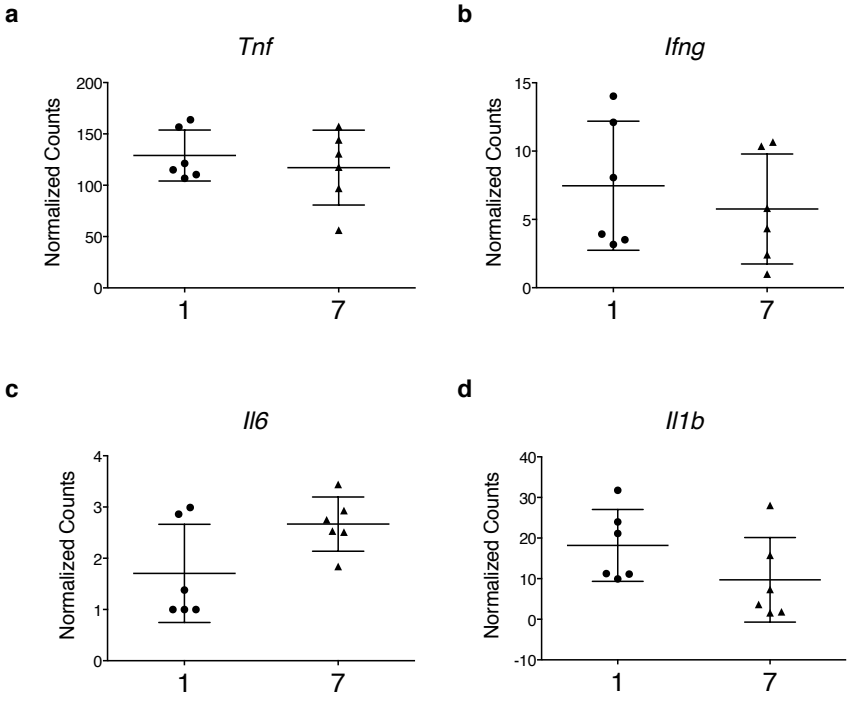
Supplementary Figure S9. Induction of colon structural changes by day 7 post-colonization is independent of colonization mode.

Colon structure was evaluated in germ-free (GF) mice and at day 7 post-colonization by application of human fecal microbiota to face and paws. (a) Representative electron microscopy images demonstrating bacterial exclusion and microvillus length. Markings indicate microvilli (Mv), epithelial cells (EC), and the black arrow indicates separation of bacteria (B) from the epithelium by the mucus (Mu) layer. (b) Microvillus length. Each data point represents analysis of one image; the horizontal line and whiskers depict the mean±standard deviation. Data was collected over 2-5 independently executed experiments. Significance was determined using an unpaired, two-tailed Mann-Whitney test.


−3 −2 −1 0 1 2 3
Row Z−Score
D7-6 D7-2 D7-1 D7-4 D1-3 D1-5 D1-1 D7-3 D1-6 D1-2 D1-4 D7-5

Supplementary Figure S10. Heat map of inflammation-associated gene expression in the colonic IEL-enriched compartment.

IELs from the colon were isolated at days 1 and 7 (D1 and D7, respectively followed by sample number 1-6) post-colonization and Log2 transformed gene expression values of inflammation-associated genes determined by NanoString.



Supplementary Figure S11. Expression of cytokine genes in the colonic IEL-enriched compartment at days 1 and 7 post-colonization.

Cytokine gene expression in the IEL-enriched compartment of the colon was evaluated by NanoString. (a) *Tnf* (b) *Ifng* (c) *Il6* (d) *Il1b*. Each data point represents one mouse; the horizontal line and whiskers depict the mean±standard deviation. Significance was assessed by an unpaired, two-tailed Mann-Whitney test.

CHAPTER 4

DIFFERENTIAL INDUCTION OF ANTIMICROBIAL REGIII BY THE INTESTINAL MICROBIOTA AND *Bifidobacterium breve* NCC2950

Differential Induction of Antimicrobial REGIII by the Intestinal Microbiota and *Bifidobacterium breve* NCC2950.

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The material in this chapter has been published in Applied and Environmental Microbiology. 2013 Dec;79(24):7745. doi: 10.1128/AEM.02470-13.

Preface: The work I performed on this manuscript was carried out from May 2013 to Sept. 2013. I share primary authorship of the manuscript with J. Natividad. I was responsible for writing the manuscript with input from J. Natividad and my supervisor E. Verdu. E. Verdu and J. Natividad designed experiments. Experiments were primarily executed and analyzed by J. Natividad with technical assistance from J. Jury, JP. Motta, and H. Galipeau, and V. Philips. The probiotic biomass was supplied by C. Garcia-Rodenas. RegIII antibodies were provided by H. Kiyama. Scientific input and critical assessment of the manuscript was given by P. Bercik.

SUMMARY AND SIGNIFICANCE

This manuscript evaluates how specific bacteria and microbial communities differentially stimulate intestinal expression of the antimicrobial peptide REGIII through colonization of germ-free mice. Our findings indicate the level of REGIII induction is dependent on the specific members of intestinal microbiota. *Bifidobacterium breve* NCC2950 stimulated intestinal epithelial cell REGIII production, mediated by MyD88-Ticam1 signaling. Altogether, these results demonstrate REGIII production can be differentially induced by the microbiota, and further support microbiota-modulating approaches to enhance intestinal barrier integrity.

Title: Differential induction of antimicrobial REGIII by intestinal microbiota and *Bifidobacterium breve* NCC2950.

Running Title: Microbiota and *B. breve*-Induced REGIII Expression.

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ABSTRACT

The intestinal microbiota is a key determinant of gut homeostasis, which is achieved, in part, through regulation of antimicrobial peptide secretion. The aim of this study was to determine the efficiency by which members of the intestinal microbiota induce the antimicrobial peptide REGIII and to elucidate the underlying pathways. We showed that germfree mice have low levels of RegIII-γ in their ileum and colon compared to mice with different intestinal microbiota backgrounds. Colonization with a microbiota of low diversity (altered Schaedler flora) did not induce the expression of RegIII-γ as effectively as a complex community (specific pathogen free). Monocolonization with the probiotic *Bifidobacterium breve* NCC2950, but not with the nonprobiotic commensal *Escherichia coli* JM83, upregulated RegIII-γ expression. Induction of RegIII-γ by *B. breve* was abrogated in mice lacking MyD88 and Ticam1 signaling. Both live and heat-inactivated *B. breve*, but not spent culture medium from *B. breve*, induced the expression of *REGIIIA*, the human ortholog and homolog of RegIII-γ, in human colonic epithelial cells (Caco-2). Taken together, the results suggest that RegIII-γ expression in the intestine correlates with the richness of microbiota composition. Also, specific bacteria such as *Bifidobacterium breve* NCC2950 effectively induce REGIII production in the intestine via the *MyD88-Ticam1* pathway. Treatment with this probiotic may enhance the mucosal barrier and protect the host from infection and inflammation.

INTRODUCTION

Antimicrobial peptides secreted by intestinal immune and epithelial cells are important effectors of innate immunity. These endogenous peptides are induced during exposure to enteric pathogens in an attempt to protect the host from infection (1). It is increasingly apparent that antimicrobial peptides also play an essential role in the maintenance of intestinal homeostasis by limiting microbial-epithelium interactions and preventing unnecessary microbe-driven inflammation (2). This is particularly important in the distal gut where microbiota load and density are high.

The intestinal microbiota consists of a complex community of bacteria with various physiological and immune-modulating capacities (3). A balanced composition of symbionts and pathobionts is thought to stimulate homeostatic responses in the host (3), while shifts in this balance (dysbiosis) have been associated with inflammatory disorders of the gut, such as inflammatory bowel disease (IBD) (4). Recently, it has been shown that the intestinal microbiota provides pivotal stimuli and cues necessary for the induction of antimicrobial peptides (5).

Regenerating islet-derived III (REGIII) proteins, which belong to the family of C-type lectins, are one class of antimicrobials that are expressed in the intestine. In mice, three distinct classes of RegIII, RegIII-α, -β, and -γ, have been identified. In contrast, only REGIII-α and -β have been identified in humans. Human REGIII-α, also known as hepatocarcinoma-intestine-pancreas/pancreatic-

associated protein (HIP/PAP), shares 67% homology with murine RegIII-γ, while human REGIII-γ shares 68% homology with murine RegIII-β. REGIII proteins bind to the peptidoglycan moieties of bacteria and induce damage to the bacterial cell wall (6–8). Different intestinal cell types express REGIII proteins (6, 9). We have previously shown that modulating intestinal microbiota composition, either by colonizing with microbiota devoid of pathobionts or supplementation with a probiotic bacterium, affects the expression of *Reg3g* in mice lacking intracellular microbial recognition receptors (10). However, it is unclear whether a specific component(s) of the intestinal microbiota differentially and directly regulates the expression of REGIII proteins by various intestinal cell types.

Here, we sought to determine whether different components of the intestinal microbiota and specific probiotics differ in their capacities to stimulate the expression of antimicrobial peptide REGIII by intestinal epithelial cells (IECs). For this, we investigated the effects of colonization with both diverse communities and specific components of the microbiota on RegIII-γ expression by ileal and colonic epithelial cells. RegIII-γ levels were quantified in whole ileal and colonic tissue of germfree (GF) mice and in mice colonized with specific-pathogen-free (SPF) microbiota, altered Schaedler flora (ASF), commensal *Escherichia coli* JM83, or the probiotic *Bifidobacterium breve* NCC2950. The importance of Toll-like receptor (TLR) signaling in this process was also investigated using GF *MyD88*$^{-/-}$*; Ticam1*$^{-/-}$ mice. Furthermore, *REG3A* expression

in human colonic epithelial cells was also quantified after stimulation with *E. coli* or *B. breve*.

MATERIALS AND METHODS

Mice. *MyD88*$^{-/-}$*; Ticam1*$^{-/-}$ mice on a C57BL/6 background were kindly provided by B. A. Beutler (La Jolla, CA, USA). SPF C57BL/6 mice were purchased from Taconic. Germfree C57BL/6 and *MyD88*$^{-/-}$*; Ticam1*$^{-/-}$ mice were rederived at the McMaster University Axenic Gnotobiotic Unit (AGU) by an axenic two-cell embryo transfer technique previously described (11). Germfree mouse colonies were maintained in flexible film isolators at the AGU, and germfree status was routinely confirmed by a combination of culture- and non-culture-based techniques in fecal and cecal contents (11). Periodic serological testing was also performed for viruses, parasites, and known pathogens (Charles Rivers Laboratories). All mice had unlimited access to autoclaved food and water. Both male and female mice were used at the age of 8 to 12 weeks. All experiments were carried out in accordance with the McMaster University animal utilization protocols.

Bacterial strains. *Bifidobacterium breve* (*B. breve*) NCC2950 was obtained from the Nestlé Culture Collection (Lausanne, Switzerland) and grown under anaerobic conditions in deMan-Rogosa-Sharpe (MRS) medium supplemented with 0.05% (vol/vol) L-cysteine hydrochloride. *Escherichia coli* JM83 (American Type

Culture Collection [ATCC]) was grown under aerobic conditions in Luria broth (LB; Oxoid) medium. After 18 h at 37°C, bacterial cells were pelleted, washed in phosphate-buffered saline (PBS), resuspended at a concentration of 10^{10} CFU/ml in PBS with 20% (vol/vol) glycerol, and kept in frozen aliquots until used. Growth medium was spun down and sterile filtered to generate the spent culture medium (SCM). The heat-inactivated (HI) formulation was prepared by incubating 50-ml aliquots of *B. breve* cells at 90°C for 1 h.

Colonization and monocolonization experiments. Monocolonization of GF mice was performed in dedicated flexible film gnotobiotic isolators as previously described (11). Briefly, *Bifidobacterium breve* or *Escherichia coli* JM83 cultures were prepared and imported into the isolator following strict aseptic procedures. A total of 10^9 CFU of bacteria of each preparation was introduced by gavage into the stomach of each mouse. Three weeks after monocolonization, mice were used for experiments. Monocolonization was confirmed at sacrifice by plating both the fecal and cecal contents in deMan-Rogosa-Sharpe (MRS; Oxoid) agar supplemented with 0.05% (vol/vol) L-cysteine hydrochloride (Sigma-Aldrich) and mupirocin (Sigma-Aldrich) under anaerobic conditions for *B. breve* and in LB agar under aerobic conditions for *E. coli*. ASF consisted of *Lactobacillus acidophilus*, *Lactobacillus salivarius*, *Bacteroides distasonis*, a spiral-shaped bacterium, and four fusiform, extremely oxygen sensitive bacteria (12). ASF colonization was achieved by cohousing germfree mice with an ASF-colonizer

from McMaster's standard colony and was confirmed by a combination of culture and molecular techniques (10).

Colonic epithelial cell line assays. The Caco-2 cell line was obtained from the ATCC and used from passages 19 to 25. Cell monolayers were maintained in cell medium consisting of Dulbecco's modified Eagle medium, 20% heat-inactivated fetal bovine serum (FBS; PAA), and 1% minimal essential medium (MEM) and nonessential amino acids (Gibco) supplemented with 2mM glutamine, and cells were cultured in a humidified atmosphere with 5% CO_2. For stimulation experiments, cells were seeded in 24-well tissue culture plates and used at 70 to 80% confluence. Cells were stimulated with live or HI *B. breve* or live *E. coli* bacteria at cell-to-bacterium ratios of 1:10 and 1:100 or with SCM (10%, vol/vol). PBS-glycerol (10%, vol/vol) was used as a negative control while interleukin-22 (IL-22; 10ng/ml) (R&D Systems) was a positive control. All stimulations were performed in FBS-free medium with 1% penicillin-streptomycin (Pen-Strep) for 4 to 24 h. After stimulation, cells were washed twice with PBS containing Pen-Strep and 50 μg/ml gentamicin and stored at -80°C until used.

RNA isolation and real-time qPCR. Total RNA from the ileum and colon of mice or cell lines was isolated using an RNeasy Mini Kit (Qiagen). Potential DNA contamination was removed by column DNase treatment (Qiagen). RNA quantity and integrity were checked with a NanoDrop instrument (Thermo

Scientific) and agarose gel electrophoresis. Only samples with intact RNA were used for subsequent cDNA synthesis with iScript reverse transcriptase (Bio-Rad). A total of 500μg of input RNA was used for each sample. Quantitative real-time PCR (RT-qPCR) was performed on an iQ5 Real-Time Detection System (Bio-Rad) with SSofast Evagreen Supermix (Bio-Rad). Primers used were as follows: murine *Reg3g* fwd, 5'-CGTGCCTATGGCTCCTATTGCT-3'; murine *Reg3g* rev, 5'-TTCAGCGCCACTGAGCACAGAC-3'; human *REG3A* fwd, 5'-TATGGCTC CCACTGCTATGCCT-3'; human *REG3A* rev, 5'-TCTTCACCAGGGAGGACA CGAA-3'; *GAPDH* fwd 5'-CCATGGAGAAGGCTGGGG-3'; *GAPDH* rev 5'-CAAAGTTGTCATGGATGACC-3'. The iQ5 manager software (Bio-Rad) was used to calculate the relative fold change in expression normalized to *GAPDH* expression by the $2^{-\Delta\Delta CT}$ (where CT is threshold cycle) method. All procedures were performed according to the manufacturer's instructions.

IF for RegIII-γ proteins. RegIII-γ protein expression in the mouse intestine was evaluated using immunofluorescence (IF) in formalin-fixed, paraffin-embedded tissue sections. Sections were cut (5 μm), deparaffinized, and then blocked with phosphate-buffered saline (PBS)-bovine serum albumin in 2% Tween 20 at 0.05% for 1 h. Samples were stained overnight (4°C) with rabbit anti-mouse RegIII-γ antibodies (1:100 dilution) (13) and for 1 h with secondary antibody at room temperature [goat anti-rabbit IgG(H+L); 1:250 dilution] (Molecular Probes) and mounted in Prolong Gold with 4',6'-diamidino-2-phenylindole (DAPI). For

quantification, specific fluorescence intensity from three different microscopic fields per animal was acquired using a Nikon Eclipse 90i instrument. All individual fields have been normalized by using the same fluorescence acquisition settings and by tissue autofluorescence. Specific RegIII-γ fluorescence staining was quantified using ImageJ software (NIH) and was reported per unit surface of tissue. Data were represented as fold increase of signal intensity compared to the control group (germfree C57BL/6 mice), which was arbitrarily reported as 1.

Statistics. Data are presented as either dot plots or bar graphs (means±standard deviations [SD]). Statistical analysis was performed by using one-way analysis of variance (ANOVA), followed by the Bonferroni test, or by using a two-tailed Student *t* test, when applicable. All statistical testing was performed using GraphPad Prism, version 6 (GraphPad Software Inc.). A *P* value of <0.05 was considered significant.

RESULTS

Germfree mice had lower RegIII-γ expression levels than SPF colonized mice. Different regions of the gastrointestinal tract have unique microenvironments characterized by specialized cell types, microbial diversity, and load. Thus, we first sought to examine ileal and colonic expression of RegIII-γ in the absence of microbiota and to determine whether microbiota with different levels of diversity had an impact on its expression. We measured *Reg3g*

in the ileum and colon of GF- and ASF-colonized and SPF C57BL/6 mice by quantitative real-time PCR. GF mice showed significantly lower *Reg3g* expression than SPF mice (Fig. 1). RegIII-γ protein expression was also examined by immunofluorescence (IF) staining. Consistent with RNA results, RegIII-γ protein expression was significantly lower in GF mice than in SPF mice, and the pattern of expression was different in the ileum from that in the colon (Fig. 2). In the ileum of SPF mice, RegIII-γ was expressed by IECs located both at the crypt and the villi, with the highest fluorescence often localized within the crypt. On the other hand, colonic expression of RegIII-γ in SPF mice was specifically expressed by colonocytes. Overall, our results show that RegIII-γ expression varies between the ileum and colon and is influenced by the type of microbiota it is colonized with.

Monocolonization with *B. breve* but not *E. coli* increased RegIII-γ expression. There are important interindividual differences in gut microbial communities (14, 15), and this may have functional implications. We investigated this using a model of gnotobiotic monocolonization. The effects of *Bifidobacterium breve* NCC2950, previously found to restore *Reg3g* in mice lacking Nod signaling (10), and of *Escherichia coli* JM83 on RegIII expression in the intestine were explored. Three weeks after monocolonization, mice were aseptically exported from flexible film isolators, and tissue was collected to measure RegIII-γ expression. Successful monocolonization was confirmed by selective plating of both fecal and

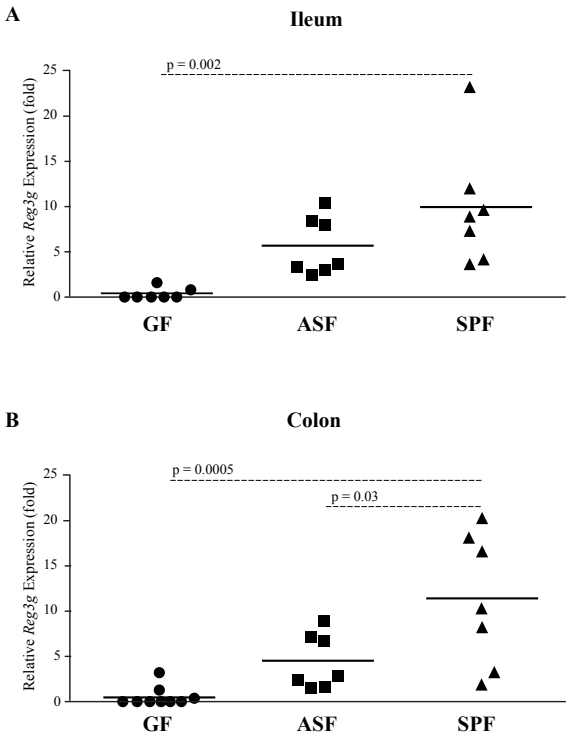

Figure 1. *Reg3g* RNA expression in ileum and colon of GF, ASF-colonized, and SPF mice.

SPF mice had higher *Reg3g* RNA expression levels than ASF-colonized mice. Total RNA from ileum and colon of 8- to 12-week-old mice was extracted, and *Reg3g* expression was measured by RT-qPCR. RNA expression was normalized to *Gapdh*, and results are shown relative to the mean of GF mice, which is set to 1. Each data point represents an individual mouse. Data were pooled from two to three independent experiments. *P* values are calculated using ANOVA, followed by a Bonferroni *post hoc* test.

cecal contents collected at 21 days postcolonization and by fluorescence in situ hybridization (FISH) staining (see Fig. S1 in the supplemental material). *B. breve*- and *E. coli*-monocolonized mice displayed similar bacterial loads in stool and cecal content (Fig. 3). However, only *B. breve*-monocolonized mice exhibited higher expression of ileal and colonic REGIII-γ than GF controls, suggesting that induction of REGIII-γ was strain specific (Fig. 4 and 5).

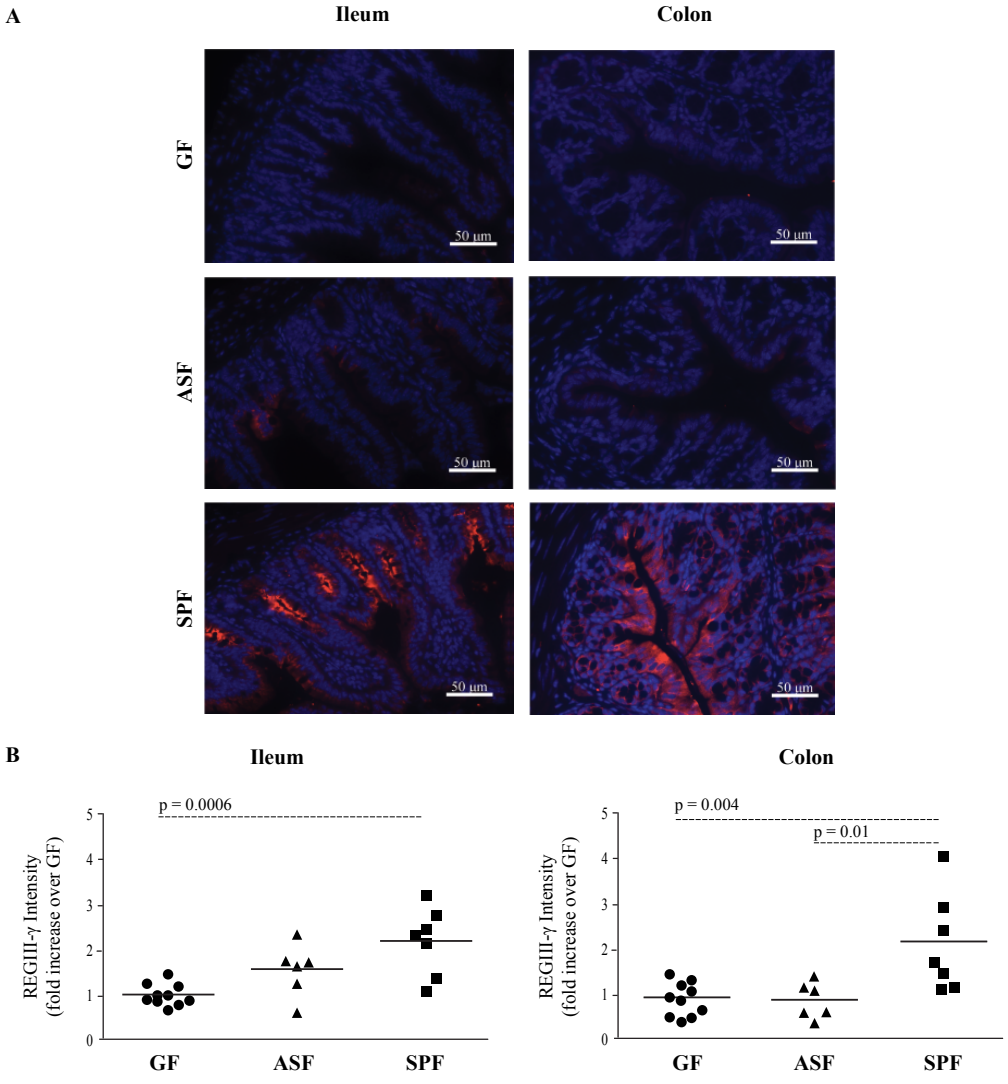

Figure 2. REGIII-γ protein expression in ileum and colon of GF, ASF-colonized, and SPF mice.

SPF mice had higher REGIII-γ protein expression in ileum and colon than GF mice. (A) Representative immunofluorescence staining (anti-REGIII-γ; red) in each group. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). (B) Quantification of REGIII-γ staining intensity was expressed as fold increase relative to the mean of GF mice, which is set to 1. Each data point represents an individual mouse. Data were pooled from two to three independent experiments. *P* values were calculated using ANOVA, followed by a Bonferroni *post hoc* test.

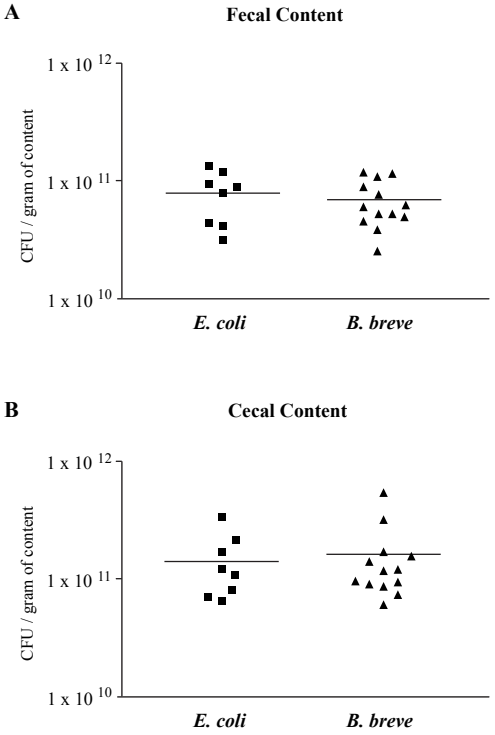

Figure 3. Bacterial load in the feces and cecum of *B. breve*- and *E. coli*-monocolonized mice.

GF mice were monocolonized with either *B. breve* or *E. coli*. At 21 days postmonocolonization, the density of bacteria in the stool and cecal content was determined by selective plating. Data are pooled from two to three independent experiments.

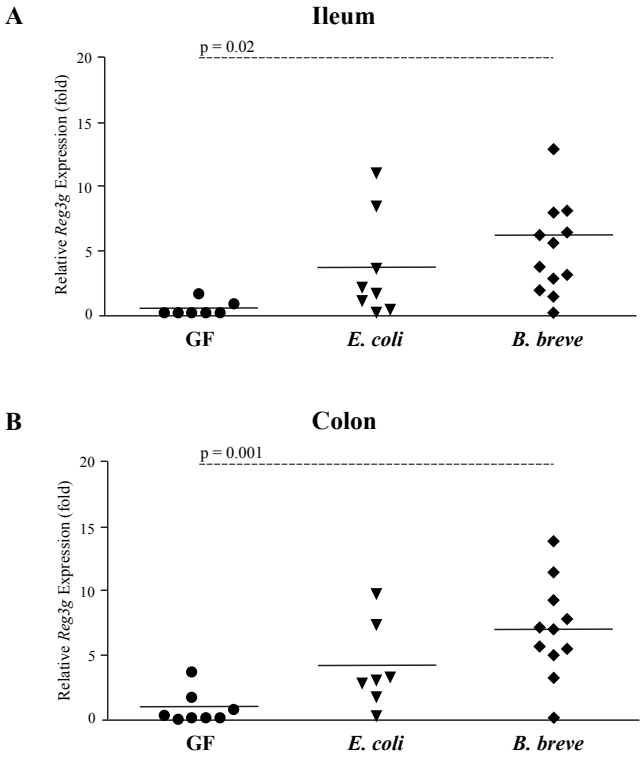

Figure 4. *Reg3g* RNA expression in ileum and colon of GF and *B. breve*- and *E. coli*-monocolonized mice.

Monocolonization with *B. breve* induced higher *Reg3g* RNA expression than monocolonization with E. coli. At 21 days postmonocolonization, total RNA from ileum and colon was extracted, and *Reg3g* expression was measured by RT-qPCR. RNA expression was normalized to *Gapdh*, and results are shown relative to the mean of GF mice, which is set to 1. Each data point represents an individual mouse. Data were pooled from two to three independent experiments. *P* values are calculated using ANOVA, followed by a Bonferroni *post hoc* test.

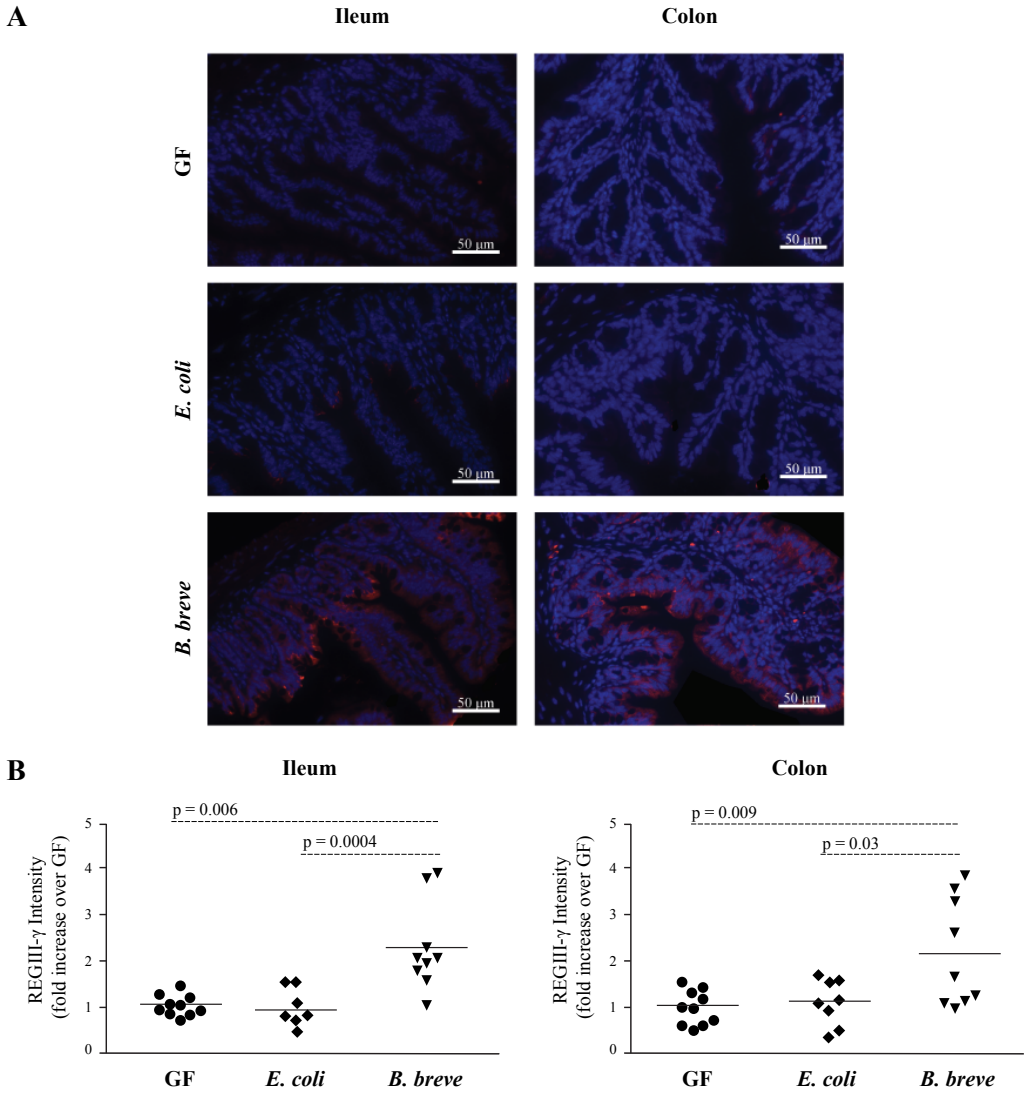

Figure 5. REGIII-γ protein expression in ileum and colon of GF and *B. breve*- and *E. coli*-monocolonized mice.

B. breve-monocolonized mice showed higher REGIII-γ protein expression than *E. coli*-monocolonized mice. (A) Representative immunofluorescence staining (anti-REGIII-γ red) in each group. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). (B) Quantification of REGIII-γ staining intensity was expressed as fold increase relative to the mean of GF mice, which is set to 1. Each data point represents an individual mouse. Data were pooled from two to three independent experiments. *P* values are indicated on the figure and were calculated using ANOVA, followed by a Bonferroni *post hoc* test.

Monocolonization with *B. breve* did not increase RegIII-γ expression in *MyD88*$^{-/-}$*; Ticam1*$^{-/-}$ mice. To determine the host factors that govern the *B. breve*-induced RegIII-γ expression, we used mice that lack myeloid differentiation primary response 88 (Myd88) and TIR-containing adaptor molecule (Ticam1) genes, which are relevant downstream signaling adaptor proteins for all TLRs. Similar to wild-type GF C57BL/6 mice, GF *MyD88*$^{-/-}$*; Ticam1*$^{-/-}$ mice displayed low levels of ileal and colonic RegIII-γ expression. Levels of RegIII-γ expression in *MyD88*$^{-/-}$*; Ticam1*$^{-/-}$ mice, however, remained unchanged after *B. breve* monocolonization (Fig. 6 and 7). Together, these results suggest that microbial induction of RegIII-γ is mediated by MyD88-Ticam-dependent pathways, such as TLR signaling.

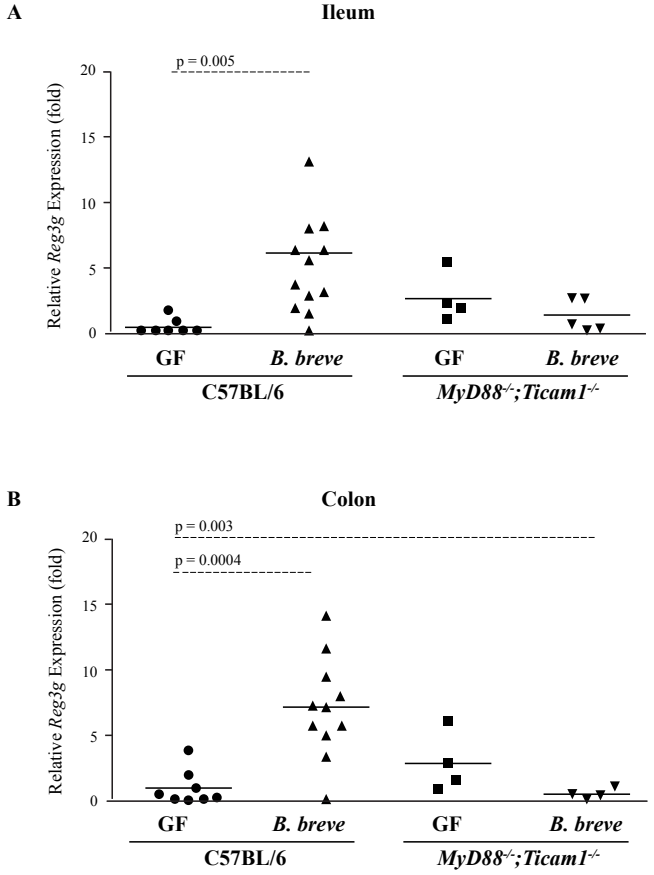

Figure 6. *Reg3g* RNA expression in ileum and colon of *Myd88*$^{-/-}$*; Ticam1*$^{-/-}$ mice monocolonized with *B. breve*.

B. breve-monocolonized *Myd88*$^{-/-}$*; Ticam1*$^{-/-}$ mice displayed *Reg3g* RNA expression levels similar to those of GF *Myd88*$^{-/-}$*; Ticam1*$^{-/-}$ mice. At 21 days postmonocolonization, total RNA from ileum and colon was extracted, and *Reg3g* expression was measured by RT-qPCR. RNA expression was normalized to *Gapdh*, and results are shown relative to the mean of GF mice, which is set to 1. Each data point represents an individual mouse. Data were pooled from two to three independent experiments. *P* values are calculated using ANOVA, followed by a Bonferroni *post hoc* test.

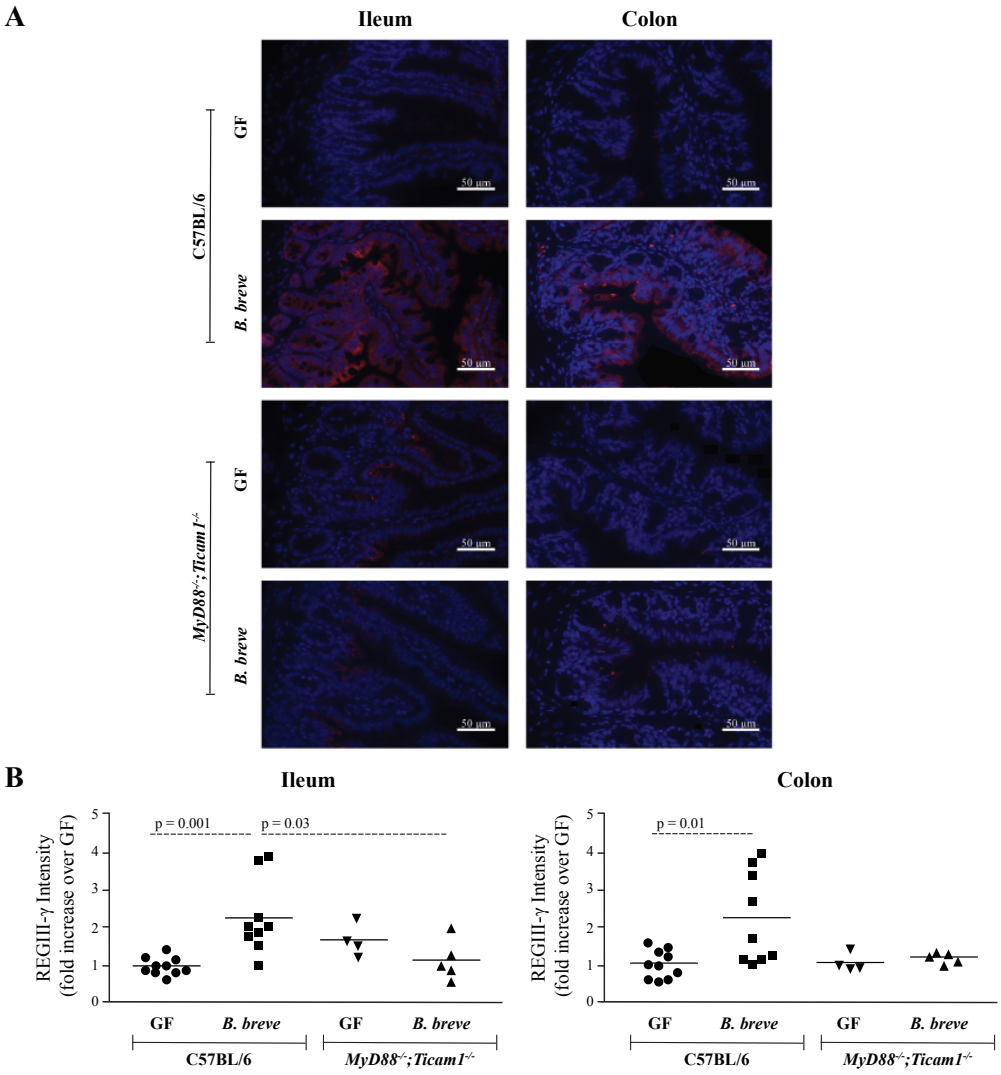

Figure 7. REGIII-γ protein expression in ileum and colon of *Myd88*$^{-/-}$*; Ticam1*$^{-/-}$ mice monocolonized with *B. breve*.

B. breve-monocolonized *Myd88*$^{-/-}$*; Ticam1*$^{-/-}$ mice displayed levels of REGIII-γ protein expression similar to those of GF *Myd88*$^{-/-}$*; Ticam1*$^{-/-}$ mice. (A) Representative immunofluorescence staining (anti-REGIII-γ; red) in each group. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). (B) Quantification of REGIII-γ staining intensity was expressed as fold increase relative to the mean of GF mice, which is set to 1. Each data point represents an individual mouse. Data were pooled from two to three independent experiments. *P* values are calculated using ANOVA, followed by a Bonferoni *post hoc* test.

Live *B. breve* induced the expression of *REG3A* in human colonic epithelial cells. To quantify expression of *REG3A*, the human ortholog and homolog of murine *Reg3g*, we performed *in vitro* experiments using Caco-2 and HT-29 human colon epithelial cell lines. To optimize the expression of *REG3A* induced by bacteria, cells were incubated for 2, 4, 6, and 24 h at cell-to-bacterium ratios of 1:10 and 1:100. Results show that 4 h of incubation at a cell-to-bacterium ratio of 1:100 led to the highest *REG3A* expression level (see Fig. S2 in the supplemental material). Using these parameters, the amount of *REG3A* expression was measured, and our results showed that colonic epithelial cells significantly upregulated expression in response to *B. breve* stimulation but not to stimulation with *E. coli* (Fig. 8).

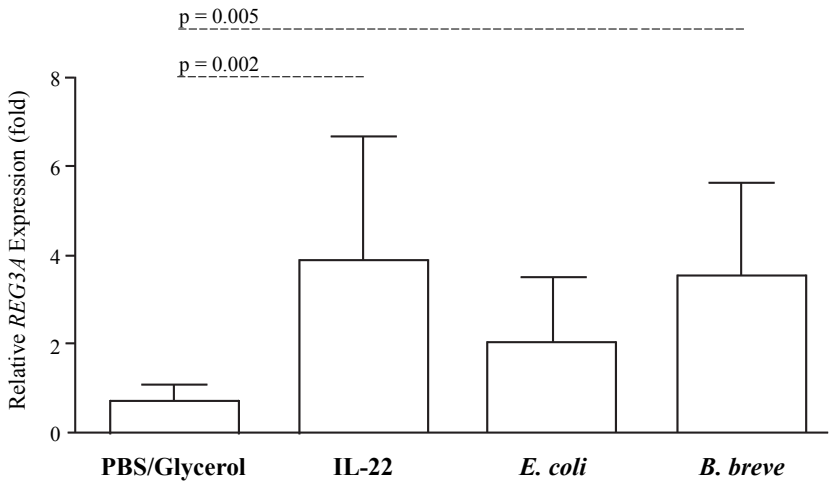

Figure 8. *REG3A* RNA expression in Caco-2 cells stimulated with IL-22 (positive control), *B. breve*, or *E. coli*.
Both IL-22 and *B. breve* induced *REG3A* RNA expression in Caco-2 cells. Total RNA from stimulated cells was extracted, and *REG3A* expression was measured by RT-qPCR. RNA expression was normalized to *GAPDH*, and results are shown relative to the mean of PBS-glycerol-stimulated cells, which is set to 1. Data were pooled from three to four independent experiments with two to four wells per group (means±SD). *P* values are calculated using ANOVA, followed by a Bonferroni *post hoc* test.

We then asked whether *B. breve* actively secretes metabolites able to upregulate *REG3A* expression from IECs. For this, Caco-2 cells were stimulated with live, HI, and SCM formulations of *B. breve*. Our results showed that only live and HI preparations induced higher *REG3A* expression levels than the PBS-glycerol control, suggesting that the whole *B. breve* cell is required for the induction, independently of its metabolic activity (Fig. 9).

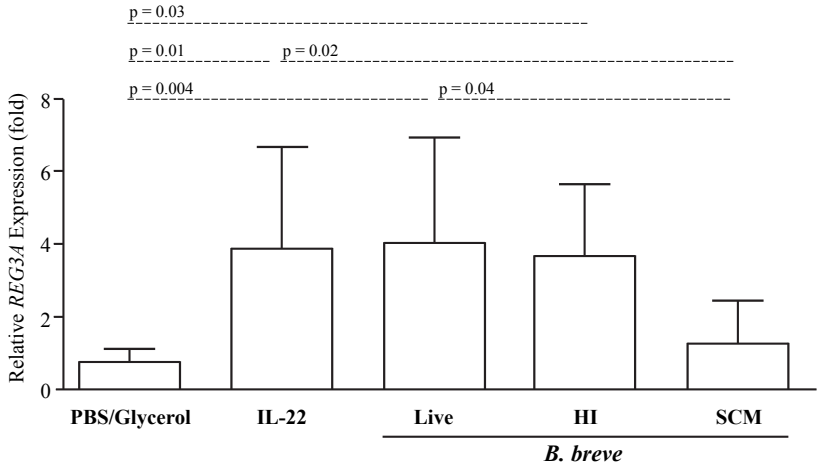

Figure 9. *REG3A* RNA expression in Caco-2 cells stimulated with live and heat-inactivated (HI) *B. breve* and *B. breve* spent culture medium (SCM).
Both live and HI B. breve (live or HI) induced *REG3A* RNA expression in Caco-2 cells. Total RNA from stimulated cells was extracted, and *REG3A* expression was measured by RT-qPCR. RNA expression was normalized to *GAPDH* expression, and results are shown relative to the mean of PBS-glycerol-stimulated cells, which is set to 1. Data were pooled from three to four independent experiments, with two to four wells per group (means±SD). *P* values are calculated using ANOVA, followed by Bonferroni *post hoc* test.

DISCUSSION

Antimicrobial REGIII proteins play an important role in maintaining gut homeostasis through spatially segregating bacteria, preventing potentially harmful immune responses, and protecting the host from infection (16–18). In parallel with previous findings (19–22), this study shows that the intestinal microbiota affects the level of REGIII expression in the intestine. However, the level of expression differs depending on the region of the gut examined and the nature of microbiota to which it is exposed. We demonstrated that the probiotic *Bifidobacterium breve* NCC2950 but not the commensal *Escherichia coli* JM83

significantly induced REGIII expression *in vivo* in mice and *in vitro* in a human intestinal cell line, and this upregulation was independent from the metabolic activity of the strain and mediated through MyD88-Ticam1 signaling. Collectively, these results indicate that regulation of REGIII depends on the richness and specific components of the intestinal microbiota.

Colonization with the community of eight strains of bacteria that compose the ASF did not induce the same level of RegIII-γ expression as observed in SPF mice. Colonization with ASF has previously been shown to effectively reverse GF-related phenotypes (23); however, ASF-induced phenotypes are not always identical to those found in SPF mice (11, 24). This may be related to variability in SPF composition; the SPF mice used in this study came from Taconic and, unlike SPF mice from other suppliers, contain segmented filamentous bacteria (SFB), a potent inducer of T-helper 17 (Th17) cells (25). IL-22 produced by Th17 cells (26) induces the expression of RegIII-γ in both murine and human colonic epithelial cells (17, 27). In fact, monocolonization with SFB led to increased RegIII-γ production, comparable to that of ASF-colonized BALB/c mice (28). It has been shown that reducing microbiota diversity with broad-spectrum antibiotics decreases RegIII-γ expression (16, 22). Thus, in conjunction with previous work, our results support the notion that microbiota composition, especially with regard to the presence of pathobionts, is an important factor in RegIII-γ regulation.

We have previously shown that supplementation of *B. breve* upregulated *Reg3g* in *Nod1*$^{-/-}$*; Nod2*$^{-/-}$ mice with lower baseline expression of this antimicrobial peptide (10), but the mechanisms underlying this specific probiotic stimulation were unclear. In this study, we monocolonized wild-type C57BL/6 mice with *B. breve* or *E. coli* and found that *B. breve* but not *E. coli* significantly increased RegIII-γ levels in the ileum and colon. Although ileal bacterial counts were not performed, fecal and cecal content showed similar bacterial loads of *B. breve* and *E. coli*, suggesting that the effects on RegIII-γ expression were not due to differential capacity of these two strains to colonize the gut. It should be noted that although *B. breve* stimulated RegIII-γ, the level of induction was lower than in SPF mice. In accordance with our findings, monocolonization with *Bacteroides thetaiotaomicron*, but not with noninvasive *Listeria innocua*, resulted in increased RegIII-γ expression in the small intestine that did not reach the level seen in SPF mice (6). Other investigators have also determined the effect of specific mono- or dicolonizations on RegIII-γ production in the colon and cecum, with variable results (28, 29). Overall, the data support the conclusion that the net effect of intestinal bacteria on RegIII-γ expression will be modulated by the presence of specific strains in the microbiota that include both commensals and potential pathobionts. One important aspect in colonization studies relates to variability in experimental design and the time point of tissue sampling. Dynamic changes in the microbiota load and diversity, as well as in immune responses, occur immediately after colonization (30). We chose to evaluate RegIII-γ at a steady

state (21 days) postcolonization (31) since we were interested in defining REGIII responses under stable conditions. Indeed, REGIII levels have been shown to peak at 4 days postcolonization in the small intestine and to stabilize by day 16. Therefore, in addition to strain specificity, the time point chosen to determine colonization effects in antimicrobial peptides should be carefully defined.

Many cell types in the gastrointestinal tract are capable of producing REGIII proteins, including intestinal epithelial cells (IECs) and γδ intraepithelial lymphocytes (6, 32). Studies have proposed that IECs, particularly enterocytes, are producers of RegIII-γ in the colon (28, 33). In this study, we used immunofluorescence to investigate the main source of RegIII-γ after microbial exposure *in vivo*. We found that IECs are the cell types that predominantly express RegIII-γ in both the ileum and colon. Furthermore, our *in vitro* studies confirmed that incubation of human colonic epithelial cells with *B. breve*, but not *E. coli*, induced expression of *REG3A*, the human ortholog and homolog of *Reg3g*.

There is evidence to suggest RegIII-γ expression in the intestine is regulated by MyD88-mediated TLR signaling (18, 22, 34–36). *B. breve* has previously been shown to stimulate TLR2/MyD88 responses in $CD103^+$ dendritic cells (DC). Thus, it is probable that *B. breve* potentially induces responses in epithelial cells through TLR2/MyD88 signaling as well (37). We found that RegIII-γ expression in *MyD88-Ticam1* double knockout mice monocolonized with *B. breve* was low and comparable to that of GF mice, indicating that *B.*

breve-induced RegIII-γ production requires TLR signaling. Direct signaling of *B. breve* through epithelial TLRs is consistent with the epithelial cell-autonomous model of RegIII-γ expression (38). The results may also explain our previous results in *Nod1*$^{-/-}$*; Nod2*$^{-/-}$ mice in which *B. breve* led to normalization of *Reg3g* expression, likely through preserved TLR signaling in these mice. Recently, a new model of RegIII-γ production that involves IL-22 has been proposed in which luminal bacteria interact with TLR expressed by DC, leading to the release of cytokines which then prime innate lymphoid cells (ILC) to release the cytokine IL-22 (38). In our previous study, we did not detect an increase in IL-22 after *B. breve*-induced RegIII-γ expression (10). We propose that *B. breve*-induced RegIII-γ expression may occur in the absence of IL-22 supplementation through an epithelial cell-autonomous manner that involves the *MyD88-Ticam1* pathway although, in this case, induction may be more moderate than in the presence of IL-22.

A number of studies have demonstrated that anti-inflammatory effects of probiotics can be elicited without live bacteria (4, 39, 40). Likewise, induction of antimicrobial human β-defensin 2 can be mediated by either live *Escherichia coli* Nissle 1917 or its structural flagellum protein (41). Here, we examined different probiotic preparations on *REG3A* expression by colonocytes. We found that live and heat-inactivated *B. breve* increased *REG3A* expression, whereas the spent culture medium did not induce any changes. These results suggest that a specific

component of the structure of *B. breve*, and not its secreted metabolites, is responsible for *REG3A* epithelial expression.

In conclusion, we demonstrated that the effects of the microbiota on REGIII expression in the intestine correlate with microbial composition and that the effect is strain and formulation specific. We determined that the probiotic *B. breve* NCC2950 upregulates RegIII-γ expression through MyD88-Ticam1 signaling. We have previously shown that preventive administration of *B. breve* NCC2950 to genetically susceptible mice not only increased *Reg3g* expression but also ameliorated the severity of subsequent colitis (10). Based on these findings, we hypothesize that treatment with *B. breve* may regulate RegIII-γ production in a controlled manner that enhances barrier integrity and protects from inflammation. Our results support the use of microbiota-modulating strategies to target homeostatic regulation of antimicrobial peptides. This could be of benefit for IBD patients, their first-degree relatives, and patients undergoing chemotherapy or radiation therapy to prevent intestinal injury.

ACKNOWLEDGMENTS

This work was supported in entirety by a Grant in Aid from the Crohn's and Colitis Foundation of Canada (E.F.V.). E.F.V. holds a Canada Research Chair in Intestinal Inflammation, Microbiota and Nutrition. J.-P.M holds a Canadian Institute of Health Research fellowship and awards from Group for Research and

studies on Mediators of Inflammation and CECED (Digestive Epithelial Cells Study Group).

We thank K. D. McCoy for the initial GF rederivation of *MyD88*$^{-/-}$; *Ticam1*$^{-/-}$. We thank Joseph Notarangelo, Sarah Armstrong, and Sheryll Competente from the Axenic Gnotobiotic Unit at McMaster University for their assistance in gnotobiotic experiments. We thank Valerie Petit for helping us set up the cell culture experiments. We thank Hiroyuki Konishi for sending the antibodies against REGIII-γ.

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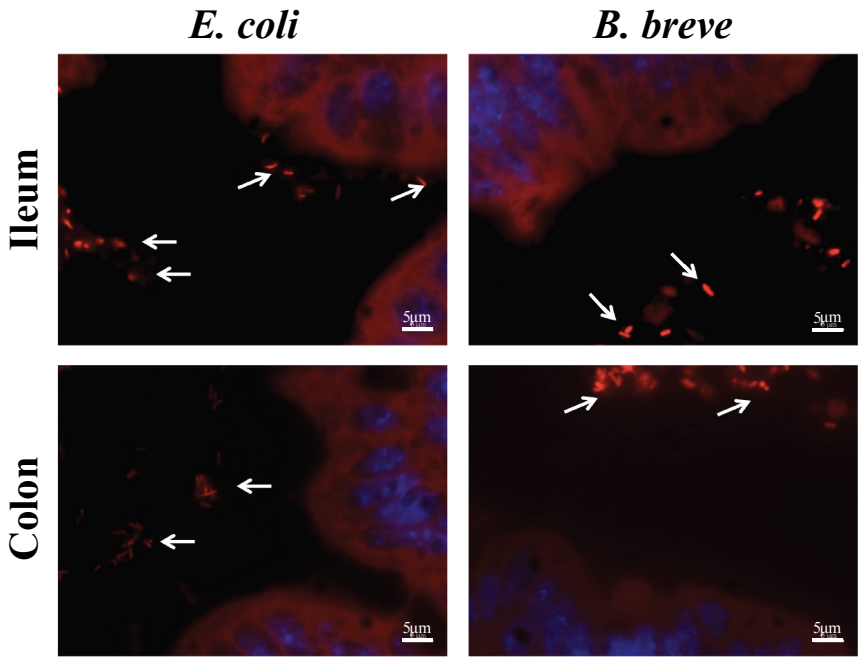
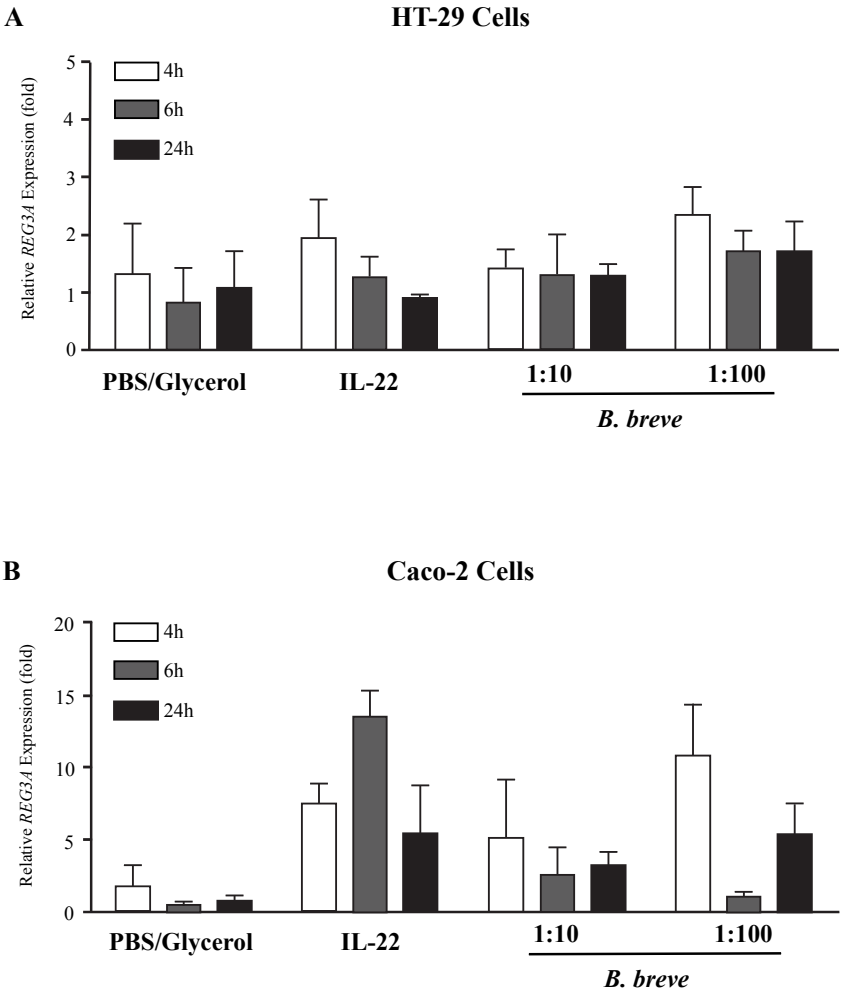
SUPPLEMENTAL FIGURES



Supplemental Figure 1. Visualization of bacterial localization relative to the epithelial surface by FISH.
Closer association of bacteria with the epithelial surface was observed in the ileum of *E. coli*-monocolonized mice compared to *B. breve*-monocolonized mice. Intestinal sections were fixed in Carnoy for 24 hours then paraffin embedded. Sections were hybridized to a probe (EUB 338, GCT GCC TCC CGT AGG AGT) that recognizes the 16S rRNA genes of all bacteria (red), and counterstained with DAPI to visualize nuclei (blue). Arrows indicate the bacteria.



Supplemental Figure 2. *B. breve* induced *REG3A* in (A) HT29 and (B) Caco-2 cells in a dose- and time-dependent manner.

Caco-2 and HT-29 cells were stimulated with IL-22 or *B. breve*. *REG3A* expression was measured at different time points by RT-qPCR. Data shown represent one independent experiment. RNA expression was normalized to *GAPDH* and shown relative to the mean of PBS-glycerol stimulated cells (all time-points), which is set to 1.

CHAPTER 5

EFFICACY OF *Bifidobacterium breve* NCC2950 AGAINST DSS-INDUCED COLITIS IS DEPENDENT ON BACTERIAL PREPARATION AND TIMING OF ADMINISTRATION

Efficacy of *Bifidobacterium breve* NCC2950 against DSS-induced colitis is dependent on bacterial preparation and timing of administration.

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The material in this chapter has been published in Beneficial Microbes. 2014 Mar;5(1):79-88. doi: 10.3920/BM2013.0039.

Preface: The work in this manuscript was performed from Sept. 2011 to Aug. 2013. I am the primary author of the manuscript, which was written with the input of my supervisor, E. Verdu. My supervisor and myself designed the experiments, which were executed and analyzed by myself with assistance and technical support from J. Natividad and J. Jury. R. Martin and P. Langella provided scientific input and critically assessed the manuscript.

SUMMARY AND SIGNIFICANCE

This manuscript investigates the most efficacious form of *Bifidobacterium breve* NCC2950 to prevent or ameliorate inflammation and injury induced by dextran sulfate sodium (DSS) in conventional (specific pathogen free) C57BL/6 mice. Although *B. breve* NCC2950 did not alter severity of ongoing DSS-induced colitis or colitis reactivation, administration of live *B. breve* NCC2950 reduced susceptibility to acute DSS-induced colitis. Both live and heat inactivated formulations of *B. breve* NCC2950 also accelerated normalization of disease symptoms after acute colitis. These findings indicate live *B. breve* NCC2950 is able to inhibit intestinal injury and inflammation, as well as promote recovery, providing rationale and insight into effective clinical use. Furthermore, these findings demonstrate formulation-specific probiotic effects and support the need for careful characterization of probiotic formulations and their optimal administration.

Title: Efficacy of *Bifidobacterium breve* NCC2950 against DSS-induced colitis is dependent on bacterial preparation and timing of administration.

Running Title: *B. breve* efficacy depends on formulation and timing of administration.

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ABSTRACT

Probiotics have been proposed as a therapy for inflammatory bowel disease, but variations in strains, formulations, and protocols used in clinical trials have hindered the creation of guidelines for their use. Thus, preclinical insight into the mechanisms of specific probiotic strains and mode of administration would be useful to guide future clinical trial design. In this study, live, heat inactivated (HI), and spent culture medium (SCM) preparations of the probiotic *Bifidobacterium breve* NCC2950 were administered to specific pathogen free C57BL/6 mice before or during colitis, as well as before colitis reactivation. Five days of 3.5% dextran sulfate sodium in drinking water was used to induce colitis. Pre-treatment with live *B. breve* reduced disease severity, myeloperoxidase activity, microscopic damage, cytokine production, IL-12/IL-10 ratio, and lymphocyte infiltration in the colon. *B. breve* did not attenuate ongoing colitis. After acute colitis, disease symptoms were normalized sooner with live and HI *B. breve* treatment; however, reactivation of colitis was not prevented. These findings indicate the efficacy of a probiotic to modulate intestinal inflammation is dependent on the formulation as well as state of inflammation when administered. Overall, live *B. breve* was most efficacious in preventing acute colitis. Live and HI *B. breve* also promoted recovery from diarrhea and colon bleeding after a bout of acute colitis.

INTRODUCTION

Inflammatory bowel disease (IBD) represents a group of gastrointestinal inflammatory disorders of which the two most common forms are ulcerative colitis and Crohn's disease. Although the precise etiology of IBD is unknown, it is believed to be caused by an aberrant immune response to the intestinal microbiota in genetically susceptible hosts.

Dysbiosis, an alteration in the normal balance of the gut microbiota, has been associated with chronic intestinal inflammation. IBD patients exhibit diminished bacterial diversity, increased mucosal bacterial loads, decreased levels of Firmicutes, as well as increased levels of *Enterobacteriaceae* which can stimulate inflammation (Andoh 2011, Kleessen 2002, Rajilic-Stojanovic 2013, Sha 2013, Walker 2011). Furthermore, changes in microbial functions have also been reported in IBD patients (Morgan 2012). Hence, reinstatement of the microbial balance using probiotic bacteria has been proposed as a therapy for IBD (Sanders 2013, Veerappan 2012).

Most clinical studies investigating the efficacy of probiotics in IBD are small and heterogeneity of study designs makes it very difficult to compare findings (Mallon 2007, Rolfe 2006, Sanders 2013). Meta-analyses and systematic reviews have concluded that larger scale, carefully designed, randomized, placebo-controlled trials are needed to assess the value of probiotics in IBD (Hedin 2007, Mallon 2007, Rolfe 2006). As a chronically relapsing disease, there are different states of inflammation IBD patients experience; therefore, the ability

of probiotics to prevent, treat, and maintain remission should be tested. Studies using animal models have shown that effects of probiotics can also depend on the strain used (Ritchie and Romanuk 2012), dose (Wen 2012, Whorwell 2006) and preparation; live, inactivated, and spent culture media formulations can all elicit anti-colitic effects (Kim 2012, Madsen 2001, Sokol 2008, Ueno 2011, Zakostelska 2011). To this regard, it is important that specific probiotics undergo pre-clinical characterization to provide rationale for clinical study design.

In this study, the ability of live, heat inactivated (HI), and spent culture medium (SCM) formulations of the anti-inflammatory *Bifidobacterium breve* NCC2950 to prevent and treat DSS colitis was characterized in specific pathogen free C57BL/6 mice. The anti-inflammatory capacity of *B. breve* was assessed at various states of inflammation through administration prior to colitis induction, during colitis, as well as before reactivation of colitis.

MATERIALS AND METHODS

Mice

All experiments were conducted with specific pathogen free male C57BL/6 mice from Taconic, aged 6-8 weeks old. Animals were given *ad libitum* access to low fat food and water, and were kept on a 12 hour light and dark cycle. Animals were housed at the McMaster Central Animal Facility. All experiments were performed in accordance with the McMaster University animal utilization

protocols and were conducted under the Canadian Council on Animal Care Guidelines.

Bacterial Strains

Bifidobacterium breve NCC2950 was acquired from the Nestlé Culture Collection (Lausanne, Switzerland) and grown in MRS (Difco, Detroit, USA) supplemented with 0.05% (v/v) L-cysteine hydrochloride (Sigma-Aldrich, St. Louis, USA) at 37ºC under anaerobic conditions. Cells were pelleted and resuspended at 10^{10} cfu/ml in PBS with 20% (v/v) glycerol. Growth media was recovered by centrifugation and filtered with 0.45µm filters (VWR, Radnor, USA) for use as the spent culture medium (SCM). Heat inactivated (HI) bacteria was prepared by incubating 50ml aliquots of live *B. breve* at 90^0C for 1 hour. All formulations were stored at -80^0C until use. Formulation controls included media used for the SCM, as well as the glycerol/PBS used to resuspend live and HI preparations; in figures, these will be referred to as "vehicle."

Dextran Sulfate Sodium (DSS) Model of Colitis

Colitis was induced using DSS (36,000-50,000 Da; MP Biochemicals, Illkirch, France) at 3.5% in drinking water for 5 days (Cooper 1993, Perse and Cerar 2012, Yan 2009). Mice were sacrificed two days following DSS removal, unless stated otherwise.

Probiotic Administration

To assess the ability of *B. breve* to prevent acute colitis, mice were orally gavaged daily for 14 days with 100µl of the SCM, HI, or live formulation (Supplemental Figure 1. Experimental protocols). Following probiotic administration, mice were put on DSS as outlined above. To determine the capacity of *B. breve* to attenuate ongoing colitis, mice were put on DSS and gavaged daily with 100µl of the SCM, HI, or live formulation until sacrifice after DSS removal. To assess the ability of *B. breve* to prevent colitis reactivation, mice were given an initial bout of colitis, followed by normal drinking water for 21 days to allow healing of the colon mucosa. For the subsequent 14 days mice were orally gavaged daily with 100µl of the SCM, HI, or live formulation, and then a second cycle of DSS was administered. Groups of mice were also sacrificed before and after probiotic administration to assess inflammation.

Assessment of Colitis Severity

Upon sacrifice, disease severity was evaluated by summing scores of colon bleeding, diarrhea, and rectal bleeding, each assessed on a scale of 0 to 3. Myeloperoxidase (MPO) activity in the colon, an indication of granulocyte infiltration, was measured as described by Bradley *et. al*. (1982). Microscopic damage was evaluated by blindly grading haematoxylin and eosin stained colon sections on a scale of 0 to 4 as outlined by Cooper *et. al.* (1993). Images were acquired using ImagePro Plus (Media Cybernetics, Rockville, USA). Colon tissue

supernatants were collected after 24 hours of 4mm diameter full thickness tissue culture in complete RPMI 1640 at 37ºC in 5% CO_2, 95% O_2 (Dieleman 1998, Natividad 2012). Tissue supernatants were used for Cytometric Bead Arrays (BD CBA Mouse Inflammation Kit, BD Biosciences, Franklin Lakes, USA) to quantify IL-10, IL-6, IL-12p70, MCP-1, TNFα, and IFNγ.

$CD3^+$ Lymphocyte Staining

CD3 staining of colon samples was completed using a modified protocol described previously (Bercik 2002, Verdu 2008). Briefly, paraffin embedded samples were incubated overnight at 4^0C with rabbit anti-mouse CD3 antibody (1:2,000; Dako A/S, Carpinteria, USA). Negative controls were processed without primary antibody. The secondary antibody used was HRP conjugated anti-rabbit, and visualization was performed using 3-amino-9-ethylcarbazole counterstained with Mayer's haematoxylin. Samples were examined using a light microscope (Olympus, Tokyo, Japan) at 40X magnification to count the number of stained cells in the lamina propria and epithelium of five randomly chosen crypts, in two colon sections per sample. The number of positively stained cells was averaged per sample. Images were obtained using ImagePro Plus (Media Cybernetics, Rockville, USA).

Statistics

Data are presented as box plots with whiskers depicting the 5th and 95th percentiles. Statistical testing was performed using the Kruskal-Wallis test and the Mann-Whitney test, when applicable. All statistical testing was performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, USA). $P<0.05$ is considered significant.

RESULTS

Preventive administration of live B. breve *reduced intestinal damage and inflammation.*

In order to determine if *B. breve* could prevent acute colitis, live, HI, and SCM formulations were administered daily for 14 days prior to colitis induction. All three formulations decreased disease severity scores compared to vehicle-colitis controls. However, none of the preparations decreased MPO activity, and only live *B. breve* reduced histological scores (Figure 1 and Supplemental Figure 2). Cytokines were quantified in colon tissues, and live *B. breve* significantly reduced production of the pro-inflammatory cytokines TNFα, IL-12p70, MCP-1, IFNγ and IL-6 (Figure 2). Although anti-inflammatory IL-10 was also reduced by live *B. breve*, the IL-12/IL-10 ratio was lower in *B. breve* treated mice than in the vehicle-colitis control ($1.2x10^{-9}$ and 0.47, respectively) indicating a shift to an anti-inflammatory state. Live *B. breve* also led to significantly less $CD3^+$ cells present in the colon (Figure 3).

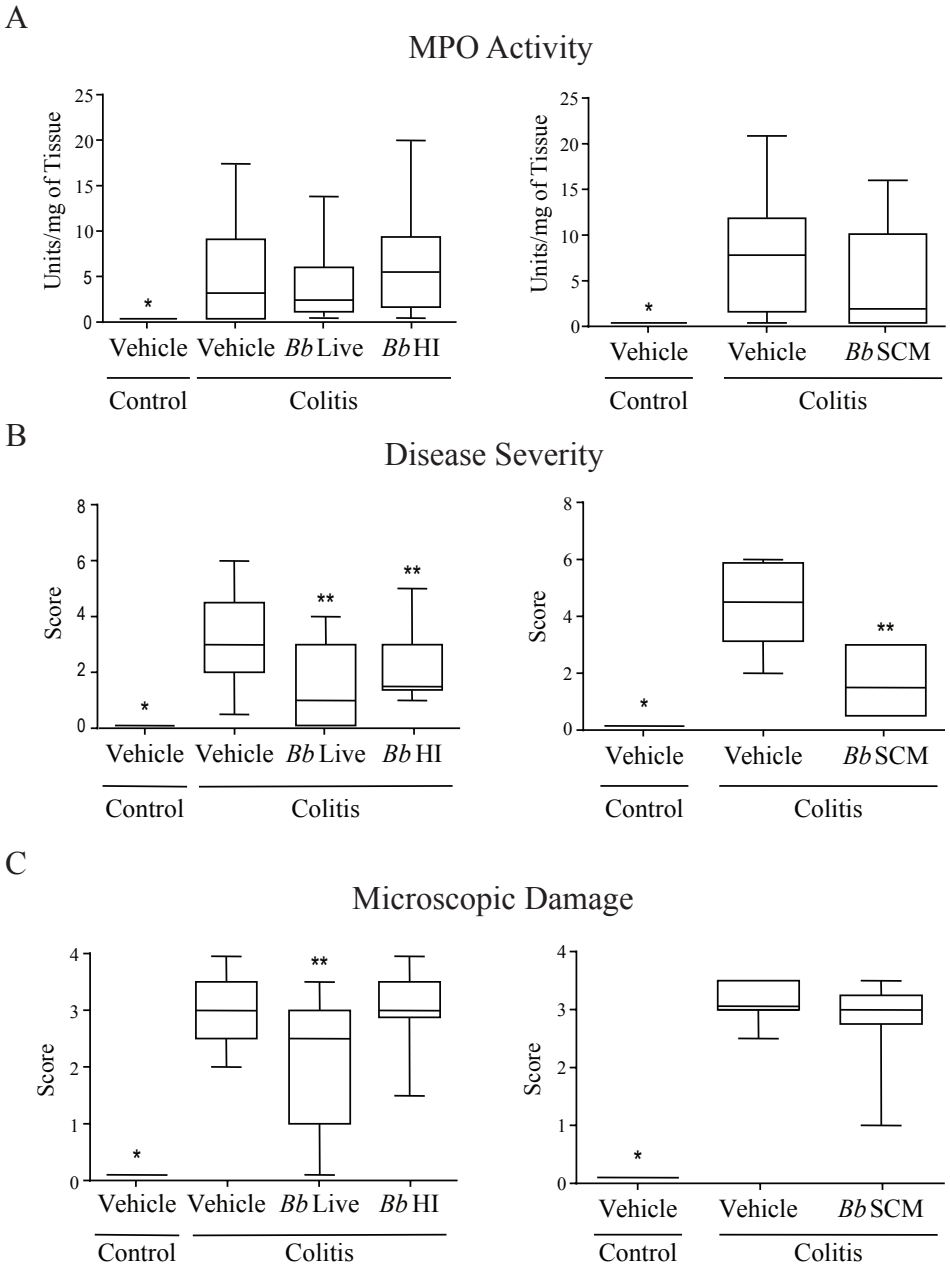

Figure 1. Effect on (A) myeloperoxidase activity, (B) disease severity and (C) microscopic damage in dextran sulphate sodium-treated mice.

Phosphate buffered saline-glycerol (n=11) was used as the vehicle control for live *Bifidobacterium breve* (*Bb* Live; n=15) and heat inactivated *B. breve* (*Bb* HI; n=14). MRS medium (n=13) was the vehicle for spent culture medium (*Bb* SCM; n=13). **P*<0.05 vs. all treatments; ***P*<0.05 vs. vehicle-colitis controls.

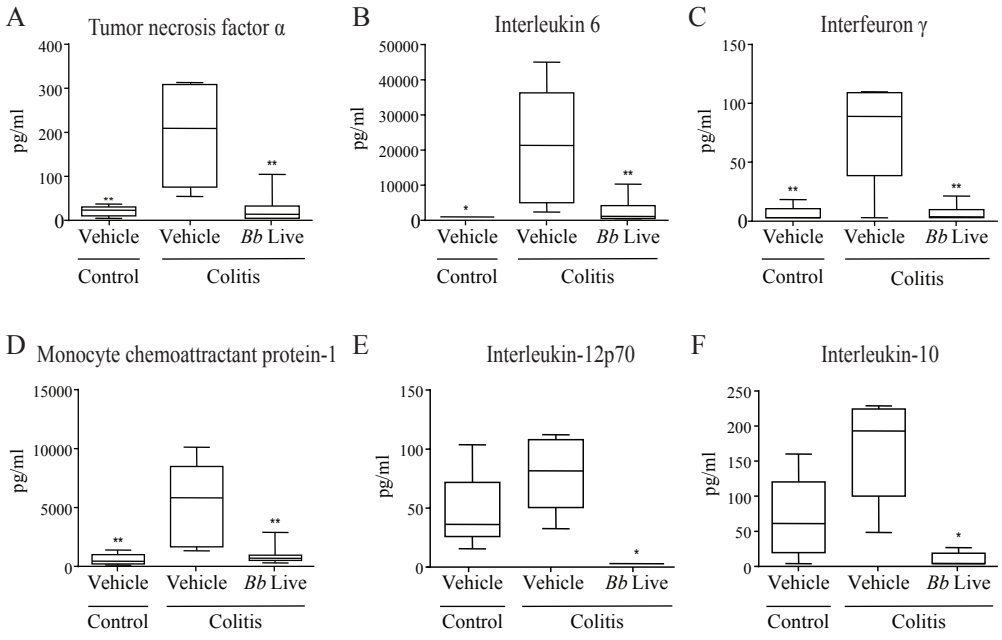

Figure 2. Augmentation of cytokines in dextran sulphate sodium-treated mice.

Phosphate buffered saline-glycerol (n=5) was the vehicle for live *Bifidobacterium breve* (*Bb* Live; n=15) **P*<0.05 vs. all treatments; ***P*<0.05 vs. vehicle-colitis controls.

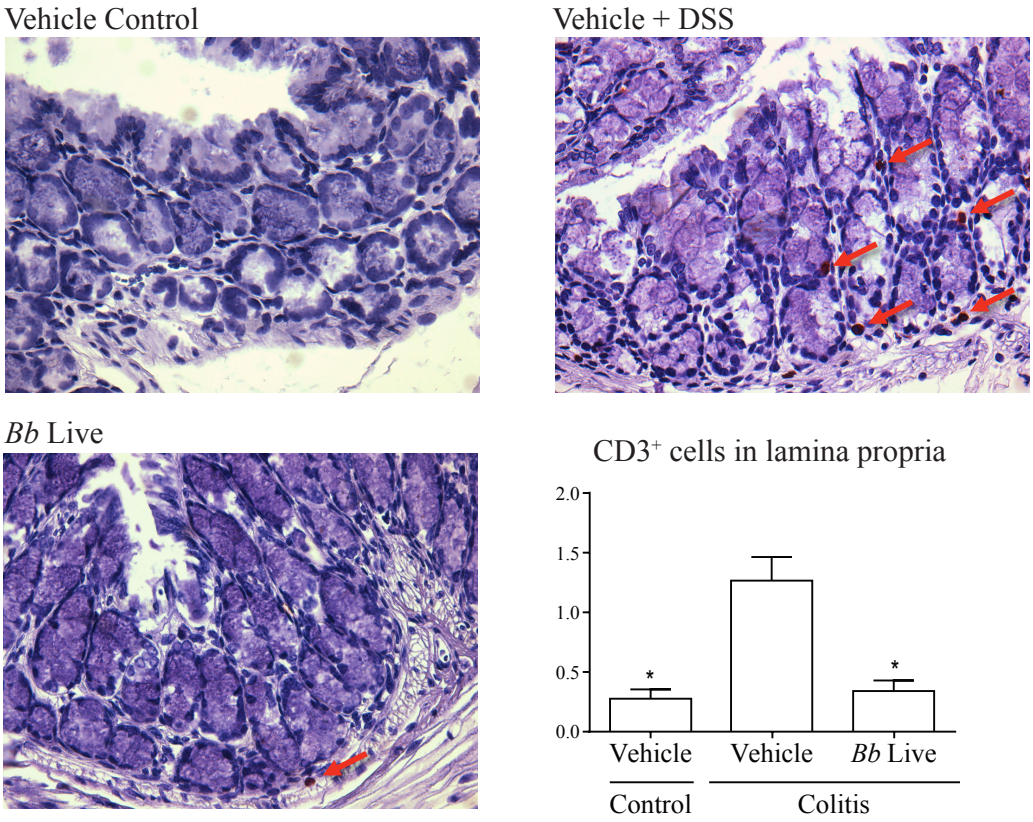

Figure 3. Effect on the amount of $CD3^+$ lymphocytes in the colon mucosa of dextran sulphate sodium (DSS)-treated mice.
Phosphate buffered saline-glycerol (n=5) was the vehicle for live *Bifidobacterium breve* (*Bb* Live; n=15) **P*<0.05 vs. vehicle-colitis controls.

B. breve *did not attenuate ongoing colitis*

B. breve was administered in conjunction with DSS to ascertain if severity of ongoing colitis could be reduced. None of the *B. breve* formulations diminished MPO activity, histology scores, or disease severity compared to vehicle-colitis controls (Figure 4 and Supplemental Figure 3).

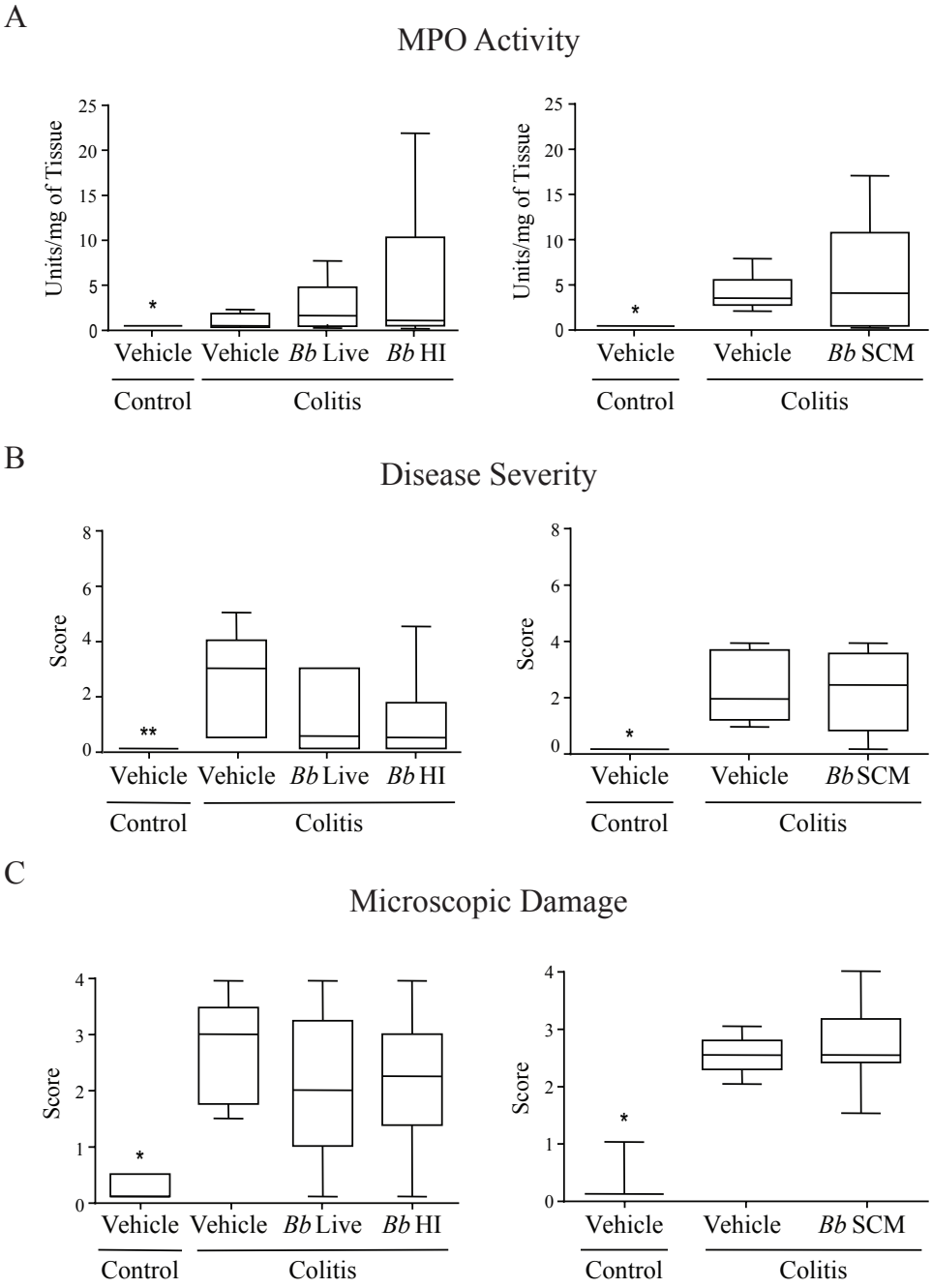

Figure 4. Effect on on-going colitis as determined by (A) myeloperoxidase activity, (B) disease severity and (C) microscopic damage.

Phosphate buffered saline (PBS)-glycerol was the vehicle for *Bifidobacterium breve* (*Bb* Live; n=10) and heat inactivated *B. breve* (*Bb* HI; n=10). MRS medium (n=9) was the vehicle for spent culture medium (*Bb* SCM; n=10). *P<0.05 vs. all treatments; **P<0.05 vs. vehicle-PBS-glycerol colitis and *Bb* Live.

B. breve *did not prevent reactivation of colitis but did accelerate clinical recovery after the first bout of colitis.*

Following recovery from colitis, *B. breve* was administered to ascertain if reactivation of colitis could be prevented. At commencement of probiotic administration, there was no detectable MPO activity, however mild microscopic damage and disease symptoms were observed in mice previously treated with DSS (Figure 5 and Supplemental Figure 4). Following administration of *B. breve* and prior to colitis reactivation, MPO activity and microscopic damage were unaffected but disease severity was attenuated in mice that had received live and HI formulations (Figure 6 and Supplemental Figure 5). After colitis reactivation, the live and HI preparations reduced MPO activity, but no further beneficial effects were observed (Figure 7 and Supplemental Figure 6).

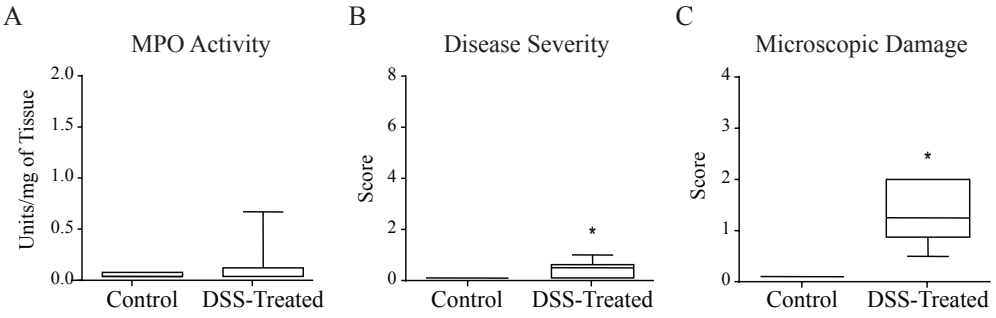

Figure 5. Effect on (A) myeloperoxidase activity, (B) disease severity and (C) microscopic damage at 21 days post-dextran sulphate sodium (DSS; n=10) treatment (day 26).

Control (n=8). **P*<0.05 vs. control.

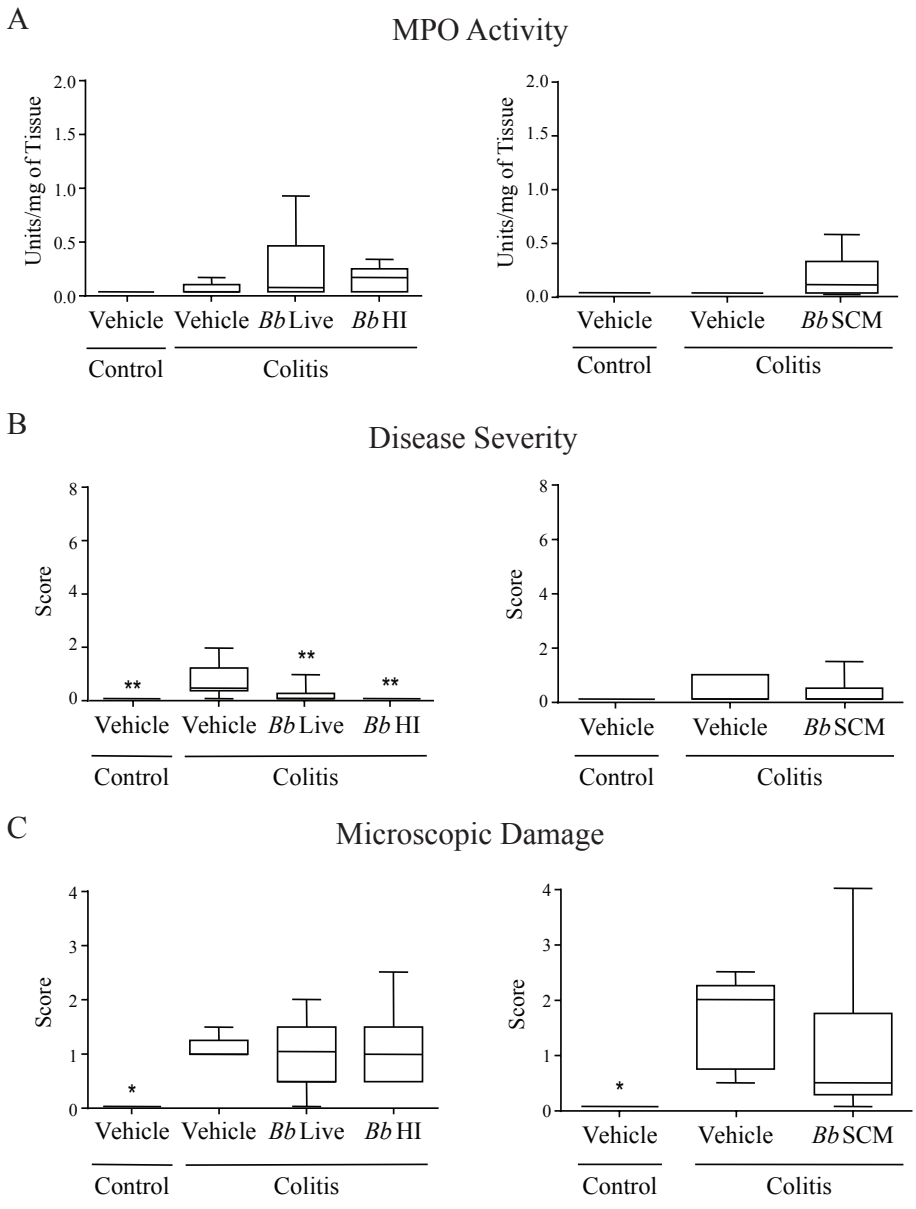

Figure 6. Effect on (A) myeloperoxidase activity, (B) disease severity and (C) microscopic damage following acute colitis (day 40).

Phosphate buffered saline-glycerol (n=5) was the vehicle for live *Bifidobacterium breve* (*Bb* Live; n=9) and heat inactivated *B. breve* (*Bb* HI; n=7). MRS medium (n=5) was the vehicle for spent culture medium (*Bb* SCM; n=9). **P*<0.05 vs. all treatments; ***P*<0.05 vs. vehicle-colitis controls.

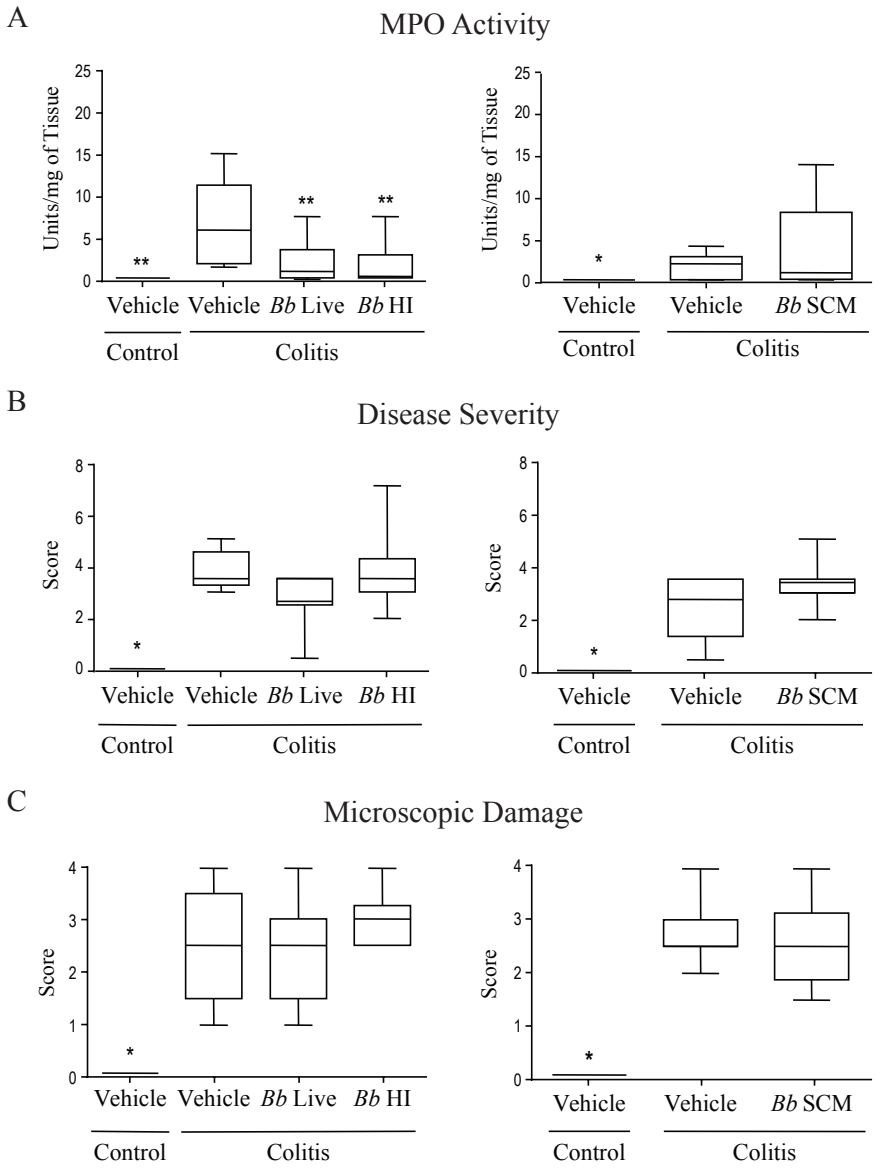

Figure 7. Effect on (A) myeloperoxidase activity, (B) disease severity and (C) microscopic damage after colitis reactivation (day 47).

Phosphate buffered saline-glycerol (n=5) was the vehicle for live *Bifidobacterium breve* (*Bb* Live; n=9) and heat inactivated *B. breve* (*Bb* HI; n=9). MRS medium (n=8) was the vehicle for spent culture medium (*Bb* SCM; n=10). **P*<0.05 vs. all treatments; ***P*<0.05 vs. vehicle-colitis controls.

DISCUSSION

In this study, we showed that probiotic *Bifidobacterium breve* NCC2950 administered in a preventive manner decreased severity of experimental colitis, but did not diminish established inflammation. Despite the prevention of a first bout of colitis and an accelerated clinical recovery after colitis, *B. breve* did not inhibit colitis reactivation. Overall, these results show that probiotic efficacy varies depending on formulation and state of inflammation in which it is administered.

Prevention of colitis by the anti-inflammatory *Bifidobacterium breve* NCC2950 strain was most effective when using live bacteria. Although the spent culture medium (SCM) and heat inactivated (HI) formulations of *B. breve* decreased disease symptoms, none of the other inflammatory parameters measured were improved. A previous study testing the protective capacity of VSL#3, a mixture of probiotic bacteria containing one *B. breve* strain, showed DNA, live, and irradiated preparations, but not the HI, diminished colitis severity (Rachmilewitz *et al.*, 2004). Lammers *et al.* (2003) have shown stimulation of peripheral blood mononuclear cells (PBMCs) *in vitro* with genomic DNA of *B. breve* BBSF very effectively induced anti-inflammatory IL-10 and IL-1β. Moreover, intraperitoneal administered SCM from *B. breve* C50 was also able to protect from colitis, as were SCM-conditioned dendritic cells (Heuvelin *et al.*, 2009). It is unlikely DNA present in the SCM used in this study would directly interact with immune cells prior to colitis induction, which may explain why the

SCM was ineffective at preventing inflammation. On the other hand, live *B. breve* given before colitis reduced microscopic damage and disease symptoms. Consistent with previous findings in *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice, live *B. breve* NCC2950 did not diminish oxidative burst activity of polymorphonuclear (PMN) cells (Natividad *et al.*, 2012). We also found $CD3^{+}$ lymphocyte counts and a variety of pro-inflammatory cytokines were decreased in the colon mucosa of live *B. breve* pre-treated mice. Despite reduced absolute levels of the anti-inflammatory cytokine IL-10, the IL-12/IL-10 ratio was low, indicating a relatively anti-inflammatory state. Other studies have shown expression of NFκB and other inflammation-related genes in colon epithelial cells decrease with exposure to live *B. breve* (Grimoud *et al.*, 2010, Heuvelin *et al.*, 2009, Ohtsuka *et al.*, 2012); therefore, inhibition of inflammatory genes may be the mechanism through which the live *B. breve* tested here decreased pro-inflammatory cytokines. An earlier study demonstrated the ability of *B. breve* Yakult to prevent colitis through induction of IL-10 producing Tr1 cells in the colon, suggesting an enhancement of regulatory immune cells may also contribute to the anti-inflammatory effects of *B. breve* (Jeon *et al.*, 2012). Previously, live *B. breve* NCC2950 was shown to stimulate production of the antimicrobial peptide RegIII-γ, enhance barrier function, and reduce susceptibility to DSS colitis in mice with defective bacterial sensing (*Nod1*$^{-/-}$*;Nod2*$^{-/-}$) (Natividad *et al*., 2012). Collectively these results suggest that different strains of *B. breve* exhibit a variety of immuno-modulatory effects, and *B. breve* NCC2950 in particular seems to strengthen innate immune

mechanisms and barrier defense leading to higher resistance to subsequent intestinal injury.

B. breve was administered during colitis in order to evaluate curative effects, however none of the formulations decreased colitis severity. Similarly, VSL#3, which has been effective in preventing colitis (Mencarelli *et al.*, 2011, Sood *et al.*, 2009), was also unable to attenuate acute DSS-induced colitis (Gaudier *et al.*, 2005). Administering a probiotic with barrier and innate immune stimulating capacity can be an effective strategy to prepare the barrier for an insult like DSS. However, we hypothesize that *B. breve,* and perhaps other live probiotics, are unable to attenuate inflammation during colitis once significant epithelial breaks are present, allowing bacterial translocation that can result in deleterious systemic effects. Altogether these observations strengthen the concept that probiotic bacteria are better able to prevent injury and inflammation than treat it.

We investigated the effects of *B. breve* on mucosal healing following an episode of acute colitis. Three weeks post-DSS, the colonic epithelium was once again intact but underlying tissue damage was still observed and accompanied by persistence of mild diarrhea. From that point, *B. breve* was administered for 14 days. Live and HI treatments reduced severity of diarrhea, indicating an ability to accelerate resolution of disease symptoms. Following probiotic treatment, colitis was reactivated with a second bout of DSS. Live and HI preparations reduced PMN cell activity, but no other anti-colitic effects were observed. Overall, none of

the formulations were able to consistently reduce multiple inflammatory parameters. VSL#3, however, has been shown to reduce severity of reactivated TNBS-induced colitis by decreasing pro-inflammatory responses of lamina propria mononuclear cells as well as enhancing IL-10 production and regulatory immune cell populations (Di Giacinto *et al.*, 2005). It is possible that reactivation is prevented with VSL#3 because it contains multiple strains which may provide additional adaptive regulatory cell induction abilities in comparison to *B. breve* alone.

In conclusion, we showed that timing of probiotic administration, as well as the formulation, can influence its efficacy. Live *Bifidobacterium breve* NCC2950 ameliorated DSS colitis when given in a preventive manner, but was unable to treat ongoing colitis. Live and HI *B. breve* accelerated recovery of disease symptoms after acute inflammation, however colitis reactivation was not inhibited. Overall, live *B. breve* NCC2950 has demonstrated the potential to prevent colitis, and thus, should be tested clinically.

ACKNOWLEDGEMENTS

This work was supported in entirety by a Grant in Aid from the Crohn's and Colitis Foundation of Canada (E. F. Verdu). Dr. Verdu holds a Canada Research Chair in Intestinal Inflammation, Microbiota and Nutrition. R. Martin is a recipient of a grant in the frame of FPARIS collaborative project selected and supported by the Vitagora Competitive Cluster and funded by the French FUI

(Fond Unique Interministériel; FUI: n°F1010012D), the FEDER (Fonds Européen de Développement Régional; Bourgogne: 34606), the Burgundy Region, the Conseil Général 21 and the Grand Dijon.

We would like to thank Jun Lu for assisting with cytometric bead assays, as well as Anne Bruttin and Myriam Steinmann at Nestlé Research Centre (Lausanne, Switzerland) for preparation of the *B. breve*.

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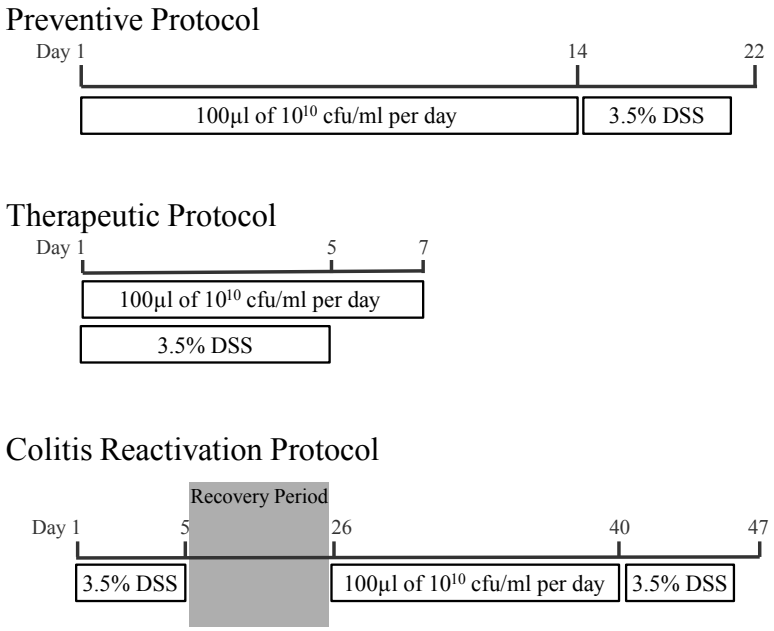
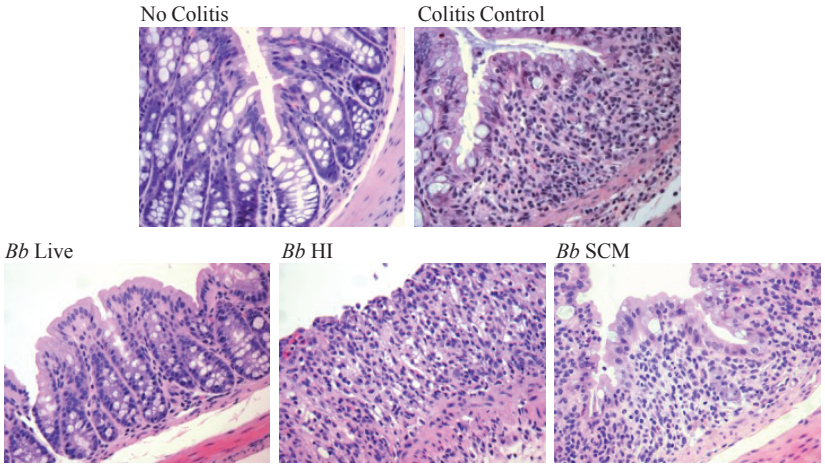
by strengthening the gut barrier function and changing the gut microenvironment. PloS One 6(11), e27961.

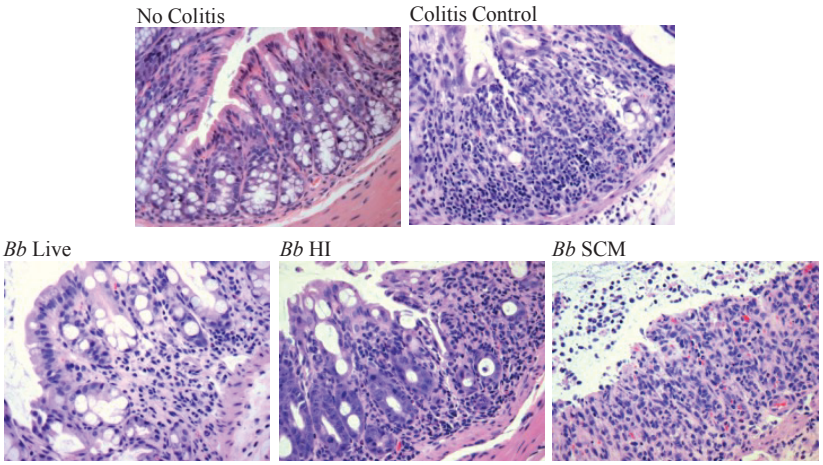
SUPPLEMENTAL FIGURES



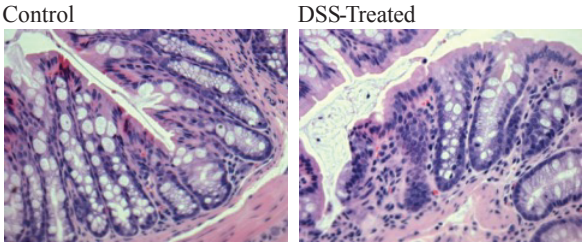
Supplemental Figure 1. Experimental protocols.



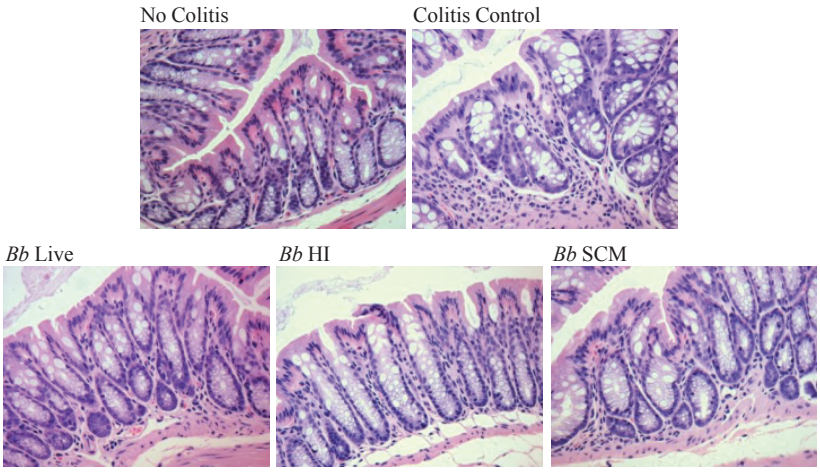
Supplemental Figure 2. Live *Bifidobacterium breve* prevented microscopic damage in dextran sulphate sodium-treated mice.



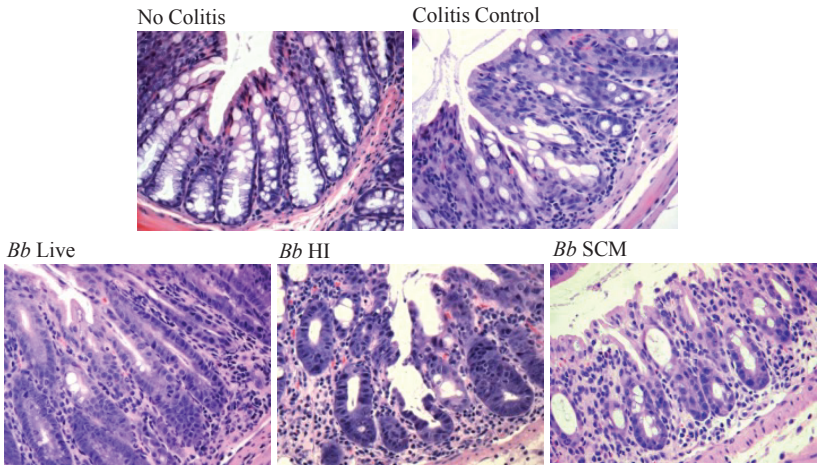
Supplemental Figure 3. *Bifidobacterium breve* did not attenuate dextran sulphate sodium-induced microscopic damage.



Supplementary Figure 4. Mild microscopic damage is observed 21 days post-dextran sulphate sodium (day 26).



Supplementary Figure 5. *Bifidobacterium breve* did not diminish the mild microscopic damage observed after colitis.



Supplemental Figure 6. *Bifidobacterium breve* did not prevent microscopic damage induced by colitis reactivation (day 47).

CHAPTER 6

DISCUSSION

6.1 Summary

Maintenance of homeostasis, the ability to maintain a relatively stable internal environment to changes induced by external factors, is essential for health. The importance of homeostasis is exemplified in the gastrointestinal (GI) tract, the largest interface between the host and environment. The intestinal barrier, in particular, faces the largest antigenic load but must permit uptake of nutrients. Using immune and physiological mechanisms, the intestinal barrier interacts with both host immunity and the gut microbiota in order to maintain homeostasis. Dysfunction of the intestinal barrier can lead to abrogation of homeostasis, which can contribute to development of chronic inflammatory intestinal disorders (CIIDs).

The integral role of gut microbiota on immune development and function has been previously characterized and is discussed in **Appendix I** (El Aidy et al. 2012; Hapfelmeier et al. 2010; Kozakova et al. 2016). However, the impact of microbiota on the establishment and modulation of intestinal barrier function remains poorly understood. Determining how microbiota affects the many aspects of barrier function is essential to elucidating the pathological consequences of aberrant microbiota-barrier interactions. *Thus, the overall aim of my thesis was to determine whether and how the microbiota affects intestinal barrier function and integrity.* This central aim is addressed by the findings presented in **Chapters 3-5**. In **Chapter 3**, I determined that the microbiota induces maturation of colonic barrier structure and paracellular permeability to the physiological state within a

week of colonization, increasing integrity and resilience towards chemically induced injury (Supplemental Figure 1, **Appendix II**). These results demonstrate the colonizing microbiota plays an integral role in establishing colonic permeability function. In **Chapter 4,** I found innate immune function of the barrier is differentially impacted by the microbiota (Supplemental Figure 2, **Appendix II**). Lastly, the ability of the bacterium *Bifidobacterium breve* NCC2950 to alter susceptibility to chemically induced injury and promote recovery is presented in **Chapter 5** (Supplemental Figure 3, **Appendix II**). Collectively, this work provides novel insight into how the microbiota contributes to establishment and modulation of intestinal barrier function and integrity, which will be valuable in deciphering barrier-microbiota interactions during the neonatal period and in CIIDs. Additionally, these findings support development of therapeutic strategies aimed at modulating the microbiota in order to improve barrier function and integrity.

In this final chapter, connections between studies detailed in **Chapters 3-5** and the overall significance of the results is explored, along with discussion of future directions and limitations.

6.2 Intestinal microbiota: a key regulator of intestinal barrier function.

The host and microbiota have coevolved and form a mutualistic relationship where the host provides a favourable environment and nutrients, and the microbiota aids with digestion, provides metabolites, and hinders survival of

pathogens. In order to maintain a homeostatic, mutualistic state, the intestinal barrier interacts with the microbiota and intestinal immune system, mediating a balance between local tolerance towards commensal bacteria and protection from antigens, in addition to facilitating digestion and nutrient absorption. To perform these complex functions, the intestinal barrier utilizes structural, innate immune and permeability features. Paracellular and transcellular transport across the barrier must be carefully regulated to prevent invasion of luminal antigens, and the mucus layer provides a physical and chemical barrier to microbes through incorporation of secreted antimicrobial peptides and secretory IgA (Frantz et al. 2012; Shroff, Meslin, Cebra 1995; Vaishnava et al. 2008; Vaishnava et al. 2011). Expression of pattern recognition receptors (PRRs) by intestinal epithelial cells allows for surveillance of antigens and induction of immune responses, when appropriate.

Proper barrier function is essential to maintaining homeostasis, as evidenced by barrier dysfunction in CIIDs. Many CIIDs share predominant pathophysiological factors: host genetics, alterations in the microbial community composition and other environmental factors, all of which can influence barrier function. Understanding how these epidemiological factors contribute to barrier dysfunction would provide valuable insight into pathogenesis and novel therapeutic avenues. Although genome wide association studies have identified genetic factors involved in barrier function, such as *NOD2* polymorphisms in Crohn's disease, these only contribute to a small proportion of heritability,

suggesting environmental cues are also required for development of chronic inflammation in genetically susceptible individuals (Adler et al. 2011; Kevans et al. 2015). Accordingly, a number of GI infections have also been linked to inflammatory bowel disease (IBD), which may contribute to initiation and/or perpetuation of barrier dysfunction and inflammation, as well as perturbations in the microbiota composition (Verdu and Riddle 2012).

Indeed, alterations in bacterial communities have been identified in a number of CIIDs, including IBD, irritable bowel syndrome and celiac disease. However, given the complex, bi-directional interactions between the barrier and microbiota, deciphering if barrier dysfunction and microbial imbalances are causes or consequences of disease has proven difficult; this largely stems from our lack of understanding of how the microbiota influences barrier function and integrity. In **Chapters 3** and **4**, I demonstrate that intestinal barrier structural, innate immune and permeability functions adapt in response to the microbiota**.** Through comparison of germ-free and conventional specific pathogen free (SPF) mice, I determined the microbiota is required for physiological colonic paracellular permeability, and in agreement with previous findings, is essential for RegIII-γ secretion and fortification of mucus structure (Cash et al. 2006; Johansson et al. 2015; Petersson et al. 2011). These findings suggest the microbiota influences many aspects of intestinal barrier function. To gain insight into how the microbiota impacts maturation and modulation of intestinal barrier

function, germ-free mice were colonized with select microbial communities or single bacteria.

6.2.1 Microbiota induces physiological intestinal permeability.

Colonization of the intestinal tract following birth is an important event that shapes host physiology. The integral role of the microbiota in immune development has been well characterized using gnotobiotic studies. Germ-free mice exhibit immature primary and secondary lymphoid structures, diminished abundance of αβ-IELs, IgA-expressing B cells, T cells, particularly T_H17, and in the colon specifically, regulatory T cells are decreased but hyperresponsive invariant natural killer T cells are increased (Atarashi et al. 2011; Ivanov et al. 2009; Macpherson and Uhr 2004; Olszak et al. 2012; Umesaki et al. 1993). Maturation of lymphoid structures and immune populations occurs following colonization of germ-free mice, although correction of invariant natural killer T cells hyperresponsiveness is limited to a "window of opportunity" in early life (Olszak et al. 2012). The colonizing community plays a critical role in immune development, with *Clostridium* and segmented filamentous bacteria specifically associated with induction of regulatory T cells and T_H17 cells, respectively (Atarashi et al. 2011; Ivanov et al. 2009). Alterations in the colonizing and successive microbial communities can affect immune development, and may have long-term impacts on host health. For instance, antibiotic use within the first year of life has been associated with increased susceptibility to asthma, allergy,

obesity, and IBD later in life (Rasmussen et al. 2018; Risnes et al. 2011; Shaw, Blanchard, Bernstein 2011). Thus, microbial colonization stimulates and shapes immune development; however, the impact of colonization on maturation of permeability remains largely unknown, particularly in the colon.

Careful regulation of intestinal permeability is essential for maintenance of homeostasis and health. The intestinal barrier must maintain a semi-permeable state for nutrient, water and electrolyte acquisition, as well as immune surveillance, without permitting dysregulated antigen uptake; as such, the barrier must adapt permeability in response to the local environment. Passage through the intestinal barrier is facilitated through a number of pathways, including paracellular and transcellular pathways, goblet cell-associated antigen passages (GAPs), as well as uptake by CX_3CR1^+ immune cells or M cells. GAPs permit translocation of antigens across the barrier and delivery to underlying lamina propria dendritic cells, which may be a mechanism of immune surveillance (Knoop et al. 2015; McDole et al. 2012). Germ-free mice reportedly have a high frequency of GAP formation, however, in unpublished findings from **Chapter 3**, I did not find evidence of bacteria-goblet cell associations at days 1 or 7 post-colonization with human microbiota, despite diminished mucus integrity at day 1 (assessed by immunofluorescence staining and fluorescence *in situ* hybridization of mucin-2 and the bacterial 16S gene, respectively; Supplemental Figure 4, **Appendix II**) (McDole et al. 2012). In the colon, microbiota-stimulated intestinal epithelial MyD88 signaling reportedly inhibits GAP formation during the steady

state, abrogation of which resulted in increased susceptibility to DSS-induced injury and inflammation (Knoop et al. 2015; Knoop et al. 2016). Thus, I speculate that the microbiota rapidly stimulates MyD88-mediated GAP inhibition following colonization in order to limit antigen exposure during early colonization when intestinal barrier and immune functions are immature.

Transcellular and paracellular passage across the intestinal barrier is essential for electrolyte and fluid balance as well as nutrient absorption, however, it must be carefully regulated to protect from pathogen invasion and antigen exposure. Small intestinal permeability characteristics were similar between germ-free and SPF mice, and following colonization, no changes in ileum permeability to probes were observed (Supplemental Figure 5, **Appendix II**; unpublished data). Such findings suggest that, unlike the colon, small intestinal permeability changes may be inherently programmed aspects of postnatal barrier maturation. Indeed, lactulose/mannitol tests in human newborns and transcellular permeability studies in mice have demonstrated that small intestinal permeability decreases, or tightens, within the first week of life (Catassi et al. 1995; Heyman et al. 1986; Weaver, Laker, Nelson 1984). In neonatal germ-free mice, small intestinal permeability also tightens, further supporting an inherently programmed occurrence of postnatal barrier maturation that may be independent of colonization (Heyman et al. 1986). However, *Lactobacillus rhamnosus* GG supplementation in neonatal mice accelerates the tightening of intestinal permeability to FITC-dextran probe, indicating that the process could still be

influenced by certain probiotic bacteria (Patel et al. 2012). Similarly, I also observed dynamic changes in mRNA expression of tight junction proteins in the small intestine within the first week of commensal colonization (Supplemental Figure 6, **Appendix II**; unpublished data). Although the significance of altering tight junction proteins in the absence of net permeability changes is unknown, this may be a mechanism to compensate for other microbiota-induced changes in barrier function, including mucus structure, in an attempt to maintain paracellular permeability function. Thus, it is possible that establishment of small intestinal barrier function differs from the colonic barrier, whereby inherently programmed postnatal development may impact permeability more than colonization. This may relate to the density of colonization in small intestine versus colon, but this remains speculative.

My findings detailed in **Chapter 3** reveal that paracellular permeability in the colon reaches the physiological level within a week of commensal colonization; this occurred in the absence of mucosal damage and was not associated with augmented systemic exposure to microbial antigens, indicating impairment of barrier integrity was neither a cause nor consequence of increased permeability. Decreased tight junction protein claudin-1 and a transient increase in enterocyte IL-18 production were congruent with induction of paracellular permeability. IL-18 has been previously shown to increase permeability through claudin-1 alterations (Li, Akhtar, Choudhry 2012). These findings demonstrate that, unlike in the ileum, the microbiota is required for establishment of colonic

paracellular permeability, which may be mediated by epithelial IL-18 alterations in tight junction structure. Further investigation using additional healthy human donors is required to confirm this pattern of paracellular permeability induction is a conventional occurrence following colonization. Such findings may also aid in deciphering the role of the colonizing microbiota in barrier maturation during the neonatal period.

To gain further insight into mechanisms involved in paracellular permeability induction, I conducted a study where *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice were colonized with human commensal microbiota by oral gavage. NOD2 and IL-18 have been previously linked; NOD2 activation in human monocyte-derived macrophages has been shown to promote IL-18 signaling, which in turn, promotes NOD2 signaling and cytokine production (Hedl, Zheng, Abraham 2014). Thus, it is possible NOD2 and IL-18 signaling work synergistically in colon epithelial cells following colonization to induce physiological paracellular permeability. Although germ-free *Nod1*$^{-/-}$*;Nod2*$^{-/-}$ mice exhibit inherently high paracellular permeability, no further increase was observed at day 7 post-colonization, as seen in wild-type C57BL/6 mice (Supplemental Figure 7, **Appendix II**; unpublished data). These preliminary findings suggest NOD1 and NOD2 signaling may play a role in paracellular permeability induction following colonization. To confirm a causal role of epithelial IL-18 in paracellular permeability induction, future investigations could evaluate permeability in mice with intestinal epithelial cell deficiencies in IL-18 or caspase-1, the activator of IL-18.

These findings, however, do not determine if permeability changes are derived from microbiota-enterocyte interactions or alterations in stem cell programming. Stem cell function is impacted by the microbiota; specifically, TLR4 signaling alters the rate of proliferation and goblet cell differentiation in neonatal mice, NOD2 activation enhances stem cell survival, and reactive oxygen species stimulated by the microbiota also affect proliferation and survival (Buchon et al. 2009; Nigro et al. 2014; Sodhi et al. 2010; Sodhi et al. 2012). Furthermore, colonization modifies the transcriptional profile of neonatal stem cells, subsequently leading to changes in enterocyte transcriptional profiles, including alterations in claudin-1 expression (Harper et al. 2011). Although no changes in paracellular permeability were observed at day 1 post-colonization, the tight junction protein ZO-1 was already diminished in the crypts, suggesting stem cells rapidly begin altering permeability characteristics in response to early colonizers (**Chapter 3**). Thus, it is possible the colonizing microbiota alters stem cell programming. However, IL-18 production was limited to the apical enterocytes, suggesting direct microbial interactions (possibly through NOD2) are required for IL-18 induction. Therefore, colonization may stimulate reprogramming of stem cells in such a way that promotes IL-18 responses upon microbial stimulation in enterocytes. Furthermore, colon cell turnover occurs within 5-7 days; thus, paracellular permeability induction could coincide with turnover with newly programmed cells. Comparison of stem cell transcriptional profiles between germ-free and newly colonized mice could provide insight into

transcription factors involved in regulating maturation of enterocytes, which could then be mechanistically assessed through targeted deletion.

Regardless of the mechanisms involved, limiting paracellular permeability during early colonization may protect the vulnerable, immature barrier from antigen exposure while mucus integrity, antimicrobial peptide expression, and overall barrier integrity are low (**Chapters 3** and **4**). Indeed, low colon claudin-1 expression is associated with augmented goblet cell differentiation and mucin-2 production, demonstrating a link between mucus fortification and increased paracellular permeability (Pope et al. 2014).

6.2.2 Intestinal barrier integrity is influenced by the microbiota.

Integrity of the intestinal barrier is vital for the maintenance of homeostasis and host health. Dysfunction or damage to the barrier predisposes the host to antigen invasion and inflammation, as is evidenced in CIIDs and studies using murine models of intestinal injury (Johansson et al. 2014; Natividad et al. 2012). The intestinal mucus layer is the first line of defense against luminal antigens, and as such, is a key contributor to barrier integrity. The mucus forms both a physical and chemical barrier to luminal contents, providing a medium for secreted antimicrobial elements, such as antimicrobial peptides and secretory IgA. Antimicrobial peptides, including RegIII-γ, prevent microbial invasion by inhibiting colonization of the inner mucus layer (Vaishnava et al. 2011). Maintenance of mucus integrity is an important aspect of intestinal barrier

function, as defects can permit bacterial exposure and potentially induce immune responses. Indeed, the mucus of IBD patients with active disease has been shown to be highly penetrable and contain higher abundances of bacteria (Johansson et al. 2014; Swidsinski et al. 2005). Antimicrobial peptide expression can also be impaired in IBD patients, although it is unknown if diminished antimicrobial peptide secretion correlates with mucosally adherent bacteria (Wehkamp et al. 2004). Therefore, mucus layer integrity is essential for maintaining homeostasis and limiting microbial-induced inflammation.

Consequently, the intestinal barrier alters antimicrobial peptide production and mucus integrity in response to microbial interactions. In agreement with my findings, colonization with human commensal bacteria alters mucin glycosylation patterns and fortifies the mucus layer (**Chapter 3**) (Graziani et al. 2016; D. R. Hill et al. 2017). Furthermore, the bacterial community affects mucus integrity in the steady state, with Proteobacteria and TM7 associated with a more penetrable mucus layer (Jakobsson et al. 2015). Bacterial interactions can also influence mucus production; monocolonization with acetate producer *Bacteroidetes thetaiotamicron* stimulates mucin production, but is abrogated by co-colonization with acetate consumer and butyrate producer *Faecalibacterium prausnitzii* (Wrzosek et al. 2013). Collectively, these studies demonstrate mucus production and integrity is continually influenced by the bacterial community, and may be attributable to metabolic activity of specific members.

As an important contributor to mucus integrity, changes in antimicrobial peptide production were also investigated following colonization in **Chapter 4**. In accordance with my findings, bacterial signaling in intestinal epithelial cells is required to stimulate RegIII-γ production (Frantz et al. 2012). Moreover, I also found colonization with the eight-strain altered Schaedler flora resulted in a less robust RegIII-γ response compared to conventional mice. RegIII-γ production is low in mice colonized with neonatal microbiota or upon microbial depletion with broad-spectrum antibiotics, suggesting induction and continued expression are dependent on the richness or specific members of the community (Kim et al. 2017; Vaishnava et al. 2008). Indeed, RegIII-γ production was also differentially induced by specific bacterial species; colonization with *Bifidobacterium breve* NCC2950 stimulated RegIII-γ production, whereas colonization with *Escherichia coli* JM83 was less efficient (**Chapter 4**). Conversely, a human-derived, non-pathogenic strain of *E. coli* was found to induce expression of a number of antimicrobial peptides, including the human ortholog of RegIII-γ, REG3A, following colonization of human intestinal organoids (D. R. Hill et al. 2017). Together, these findings indicate intestinal barrier antimicrobial peptide production is differentially influenced by the microbiota at a species-specific level. Thus, induction of RegIII-γ may promote barrier integrity by inhibiting colonization of the mucus and microbial invasion.

Accordingly, mucus integrity and antimicrobial peptide secretion are key elements mediating susceptibility to intestinal injury and inflammation in

experimental models (**Chapter 3**) (Frantz et al. 2012; Johansson et al. 2008; Johansson et al. 2010; Johansson et al. 2014; Natividad et al. 2012). Following human commensal colonization, establishment of physiological mucus characteristics paralleled an overall increase in barrier integrity, demonstrated by diminished exposure to bacterial antigens and enhanced resilience towards DSS-induced intestinal injury and inflammation (**Chapter 3**). DSS causes injury to the barrier through diminishment of the mucus layer and epithelial cell damage, which enables microbiota invasion and subsequent inflammation (Gaudier et al. 2005; Wirtz et al. 2007). Thus, a fortified mucus layer may inhibit epithelial exposure to DSS, and in combination with RegIII-γ, limit microbial invasion, resulting in reduced susceptibility to injury and inflammation (Brandl et al. 2007; Johansson et al. 2014). During early colonization, the intestinal barrier was highly susceptible to low-dose DSS-induced injury, which may be attributable to immaturity of the mucus structure. However, the microbiota also influences resilience of the intestinal barrier following colonization, independently of colonization duration; adult mice colonized with neonatal microbiota exhibit high susceptibility to infection-induced colitis (Kim et al. 2017). Such findings suggest members of the neonatal microbiota may be unable to promote maturation of barrier characteristics associated with integrity, emphasizing the role of the microbiota in determining barrier integrity.

Since the microbiota can greatly impact barrier integrity, microbiota-targeted therapeutic strategies have developed to modulate barrier function, such

as use of probiotics. In **Chapter 4**, *B. breve* NCC2950 was shown to induce production of RegIII-γ, and our lab has previously demonstrated improved resilience towards DSS-induced injury of mice deficient in NOD1 and NOD2 signaling occurred with *B. breve* NCC2950 supplementation (Natividad et al. 2012). Therefore, I further characterized the therapeutic potential of *B. breve* NCC2950 as a probiotic in wild-type SPF mice, and found supplementation of live *B. breve* reduced susceptibility to acute DSS-induced injury (**Chapter 5**). In contrast, acute DSS-induced injury was not prevented by IL-10 secreting *Lactobacillus lactis* (unpublished data). However, IL-10 producing *L. lactis* is protective in chronic models of inflammation, whereas *B. breve* NCC2950 was unable to prevent reactivation of DSS-induced inflammation (**Chapter 5**) (Martin et al. 2014; Steidler et al. 2000). Additionally, administration of *B. breve* NCC2950 concurrently with DSS did not attenuate acute injury (**Chapter 5**). During injury and inflammation, it is possible the intestinal barrier response to *B. breve* is altered, reducing barrier-promoting effects. Secretion of antimicrobial peptides, including RegIII, can be increased during inflammation, as evidenced in chemical and infection colitis models as well as IBD patients with active disease (Brandl et al. 2007; Granlund et al. 2011; Ismail et al. 2009; Ogawa et al. 2003; Wehkamp et al. 2004). Thus, promotion of antimicrobial peptide production may be insufficient to attenuate inflammation. Indeed, probiotic mixtures that have shown efficacy during active inflammation are associated with promotion of mucus production (Caballero-Franco et al. 2007; Toumi et al. 2013). Although

colitis reactivation was not prevented by *B. breve* NCC2950, both the live and heat-inactivated formulations normalized clinical symptoms following acute DSS (**Chapter 5**). Thus, I hypothesize that *B. breve* NCC2950 stimulates RegIII-γ production and promotes bacterial exclusion, providing protection and enhancing recovery from injury (Supplemental Figure 8, **Appendix II**); however, once the structural integrity of the barrier has been compromised and inflammation has become established, RegIII-γ-mediated bacterial exclusion is insufficient to prevent invasion (**Chapter 4**) (Natividad et al. 2012; Vaishnava et al. 2011). Together, these results suggest the intestinal inflammatory milieu and probiotic species-specific mechanism of action impacts the resilience towards injury and inflammation.

These findings demonstrate the microbiota greatly impacts resilience towards intestinal injury and inflammation in association with modulation of RegIII-γ production and mucus fortification. Furthermore, efficacy of microbiota-targeted strategies to promote barrier function and reduce inflammation was demonstrated, supporting their use as clinical therapies. However, effects on barrier function and resilience are dependent upon the type of bacterial-derived stimulus encountered, mechanism of action, as well as the existing intestinal inflammatory milieu; thus, microbiota-based therapeutic strategies should be comprehensively characterizer prior to clinical investigation.

6.2.3 Human microbiota colonization of adult mice: a model for barrier function assessment.

Derivation of mice into a germ-free status has proven to be a valuable tool in elucidating of the effects of the microbiota on host physiology. The colonization of germ-free mice is commonly used to investigate the role of specific microbial communities or select bacteria on immune development and function, obesity and malnutrition, the gut-brain-axis (Bercik et al. 2011; El Aidy et al. 2012; Macpherson and Uhr 2004; Turnbaugh et al. 2009). As differences in microbiota composition were recognized in inflammation and disease, colonization of germ-free mice with human microbiota became one of the foremost models of evaluating effects of specific microbial communities on host physiology as well as potential therapeutic targets (Caminero et al. 2016; De Palma et al. 2017; Johansson et al. 2015; Natividad et al. 2015; Turnbaugh et al. 2009). Microbiota establishment and metabolic profiles, as well as immune development, have been characterized in human microbiota colonized mice (El Aidy et al. 2012; Johansson et al. 2015; Marcobal et al. 2013; Turnbaugh et al. 2009). As a key regulator of antigen exposure and immune responses, proper regulation of barrier permeability and maintenance of integrity is of great importance. Deviations in barrier permeability and integrity from conventional mice upon colonization with human microbiota could greatly impact findings and limit the translatability of studies. Therefore, in **Chapter 3,** I evaluated permeability and barrier structural characteristics following human commensal

microbiota colonization of germ-free mice, and found them to be comparable to SPF mice within a week of colonization. My findings that human commensal microbiota colonization of adult germ-free mice induces physiological barrier function support its use as a model for evaluating potential modulators of intestinal barrier function.

6.2.3.1 Limitations of adult colonizations.

To elucidate the impact of commensal microbiota independently of inherently programmed postnatal barrier maturation and effects of weaning, adult mice were used in my colonization study outlined in **Chapter 3**. Furthermore, to ensure consistent microbiota composition between mice and development of a complex microbial community, *de novo* colonization with adult microbiota was implemented. While my methods were warranted to obtain my objective and my results provide valuable information, in light of these factors, I recognize the barrier maturation dynamics observed in my study may not reflect that of a neonate. Differences in microbial succession have been noted between my colonizations with adult microbiota and vertical transmission. In mice, *Streptococcus* is dominant at postnatal day 1, and remains abundant while expansion of *Lactobacillus* occurs at days 3 and 9, coinciding with a transient loss of diversity (Pantoja-Feliciano et al. 2013). In contrast, I did not observe early expansion of Streptococcaceae or Lactobacillaceae. Expansion of Clostridiales, Porphyromonadaceae, Verrucomicrobiaceae, Ruminococcaceae and

Lachnospiraceae occurs by postnatal day 21 in neonates, coinciding with weaning (Kim et al. 2017; Pantoja-Feliciano et al. 2013). In my study, Clostridia are abundant at day 1 post-colonization and expansion of the other aforementioned bacteria is observed by day 7. Differing inoculum compositions, diets and host sources may contribute to such discrepancies in succession. Indeed, the fecal microbiota of vaginally delivered human infants harbours high abundances of *Prevotella* and *Lactobacillus* during early colonization (Dominguez-Bello et al. 2010). Overall, this evidence suggests the dynamics of vertically transmitted early colonizers is lacking in my study of adult microbiota colonization; however, microbiota taxonomic structure in my *de novo* human microbiota colonized adult mice resembles that of conventional mice post-weaning. Thus, despite differing microbial succession patterns during early colonization, prominent members of the adult microbiota are established in my *de novo* adult human commensal colonized mice, along with physiological barrier function. Therefore, colonization of adult mice with human microbiota is a practical, reductionist model for investigating effects of the microbiota on intestinal barrier function independently of age-related dietary factors and postnatal maturation.

Additionally, in **Chapter 3**, it was noted that mice colonized for 7 days did not exhibit the same level of resilience to low dose DSS-induced injury and inflammation as conventionally raised SPF mice. This may be due to the more proinflammatory colonic immune milieu that results from colonization in adulthood (El Aidy et al. 2013; Olszak et al. 2012). Furthermore, it should be

considered that the immune system is also developing following colonization. Thus, differing immune responses between 7-day colonized and conventional mice may be accountable for different levels of resilience. In turn, there may be immune responses absent in 1-day colonized mice that contribute to resistance towards chemically induced injury and inflammation observed in mice colonized for 7 days. Also, adaptive immune development in mice colonized with human microbiota is not equivalent to development in conventionally raised mice; T cells are reduced throughout the small intestine and MLNs, and activation markers, T_H17 responses, αβ-IELs, B cells and IgA production are also diminished in the small intestine (Chung et al. 2012). Therefore, the conventional mice may have a more effective immune response towards microbial invasion following DSS-induced injury.

6.3 Microbial sensing mediates intestinal barrier function.

PRRs such as NOD1, NOD2 and TLRs have been implicated in facilitating barrier function changes in response to the microbiota. Indeed, as detailed in **Chapter 3**, increased mRNA expression of NOD1, TLR2, TLR5, MyD88, and a number of proteins involved in PRR signaling cascades paralleled barrier mucus and permeability changes post-colonization. As discussed above, I also found evidence NOD1 and NOD2 signaling may facilitate colon paracellular permeability changes following colonization. TLR-MyD88 signaling has previously been shown to induce mucin-2, RELM-β and RegIII-γ production

(Frantz et al. 2012; Gong et al. 2010; Vaishnava et al. 2008). Accordingly, I also found intestinal RegIII-γ induction was regulated by TLR adaptor proteins MyD88 and Ticam1 (**Chapter 4**). Moreover, RegIII-γ production could be stimulated *in vitro* by live and heat-inactivated *B. breve* NCC2950, but not the filtered growth media, suggesting structural components of *B. breve* NCC2950 elicit RegIII-γ production (**Chapter 4**). In the steady state, NOD2 signaling also stimulates RegIII-γ production, and our lab has previously demonstrated mice deficient in NOD1 and NOD2 signaling have impaired intestinal RegIII-γ production, which can be promoted with live or heat-inactivated *B. breve* NCC2950 supplementation (Natividad et al. 2012; Vaishnava et al. 2008). Collectively, these studies demonstrate microbial stimulation of PRRs modulates structural, innate immune and permeability aspects of intestinal barrier function.

6.4 Conclusions

The work presented in this thesis demonstrates the microbiota impacts establishment and modulation of intestinal barrier function and integrity. Such work supports the utility of human microbiota colonization of adult germ-free mice as an experimental model and also provides a knowledge basis from which altered barrier function can be better understood. In future investigations, targeted disruption of barrier maturation dynamics may aid in deciphering the complex dynamics of neonatal colonization and establishment of barrier function (or

dysfunction). Furthermore, these findings also provide rationale for the therapeutic use of microbiota-targeted strategies to fortify barrier function.

APPENDIX I

IMPORTANCE OF THE MICROBIOTA IN EARLY LIFE AND INFLUENCE ON FUTURE HEALTH

Importance of the Microbiota in Early Life and Influence on Future Health

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This book chapter has been published in The Gut Brain Axis: Dietary, Probiotic, and Prebiotic Interventions on the Microbiota (Academic Press):
Verdu, E.F., Hayes, C.L., O'Mahony, S.M. (2016). Importance of the microbiota in early life and influences on future health. In N. Hyland & C. Stanton (Eds.), *The Gut Brain Axis: Dietary, Probiotic, and Prebiotic Interventions on the Microbiota* (159-184). Academic Press.

Title: Importance of the Microbiota on Early Life and Influence in Future Health

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ABSTRACT

The infant gut microbiome is dynamic, with radical shifts in its composition occurring during early life. Many factors are capable of influencing this colonization. Disruption of the developmental patterns prior to the attainment of a more stable adult-like gastrointestinal microbiota is increasingly being linked to adverse health outcomes. The impact of the microbial composition of our gastrointestinal tract on systems outside the gut is also becoming apparent and has attracted much recent attention. Many disease states are now associated with a dysbiosis of the gut bacteria with the origin of such being related to early life colonization patterns. Here we highlight the factors that are influenced by microbiota colonization in early life and the potential outcomes with regard to future health.

Keywords: Anxiety; Brain–gut microbiota axis; Celiac disease; Depression; Early life; Germ-free; Inflammatory bowel disease; Irritable bowel syndrome; Microbiota.

INTRODUCTION

Just as in other mammals, humans harbor enormous quantities of commensal microorganisms on the external and internal surfaces of our bodies. Within a particular habitat, for example, in the gut, the collection of these commensals is referred to as microbiota, the collective genome being termed the microbiome (Collins et al., 2012; Cryan and Dinan, 2012). Within our gut a unique combination of different populations of organisms exists. These are mainly bacteria but also archaea, viruses, and protozoa—roughly approximating 3–10 times the number of human cells (Blaser and Webb, 2014). With the advent of metagenomic high-throughput platforms, a better understanding of the composition of a healthy human gut microbiome has been achieved, albeit quite likely that multiple, individual-specific, "healthy" compositional states exist (Clarke et al., 2014a). The gastrointestinal (GI) microbiota is quickly being recognized as a vital player in health and disease. A well-balanced microbial community within the gut is known to confer many health benefits on the host, whilst many GI (Verdu, 2012; Clarke et al., 2014b; De Palma et al., 2014a; Galipeau and Verdu, 2014; Moloney et al., 2014; Verdu et al., 2015) and extra-GI diseases (Cryan and O'Mahony, 2011; Bercik et al., 2012; Desbonnet et al., 2014; Burokas et al., 2015; Gulden et al., 2015; Lopez-Cepero and Palacios, 2015; Melli et al., 2015) are associated with dysbiosis. The origin and development of a "healthy" gut microbiota starts in early life, which is designated as an important neurodevelopmental time window. This provides an opportunity for the

colonizing microbiota to influence immature systems, such as the central nervous system (CNS), and can permanently impact host health and well-being. This is abetted by the bidirectional communication system, the microbiota–gut–brain axis, which includes the stress axis, the immune system, and neuronal elements such as the vagus nerve (Cryan and O'Mahony, 2011; De Palma et al., 2014b). During early life this axis is also developing and is, in itself, open to modification by the gut microbiota (Sudo et al., 2004; O'Mahony et al., 2014). Stress during early life is known to be a predisposing factor for psychiatric disease in adulthood (Lupien et al., 2009) and has long-term effects on the colonization of the gut (O'Mahony et al., 2009). Conversely, disruption of the microbiota in early life, or its absence, leads to altered pain sensitivity (Amaral et al., 2008; O'Mahony et al., 2009), development of inflammatory bowel disease (IBD) (Shaw et al., 2010), asthma (Canova et al., 2015; Pitter et al., 2015), diabetes (Candon et al., 2015), and obesity (Saari et al., 2015). In this chapter, we focus on the importance of the microbiota that colonize the GI tract in early life and events that can affect which bacteria take up residence (Fig. 9.1). We consider the systems that are concomitantly developing and ones that can be permanently affected by the pattern of colonization events. We highlight disorders that are associated with aberrant microbiota composition and microbiota–gut–brain axis signaling. Finally, we integrate these findings to define the implications for future health from a preventative, diagnostic, and therapeutic perspective.

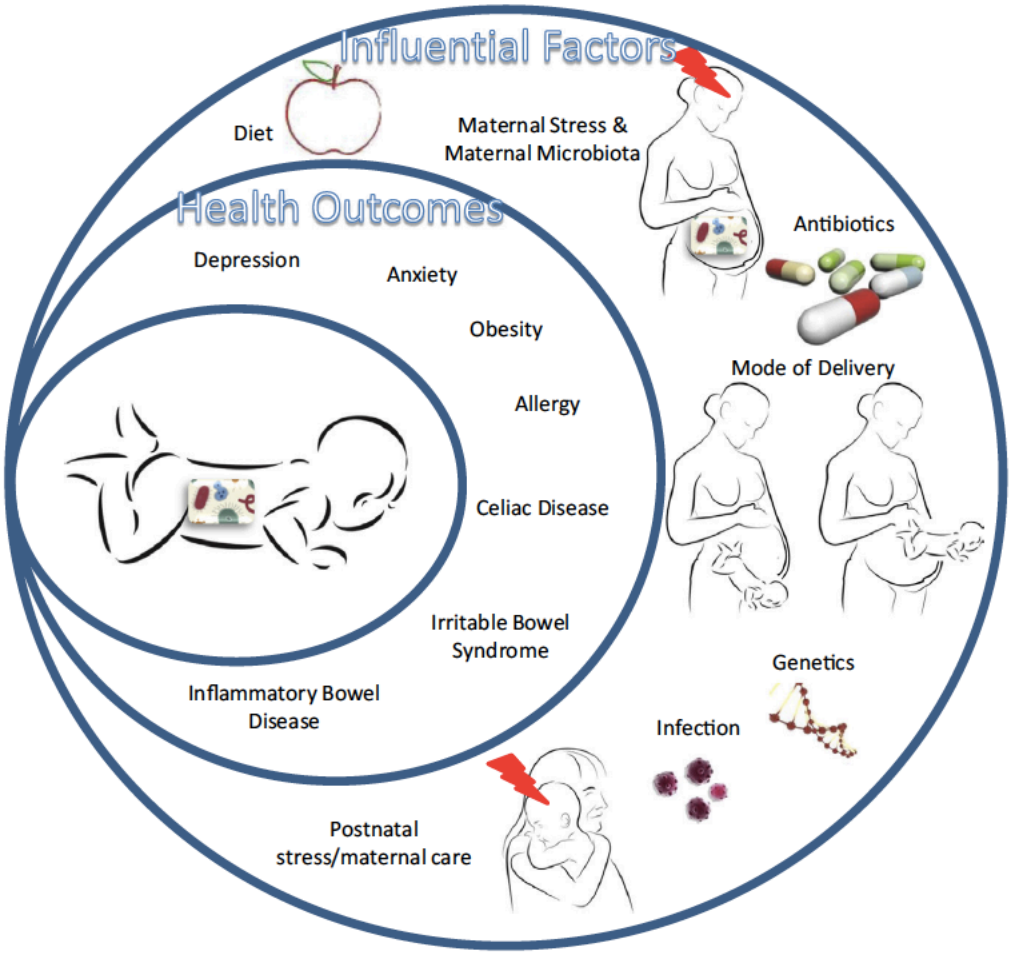

Figure 9.1. Some of the potential factors that can influence colonization of the infant gut in early life.
It is now apparent that these factors can have an impact both pre- and post-natally. Disruption of colonization by these factors may have long-term impacts on the future health of the child.

THE DEVELOPING MICROBIOTA IN EARLY LIFE

Prenatal Microbiota Colonization

Whilst the microbiota changes and evolves throughout the life span (Borre et al., 2014b; O'Mahony et al., 2015), here, we focus largely on the early life

period. Previously held convictions that the fetus and intrauterine environment are sterile are now being challenged (El Aidy et al., 2013; Funkhouser and Bordenstein, 2013). Evidence put forward in support of this proposition include the demonstration that bacteria can be isolated from the meconium of healthy neonates (Jimenez et al., 2008), potentially as a result of prenatal colonization (Mshvildadze and Neu, 2010). A uterine source for these meconium-derived bacterial strains is suggested in part by studies indicating a microbial presence in amniotic fluid during preterm labor (DiGiulio et al., 2008). It is equally plausible, given the time delay between birth and meconium production, that the source of such bacteria is the mother's own microbiota. However, maternal infection cannot be eliminated as the amniotic source (Matamoros et al., 2013). Spontaneously released meconium obtained from preterm babies during the first three weeks of life contained a specific microbiota that differs from those observed in early fecal samples (Moles et al., 2013). Firmicutes predominated in meconium while Proteobacteria was abundant in feces (Moles et al., 2013). In animal studies, genetically labeled *Enterococcus faecium* was detected in the amniotic fluid of pregnant rats and in the meconium of the offspring delivered via caesarean section following oral administration during pregnancy (Jimenez et al., 2008). These data suggest that maternal microbial transmission in mammals occurs during pregnancy and lactation. The placenta may be permissible to this translocation as *Lactobaccillus* and *Bifidobacteria* DNA were isolated from the placenta following delivery, however, there were no viable cells (Satokari et al., 2009). Moreover, a

unique microbiome has been identified within the placenta that appears to correlate with the oral cavity population of the mother (Aagaard et al., 2014). Several bacterial species, including *Enterococcus*, *Streptococcus*, *Staphylococcus*, and *Propionibacterium*, have also been isolated from umbilical cord blood (Jimenez et al., 2008). These data suggest that this element may also be involved in the translocation of bacteria. The implications of prenatal microbiota transfer on the developing fetus and on future health are not yet known. Given that essential modulatory systems develop in utero, this period presents as an important influential opportunity for microbiota–host interactions. To date, the most explicit preclinical example of a prenatal role for the microbiome relates to development of the blood–brain barrier, which has been shown to be altered in germ-free animals (Braniste et al., 2014). Despite the growing appreciation that the prenatal environment may not be sterile, it is still, nonetheless, accepted that the developing fetus is largely protected from bacterial infections (Costello et al., 2012).

Both the vaginal (Aagaard et al., 2012; Romero et al., 2014; Mueller et al., 2015a) and intestinal microbiota (Koren et al., 2012; Mueller et al., 2015a) undergo extensive remodeling over the course of pregnancy. As yet it is not known if there is transfer from these sites to the fetus prenatally. Specific changes include a decrease in proinflammatory Proteobacteria from the first to the third trimester and an increase in anti-inflammatory *Faecalibacterium prausnitzii* (Koren et al., 2012; Mueller et al., 2015a). Moreover, bacteria superior at energy

harvesting are enriched during pregnancy to presumably support the growth of both mother and baby (Koren et al., 2012; Mueller et al., 2015a). The maintenance of the optimum microbial balance during pregnancy is essential to the successful inoculation of the baby during delivery; hence any situation likely to perturb the maternal microbiota, such as stress or antibiotic administration, have the potential to influence optimal vertical transmission to the baby.

It has been demonstrated that maternal stress, measured by reported stress or elevated cortisol levels, is associated with a differential gut microbiota in infant offspring (Zijlmans et al., 2015). This colonization pattern was related to infant GI symptoms and allergic reactions. Maternal prenatal stress impacts on the microbiota that will essentially colonize the infant during birth, thereby helping to shape future development (Jasarevic et al., 2015a,b). Indeed, a recent study has shown that prenatal stress in mice led to decreased maternal vaginal *Lactobacillus*, which subsequently resulted in decreased transmission of this bacterium to offspring (Jasarevic et al., 2015a). These results identify the vaginal microbiota as a novel factor by which prenatal stress may contribute to reprogramming of the developing baby with possible implications for future health and well-being. Moreover, we have recently shown that prenatal stress induced long-lasting alterations in the intestinal microbiota in adult offspring (Golubeva et al., 2015). In particular, the relative abundance of distinct bacteria genera significantly correlated with certain physiological parameters and the responsiveness of the stress axis. Hence, seeding the neonatal gut with a

suboptimal microbiota can lead to long-lasting effects on overall future health. Of clinical relevance, intrapartum antibiotic use is associated with decreased bacterial diversity of the baby's first stool and lower abundance of beneficial bacteria (O'Neil et al., 2014), the long-term impact of which remains unexplored. The influence of prenatal events on the gut microbiota and subsequent impact on future health is coming to the fore, but more research is needed in order to realize the full impact.

FACTORS AFFECTING POSTNATAL MICROBIAL COLONIZATION

Environmental influences over microbiota development are substantial. Microbial nurturing and environmental conditions give rise to both a tailoring of the microbiota and a progressive sequence of dominant flora as the child matures, as well as determining health outcomes (Clarke et al., 2014a; Moloney et al., 2014). Studies indicate that the initial GI microbial community composition is determined by delivery mode and that this maternally influenced microbial signature can last from months to years (Jakobsson et al., 2013; Clarke et al., 2014a) and influences health outcomes (Parfrey and Knight, 2012; Relman, 2012). Furthermore, infants born vaginally initially harbor a subject-specific microbiota dominated by lactic acid bacteria, for example, *Lactobacillus* spp. characteristic of their mother's vaginal and fecal microbiota (Dominguez- Bello et al., 2010; Vaishampayan et al., 2010). Whilst the initial microbial residents in babies born by caesarean section also exhibit features of maternal transmission,

they tend to resemble the skin microbiota (Dominguez-Bello et al., 2010; Mueller et al., 2015b) and to be devoid of, or low in, *Bifidobacteria* spp. (Penders et al., 2006; Biasucci et al., 2008). Environmental sources from the hospital environment can also influence the initial microbiota of caesarean section–delivered infants (Dominguez- Bello et al., 2010). It also appears that alterations in bacterial richness and diversity associated with caesarean section delivery are accentuated by elective procedures (Azad et al., 2013). As a consequence of caesarean section delivery, decreased microbiota diversity and delay in colonization with beneficial *Bifidobacteria* spp. and *Bacteroidetes* persists for the first two years of life and is associated with reduced Th1 immune responses (Jakobsson et al., 2013). Importantly, however, infants delivered by caesarean section do eventually match up to their vaginally delivered counterparts in terms of stability and diversity of their adult microbiota (Maynard et al., 2012). However, more studies are necessary to assess the full implications of being born via caesarean section, the impact of altered initial colonization in vulnerable individuals, and the development of disorders in later life. Of note, preterm birth is associated with a considerably different GI microbiota profile than that observed in full-term counterparts (Barrett et al., 2013) and correlates with delays in development.

ENVIRONMENTAL INFLUENCES ON EARLY LIFE MICROBIOTA

Nutrition

A key element in shaping the microbiota in early life relates to nutritional factors. There is an increased abundance of certain *Bifidobacterium* spp., which thrive on human milk oligosaccharides (Zivkovic et al., 2011), in infants who are exclusively breastfed (Costello et al., 2012). Human milk microbiota also appears to be a direct source of *Bifidobacteria* and *Lactobacillus* in addition to its prebiotic properties (Fernandez et al., 2013). Moreover, the bacteria contained in breast milk has been noted to vary from colostrum to late lactation, by gestational age, maternal health status, and delivery mode (Khodayar-Pardo et al., 2014; O'Neil et al., 2014; Mueller et al., 2015a). The microbiota of formula-fed infants appear to have an increased diversity and a high abundance of coliforms, *Bacteroides* and *Clostridium difficile* (Penders et al., 2006; Azad et al., 2013). Moreover, a modest increase in both verbal and nonverbal IQ scores in breast-fed individuals has also been noted (Christakis, 2013). Collectively these studies indicate that particular microbes may impact brain development and, potentially, future health. Maternal nutrition may also play a role in this context with some of the reported benefits at three years of age being most pronounced in infants whose mothers consumed more than two portions of fish per week while breast-feeding (Belfort et al., 2013).

Stress

Stress, the effects of which are predominantly mediated through the hypothalamic-pituitary-adrenal (HPA) axis, has long been known to influence the composition of the gut microbiota (Tannock and Savage, 1974). However, this influence is now more widely acknowledged; in particular, stress in early life significantly alters the microbiota in adulthood (O'Mahony et al., 2009). Hence, events during early life capable of activating the stress axis can potentially impact the developing microbiota in the neonatal period and vice versa. This could ultimately lead to dysbiosis in the GI tract and an inappropriate stress response persisting into adulthood.

Early Life Antibiotic Use and Germ-Free Environment

Early life antibiotic treatment is common practice (Vangay et al., 2015) with epidemiological studies highlighting associations between antibiotic use in early infancy and occurrence of disorders such as diabetes (Candon et al., 2015), obesity (Saari et al., 2015), asthma (Pitter et al., 2015), celiac disease (Canova et al., 2015), and IBD (Shaw et al., 2010). Longitudinal studies have demonstrated profound short- and long-term effects of antibiotics on the diversity and composition of the microbiota (Rodriguez et al., 2015). For example, neonatal antibiotic usage reduces the diversity of the *Bifidobacterium* spp. and *Bacteroides* (Hussey et al., 2011; Johnson and Versalovic, 2012). There are also some recent indications that an adult-like stable and diverse microbiota might not be acquired

until adolescence, which, if confirmed, greatly extends the time window during which the microbiota can influence health outcomes (Agans et al., 2011). Germ-free mice provide a valuable model in which to assess the impact of growing up without a microbiota (Williams, 2014) and have proven useful as tools in which to assess the impact of the microbiota on CNS function (Collins et al., 2012; Farmer et al., 2014; Dinan et al., 2015; Stilling et al., 2015; Zhou and Foster, 2015; Stilling et al., 2016). Such work extends to behaviors relevant to mood, cognition, pain, and social interaction (Burokas et al., 2015). It is clear that the postnatal period is a dynamic phase of microbial development during which time there is a great deal of potential for this ecosystem to impact other concomitantly developing systems.

IMPACT OF EARLY LIFE MICROBIOTA ON THE DEVELOPMENT OF KEY HOST HOMEOSTATIC MECHANISMS

How is the intestinal barrier development shaped by the microbiota?

The intestinal barrier is the host's first line of defense against luminal pathogens and toxins, and yet, must permit tolerance toward the commensal microbiota. At birth, however, the intestinal barrier is physiologically and morphologically immature. Upon colonization the intestinal barrier undergoes changes in order to cope with microbial interactions and reach a homeostatic state that maintains gut function and provides protection to the host, thereby avoiding aberrant immune activation and inflammation (Fig. 9.2). Barrier changes induced

by colonization have been studied using germ-free animal models and have focused on the impact of commensal microbiota on immune parameters of barrier maturation (El Aidy et al., 2012). Within days of conventionalization (colonization with mouse specific pathogen free (SPF) microbiota), the structure of the mucosa in germ-free mice is altered. Specifically, crypts elongate, small intestine villi shorten, cellular infiltration increases, the lamina propria expands, and differentiation of enterocytes into Paneth cells and enteroendocrine cells increases (Smith et al., 2007; El Aidy et al., 2012). Mucin expression, mucus layer thickness, and the proportion of sialylated to sulfated mucins also increase following colonization (Linden et al., 2008; Petersson et al., 2011; El Aidy et al., 2012; Frantz et al., 2012; Larsson et al., 2012). However, these changes to the mucus layer are dependent on the type of bacteria and their metabolic products. For example, colonization with the short-chain fatty acid-producer, *Bacteroides thetaiotaomicron* results in normal mucus layer development. However, when combined with an acetate consumer and butyrate producer, *Faecalibacterium prausnitzii*, the extent of the mucus layer changes are attenuated (Wrzosek et al., 2013). Furthermore, both luminal lipopolysaccharide and peptidoglycan can increase mucus thickness independently, demonstrating that bacterial antigen signaling alone can alter the mucus layer (Petersson et al., 2011).

Innate immune aspects of the barrier develop by day four post-conventionalization of germ-free mice and include increased expression of pattern recognition receptors (PRRs) as well as secretion of antimicrobial peptides

(Vaishnava et al., 2008; Gaboriau-Routhiau et al., 2009; Vaishnava et al., 2011; El Aidy et al., 2012; Frantz et al., 2012). The induction of antimicrobial peptide production is also influenced by the type and complexity of the microbiota, with studies showing that colonization of germ-free mice with SPF microbiota leads to greater MyD88/TRIF-mediated RegIII-γ expression in the colon compared to the eight-strain altered Schaedler flora (Natividad and Verdu, 2013). Furthermore, monocolonization with the probiotic *Bifidobacterium breve* induces higher RegIII-γ expression than *Escherichia coli* JM83 (Natividad et al., 2013).

Intestinal permeability is a function of the intestinal barrier that must be carefully regulated in order to facilitate nutrient absorption and passage of electrolytes without permitting bacterial infiltration. Although luminal contents are taken up through enterocyte endocytosis in the small intestine, 97% of proteins undergo lysosomal degradation, preventing transcellular trafficking of intact proteins (Heyman et al., 1986; Fujita et al., 1990). However, transcellular passage of intact proteins is transiently increased at days three and four after birth in the small intestine and coincides with translocation of live bacteria to mesenteric lymph nodes (MLNs) and spleen in *Morganella morganii* monocolonized mice (Heyman et al., 1986; Shroff et al., 1995). Given that maternal immunoglobulins from breast milk can undergo retrograde transcytosis by binding to apical neonatal Fc receptors (nFcR) on enterocytes (Simister and Rees, 1985), transiently increased transcellular permeability is an important mechanism for luminal antigen sampling and early life protection. On the other

hand, paracellular permeability to large molecules is regulated by the structural integrity of tight junction and adherens junction protein complexes between adjacent epithelial cells. Compared to conventionally raised mice, both ileum adherens and tight junctions of germ-free mice are shorter in size, and expression of integral tight junction proteins is decreased (Kozakova et al., 2015). Translocation of live bacteria to the MLNs and spleen diminishes within one to three weeks of conventionalization and monocolonization of germ-free mice (Berg and Garlington, 1979; Shroff and Cebra, 1995). This may be due to immune maturation, since the decline in bacterial translocation coincides with production of microbe-specific IgA in *Morganella morganii* monocolonized mice (Berg and Garlington, 1979; Shroff and Cebra, 1995).

Recently, goblet cell-associated antigen passages (GAPs) have been described. These allow translocation of antigens from the lumen to the lamina propria following muscarinic acetylcholine receptor 4 activation (McDole et al., 2012; Knoop et al., 2015). GAPs form in the adult small intestine but are inhibited in the colon by bacterial stimulation of MyD88 signaling under steady-state conditions, indicating region-specific regulation by the microbiota. It is unknown whether molecules pass through goblet cells themselves or paracellularly. Colonic goblet cells of young mice have higher incidence of endocytosis compared to adults, suggesting GAP antigen uptake may be higher during early colonization if endocytosis is involved (Colony and Specian, 1987).

Together these findings suggest microbial colonization impacts intestinal barrier maturation, although the influence of different microbial communities remains largely unknown. Since impaired barrier defenses and permeability regulation are associated with a number of GI and psychological disorders, determining if barrier dysfunction originates from early life colonizing microbiota-induced alterations may further elucidate disease pathogenesis.

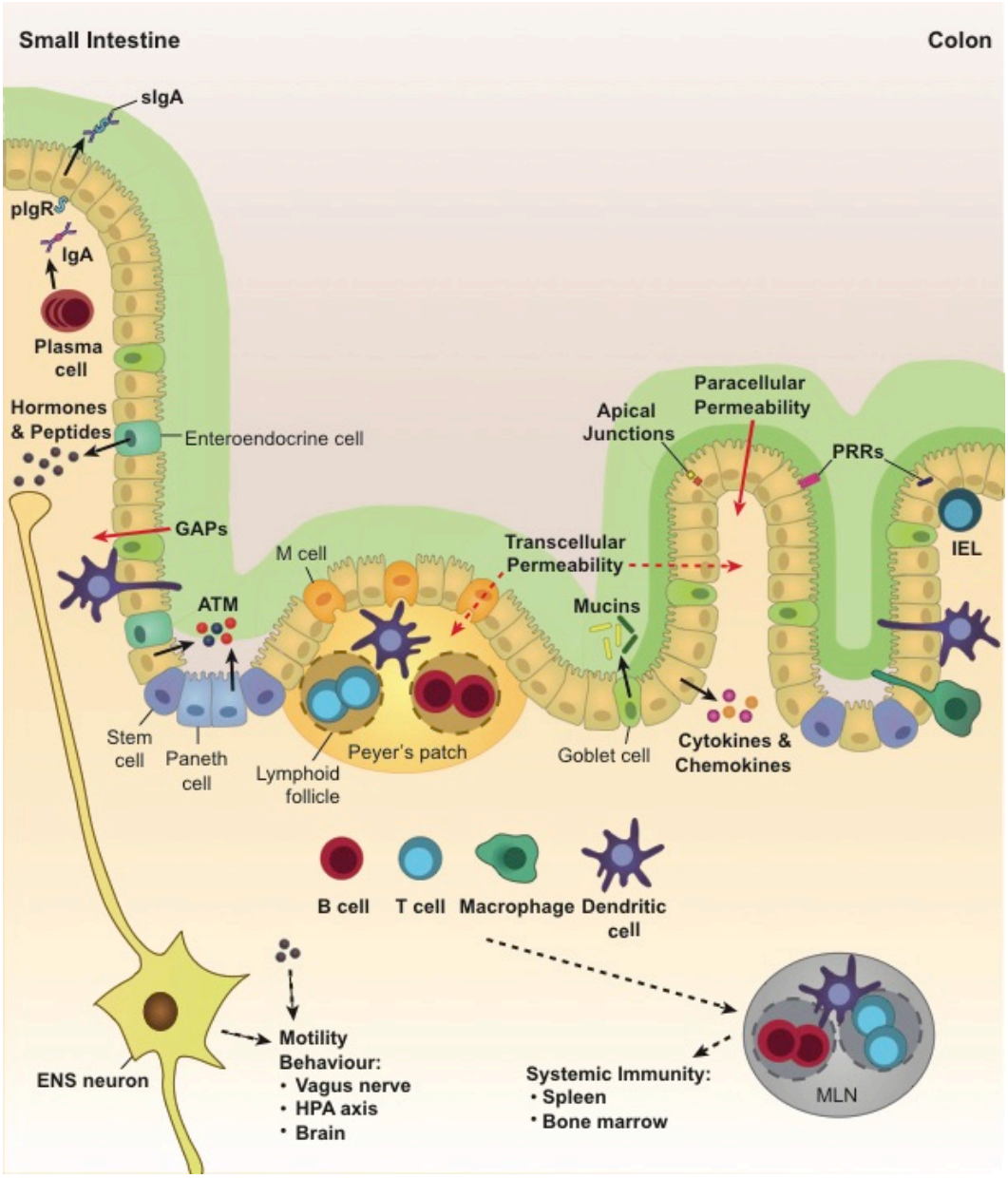

Figure 9.2. The intestinal barrier is the host's first line of defense against luminal pathogens and toxins, but must permit tolerance toward the commensal microbiota and environmental antigens, while also allowing passage of water, electrolytes, and nutrients.

To facilitate these various functions, the intestinal barrier is comprised of a semipermeable epithelial and mucus barrier that uses adaptive and innate immune features to limit translocation of pathogens and harmful antigens, and elicits measured immune responses when appropriate.

Immune Development

Colonization studies have demonstrated microbial stimulation is required for maturation of lymphoid structures (Smith et al., 2007). Germ-free mice have immature isolated lymphoid follicles (ILFs), small Peyer's patches that lack germinal centers, and an absence of distinct T and B cell follicles in MLNs and spleen. However, these mature following colonization (Adachi et al., 1997; Bouskra et al., 2008). Maturation of ILFs is impaired in the absence of nucleotide-binding oligomerization domain-containing protein (NOD) 2. Toll-like receptor (TLR) signaling is completely attenuated without NOD1 signaling to recruit T and B cells (Bouskra et al., 2008). Moreover, the *Bacteroides fragilis* product, polysaccharide A (PSA), can stimulate expansion of spleen lymphocyte follicles (Mazmanian et al., 2005). Microbial stimulation, often through MyD88 signaling, is required to induce site-specific expansion and maturation of innate immune cells and is directly related to the level of microbiota complexity (Williams et al., 2006; Fujiwara et al., 2008; Balmer et al., 2014). Following colonization, the capacity of spleen dendritic cells (DCs) and macrophages to produce proinflammatory cytokines and prime natural killer (NK) cells is enhanced, and gut chemokine CX3C receptor 1 positive cell sampling of luminal antigens increases (Chieppa et al., 2006; Niess and Adler, 2010; Ganal et al., 2012). Over the first two weeks of colonization, systemic neutrophil levels rise to compensate for microbial exposure and prevent septic bacterial infections; this is inhibited by perinatal antibiotic-induced microbiota depletion (Bandeira et al., 1990;

Deshmukh et al., 2014). Following colonization. intraepithelial lymphocyte (IEL) populations increase, particularly αβ T cell receptor-bearing cells; although maintenance of IEL populations is dependent upon stimulation of the aryl hydrocarbon receptor by dietary ligands (Li et al., 2011). Furthermore, MyD88 signaling in small intestinal epithelial cells leads to production of the bactericidal lectins, RegIIIγ and RegIIIβ, by γδ IELs, thus preventing bacterial translocation to the spleen (Ismail et al., 2011). Therefore, the microbiota stimulates expansion and maturation of innate immune cells.

Colonization also greatly influences development of adaptive immunity, altering the abundance, phenotype, and function of T and B cells. Adaptive immune presence is observed by day eight in the colon and day 16 in the small intestine of conventionalized mice when a balance between pro- and anti-inflammatory responses is induced (Williams et al., 2006; Gaboriau-Routhiau et al., 2009; El Aidy et al., 2012). However, specific members of the microbiota can influence T cell induction and dictate the balance between effector and regulatory T cells following colonization. Bacteria belonging to Firmicutes, particularly the *Clostridia* class, are instrumental in inducing balanced T cell responses (Gaboriau-Routhiau et al., 2009; Atarashi et al., 2011, 2013; Chung et al., 2012; Atarashi et al., 2013). Colonization with microbiota low in Firmicutes leads to augmented T helper (Th)17 cells in the colon, and this can be reversed by supplementation with Lachnospiraceae and Ruminococcaceae (Natividad et al., 2015). *Clostridium* clusters IV and XIVa, as well as *B. fragilis* PSA, stimulate

expansion of interleukin (IL)10 expressing regulatory T cells (Tregs), whereas segmented filamentous bacteria (SFB) induce Th17 cells (Mazmanian et al., 2005; Ivanov et al., 2009; Round and Mazmanian, 2010; Atarashi et al., 2011; Geuking et al., 2011; Lathrop et al., 2011; Round et al., 2011; Atarashi et al., 2013). However, in the spleen, PSA induces Th1 differentiation, indicating different compartmental responses to microbial stimulation (Round and Mazmanian, 2010). Colonization of germ-free mice with human microbiota or SFB fails to elicit the same level of adaptive immune maturation seen in conventionalized mice. However, cohousing human microbiota colonized, or "humanized," mice with conventionalized mice that harbor SFB normalizes effector T cell levels in the gut but not Tregs (Chung et al., 2012). Germ-free mice have low amounts of invariant NK (iNK) T cells in the spleen and liver but high amounts in the colon (Wei et al., 2010; Olszak et al., 2012). Colonization with iNK T cell antigen-bearing *Sphingomonas* rapidly increases spleen and liver iNK T cell numbers and induces maturation and responsiveness to antigens, which is not observed with iNK T cell antigen lacking *E. coli* colonization (Wei et al., 2010; Wingender et al., 2012).

B cells are present in the spleen and intestinal lamina propria prior to birth, but require microbial signals for class switching and augmented antibody production in the spleen and Peyer's patches, as well as antibody secretion into the gut lumen (Hansson et al., 2011). Furthermore, the presence of B cells in Peyer's patches and MLN diminishes without ongoing bacterial exposure, suggesting continual microbial stimulation is required for maintenance of germinal center B cell

populations (Hapfelmeier et al., 2010). Low numbers of IgM-containing cells are present in the MLN by day six of life in human infants and by day 12, IgM- and IgA-containing cells that are observed in the colon (Perkkio and Savilahti, 1980). In the small intestine, an abundance of IgA-secreting cells is observed by day 14 following monocolonization with *E. coli* (Hapfelmeier et al., 2010). Following microbiota-driven MyD88 activation, epithelial cell expression of polymeric immunoglobulin receptor and sIgA transcytosis is increased (Frantz et al., 2012). The microbiota also affects the microbial specificity of sIgA produced (Shroff et al., 1995; Hapfelmeier et al., 2010; Lecuyer et al., 2014). Thus, microbiota drives B cell maturation and antibody production, and influences IgA specificity.

The importance of early life microbiota is further demonstrated when evaluating immune development in germ-free mice colonized at older ages. Colonization at three weeks of age alters the relative abundances of systemic immune cells and cytokines and generates a proinflammatory bias of MLN cells (Hansen et al., 2012). Mice colonized in adulthood have higher colon iNK T cell accumulation and sustained expression of proinflammatory immune-related genes in the jejunum and colon, 77% of which have been linked to Crohn's disease in genome-wide association studies (Olszak et al., 2012; El Aidy et al., 2013). Therefore, delayed colonization can bias immune responses to be proinflammatory, further connecting early life microbiota disruption to development of inflammatory disorders later in life.

An important aspect of mucosal immunity is the ability to mount immune responses toward pathogens and toxins, but to also remain tolerant toward commensal microbes and environmental antigens. Following colonization, establishment of oral tolerance toward the microbiota involves adaptation of both innate and adaptive immunity. During the first two weeks of life intestinal epithelial cells are transiently desensitized to TLR4 stimulation, protecting intestinal epithelial cells from induction of inflammation (Chassin et al., 2010). Conversely, following caesarean delivery, TLR4 desensitization does not occur, suggesting that mode of colonization, or colonizing microbiota, may be important factors in establishment of tolerance and homeostasis (Lotz et al., 2006). Furthermore, activation of MyD88 signaling inhibits trafficking of lumenal antigens to the MLN and induction of proinflammatory immune responses, suggesting that commensal bacteria themselves may promote tolerance (Diehl et al., 2013). Thus, it is clear that the microbiota drives development of the immune system and establishment of oral tolerance. Moreover, the presence or absence of specific bacteria can impact the balance between proinflammatory and regulatory responses. However, further work is needed to characterize the effects of different complex microbial communities on immune maturation and how this relates to susceptibility to disease.

DEVELOPMENT OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS AND STRESS RESPONSE

The brain–gut–microbiota axis is a bidirectional communication pathway between the central nervous system and the GI tract and encompasses neural, immune, and endocrine pathways (Cryan and O'Mahony, 2011; Bercik et al., 2012; De Palma et al., 2014b; Dinan et al., 2015). Perturbation of this axis results in alterations in the stress response and behavior and has been implicated in various disease states (Bonaz and Bernstein, 2013; Moloney et al., 2014; Borre et al., 2014a; Mayer et al., 2014; De Palma et al., in revision).

The appropriate development of the HPA stress axis determines the ability of an individual to cope and adapt to stressors. The stress hyporesponsive period occurs during the first two weeks of life in the rodent, with the majority of stressors evoking a subnormal response from the axis (Lupien et al., 2009). There is also evidence that this period also exists in humans and is thought to extend throughout childhood (Raabe and Spengler, 2013). It is also thought that this hyporesponsive period may have evolved to protect the rapidly developing child from the influence of elevated glucocorticoids (Raabe and Spengler, 2013). Early life stressors are associated with increased anxiety and depressive-like behaviors as well as GI disorders associated with a stress component (Lupien et al., 2009; O'Mahony et al., 2011). Given the influence of stress on the gut–brain axis (Grenham et al., 2011), appropriate development of the stress axis is essential to the balanced functioning gut–brain axis.

HEALTH OUTCOMES RELATED TO PERTURBATION OF THE INTESTINAL MICROBIOTA IN EARLY LIFE

Defects in intestinal barrier integrity, immune surveillance, and the stress responses can be detrimental to host health. A number of disorders are associated with altered neonatal colonization that may impact intestinal barrier and immune maturation, the gut–brain axis, and, therefore, may ultimately contribute to development of GI and psychological disorders.

Disorders of the Gut

Inflammatory Bowel Disease

Altered colonization is associated with increased susceptibility to IBD. Delivery by caesarean section and childhood antibiotic use are both associated with increased risk of IBD, although some studies report only an association with Crohn's disease, a higher prevalence specifically being reported in males exposed to antibiotics (Bager et al., 2012; Virta et al., 2012; Li et al., 2014; Ungaro et al., 2014; Sevelsted et al., 2015). Ulcerative colitis (UC), on the other hand, is associated with a decrease in Firmicutes and induces expansion of Th17 cells in the colon following colonization, the effects of which can be reversed by Lachnospiraceae and Ruminococcaceae supplementation, suggesting a lack of colonizing Firmicutes contributes to Th17-driven inflammation (Jiang et al., 2014; Natividad et al., 2015). Invariant NK T cells have also been implicated in the pathogenesis of UC. In particular, the mucosal accumulation of iNK T diminishes

when colonization occurs in the neonatal period (Olszak et al., 2012). Therefore, altered colonization patterns may affect immune maturation, leading to a proinflammatory environment associated with IBD. Thus, dysbiosis and microbial dysfunction are observed in IBD pathogenesis with potential origins, in part, lying in early life colonization patterns.

Celiac Disease

Celiac disease (CD) is a chronic inflammatory disorder of the small intestine resulting from intolerance to gluten in genetically susceptible hosts. Major histocompatibility class (MHC) II alleles, HLA-DQ2 and HLA-DQ8, confer susceptibility to CD due to their affinity for gluten peptides that are deamidated in the lamina propria by host tissue transglutaminase 2 (TG2) (Shan et al., 2002; Bergseng et al., 2005). Although the risk alleles HLA-DQ2 and HLA-DQ8 have been identified, only a small subset of these individuals develop CD, which suggests that the environment also influences CD pathogenesis (Verdu et al., 2015). Despite suggestions that early life gluten exposure and breast-feeding practices may impact CD risk (Ivarsson et al., 2000; Sellitto et al., 2012), recent clinical studies have not confirmed a protective effect of breast-feeding or age of gluten introduction in high-risk children (Jansen et al., 2014; Lionetti et al., 2014; Vriezinga et al., 2014; Sevelsted et al., 2015). It remains to be determined whether an alteration in the microbiota is a modifier of CD risk, particularly in subjects with low- or moderate-genetic risk. However, disruption to the microbiota in early

life has been implicated in CD pathogenesis. Early antibiotic use has been associated with the emergence of CD (Marild et al., 2013; Canova et al., 2014), whilst other studies indicate that delivery mode is also influential, although not all studies support this link (Decker et al., 2010; Marild et al., 2013; Emilsson et al., 2015). Furthermore, infants at high risk of developing CD exhibit dysbiosis (De Palma et al., 2012; Sellitto et al., 2012). Moreover, NOD-DQ8 mice with microbiota lacking pathobionts and Proteobacteria (altered Schaedler flora, ASF) exhibit reduced sensitivity to gluten-induced immunopathology compared to conventional mice that harbor potentially opportunistic Proteobacteria (Verdu et al., 2015). Moreover, supplementation of ASF colonized mice with *E. coli* ENT CAI:5 isolated from CD patients reverts the protective effect of colonization. On the other hand, germ-free status is associated with an increased sensitivity to gluten, likely related to the lack of immune maturation induced by commensal microbes (Verdu et al., 2015). Thus, microbiota structure can either ameliorate or enhance immune responses to gluten in NOD-DQ8 mice.

Disorders of the Brain and Nervous System

Anxiety and Depression

Studies using germ-free mice have robustly and reproducibly shown that they are less anxious than their conventionally colonized counterparts (Sudo et al., 2004; Diaz Heijtz et al., 2011; Neufeld et al., 2011; Clarke et al., 2013), while a normo-anxious state can be reinstated by the introduction of a microbiota during

the critical early life window (Clarke et al., 2013). Depression, as with other psychiatric disorders, has been associated with stress in early life (Chen and Baram, 2015). Recently, increased fecal levels of Enterobacteriaceae and *Alistipes* and reduced levels of *Faecalibacterium* were noted in patients with major depressive disorder compared to healthy controls (Jiang et al., 2015). *Alistipes* species are indole positive, affecting the serotonin precursor tryptophan, while *Faecalibacterium* has anti-inflammatory properties (Jiang et al., 2015). We, and others, have shown that maternal separation, a form of early life stress, impacts the gut microbiota in this animal model (O'Mahony et al., 2009; Dinan and Cryan, 2013; Park et al., 2013). Bailey and Coe (1999) investigated the stability of the indigenous microflora before and after maternal separation in rhesus monkeys and demonstrated a significant decrease in fecal bacteria, in particular of *Lactobacilli*, three days after separation, which correlated with stress-indicative behaviors and susceptibility to opportunistic bacterial infection. These results suggest that stress during this vulnerable period of microbiota development leads to gut–brain axis dysfunction and ultimately may increase vulnerability to disease. Despite the etiology of depression being associated with both early life stress and an altered gut microbiota, it is still remains unclear as to whether there is an interaction between the two.

Obesity

Administration of antibiotics during pregnancy is associated with an increased risk of obesity (Mueller et al., 2015b). Obesity itself is linked to an altered gut microbiota characterized by an altered Firmicutes:Bacteroidetes ratio relative to lean individuals (Schele et al., 2015). Hence, there is the possibility that prenatal microbiota manipulation can have long-term effects on the health of the infant, including the propensity to gain weight. There are indications that caesarean section delivery influences adiposity and might be linked to an increased risk for both childhood and adult obesity (Goldani et al., 2011; Huh et al., 2012; Mesquita et al., 2013). Although an altered microbiota could precede the development of obesity, a causal relationship is still far from certain. Dietary factors, host physiology alterations later in life, and maternal obesity during pregnancy are also important considerations (Sirimi and Goulis, 2010; Flint, 2012). Hypothalamic dysfunction has also been implicated in this regard (Schellekens et al., 2012). Moreover breast-feeding, which confers a "healthier" microbial balance in babies, is associated with a reduced risk of obesity in both childhood and adulthood (Ejlerskov et al., 2015; Krenz-Niedbala et al., 2015).

Autism Spectrum Disorders

Autism spectrum disorders have also been linked to an abnormal composition of the gut microbiome (de Theije et al., 2011; Mulle et al., 2013). A preclinical study assessing the impact of maternal infection on both the gut and

the brain in the offspring determined that maternal immune activation leads to altered gut microbiota, gut barrier function, and behavioral alterations indicative of autistic-like behavior (Hsiao et al., 2013). Furthermore, the impact of maternal infection on the function of the microbiota–gut–brain axis was reversed by the administration of *B. fragilis* to the offspring.

CONCLUDING REMARKS

We have considered how the gut microbiota in early life sculpts the development and maturation of key host regulatory systems, disruption of which may predispose an individual to disease. Proof of principle studies in germ-free animals have clearly demonstrated a multidimensional impact of growing up without a microbiota. Domains affected by early life colonization include those relevant to GI and mental health, as well as pain disorders and obesity. There is substantial overlap between the emergence of these disorders and distortions of the gut microbiome in early life. The restorative options that can be considered to counteract early life microbiome alterations and promote a return to a "healthy" microbiome composition have been well cataloged (Salazar et al., 2014; Mueller et al., 2015a). The tools at our disposal include precision nutritional strategies, such as probiotic and prebiotic supplementation, as well as increased efforts to encourage breast-feeding and limit caesarean section delivery to appropriate medically indicated circumstances. Future developments in this emerging field are not without their challenges, not least whether preclinical data will translate to the

clinic. In any case, it is no longer sufficient to consider the host without regard to the microbiome. As our appreciation of early life microbiota grows, the potential for effective and earlier interventions for disease prevention is a tangible prospect. Translating this potential is undoubtedly a challenge, but also an extremely worthwhile multidisciplinary venture.

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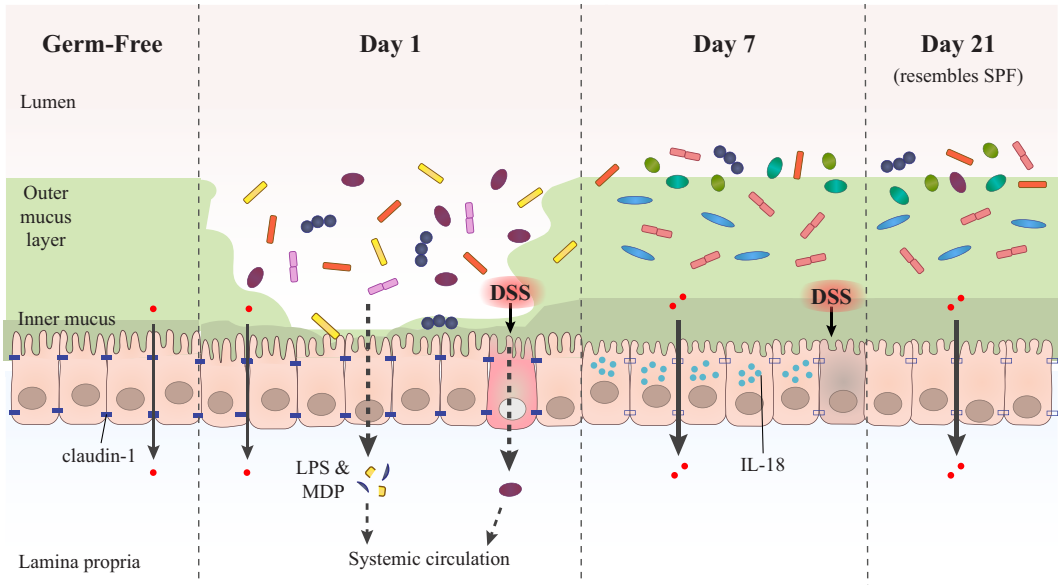
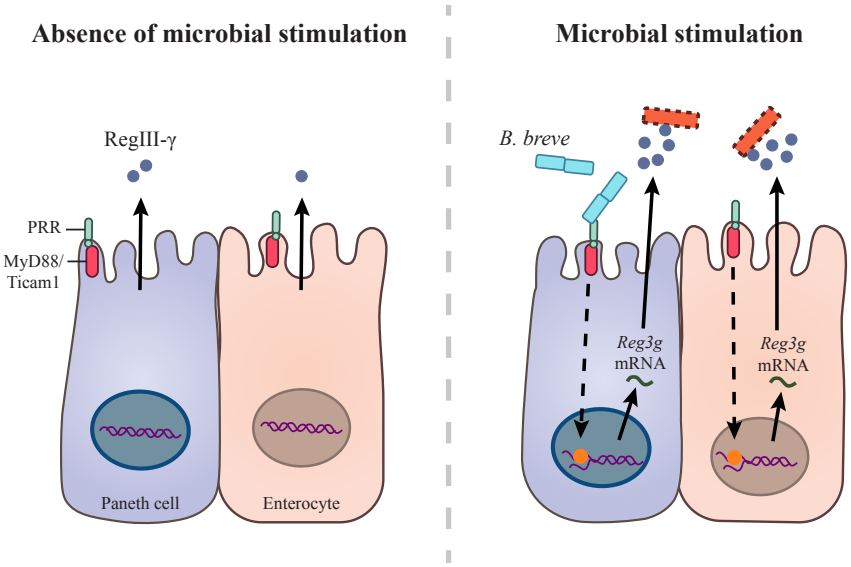
APPENDIX II

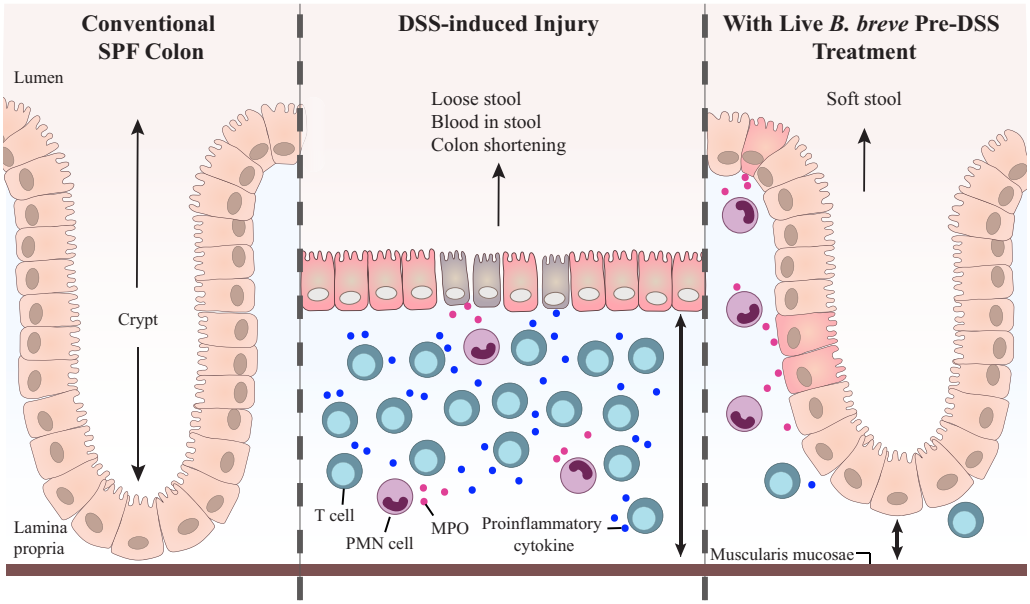
SUPPLEMENTAL FIGURES



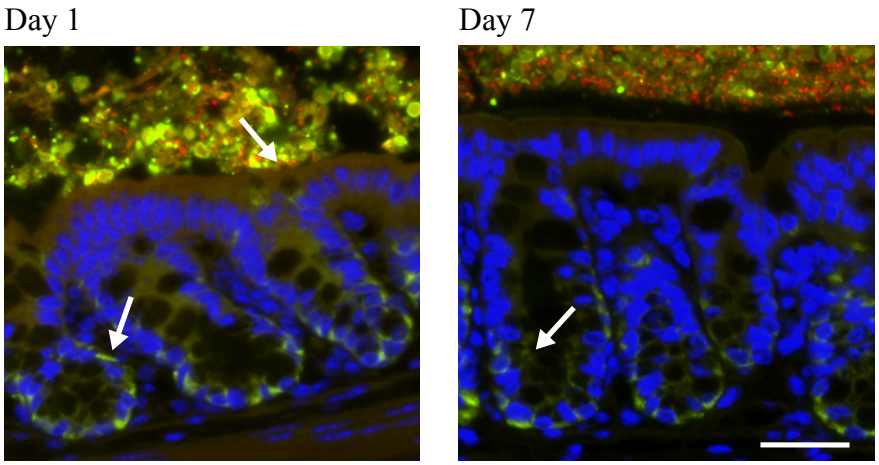
Supplemental Figure 1. Colonization enhances colonic barrier integrity through maturation of structure and function to the physiological state.



Supplemental Figure 2. Differential induction of antimicrobial RegIII-γ by intestinal microbiota and *Bifidobacterium breve* NCC2950.

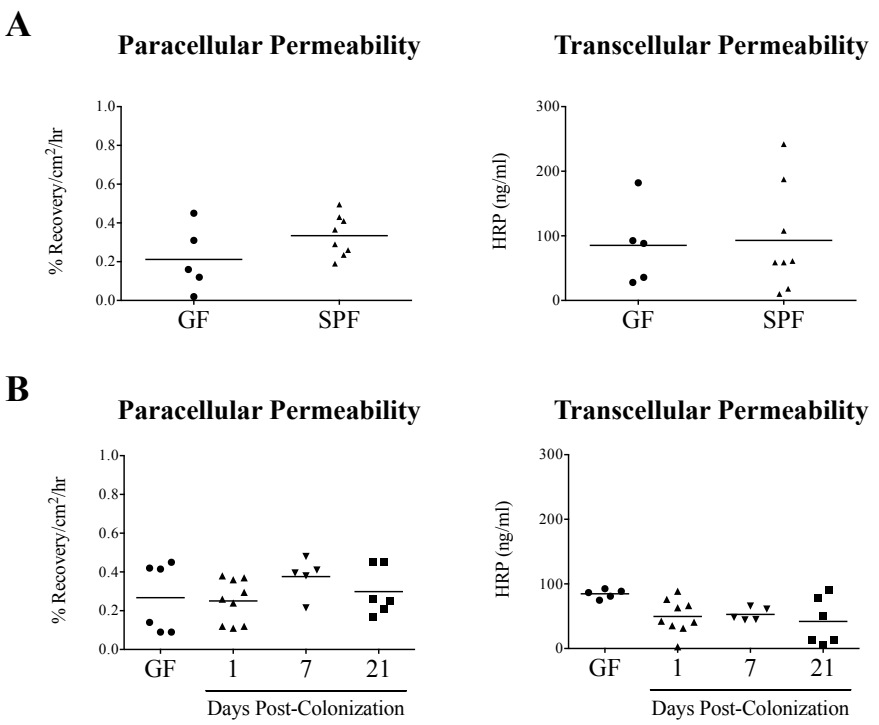


Supplemental Figure 3. *Bifidobacterium breve* NCC2950 reduces susceptibility to acute chemically induced intestinal injury and inflammation.

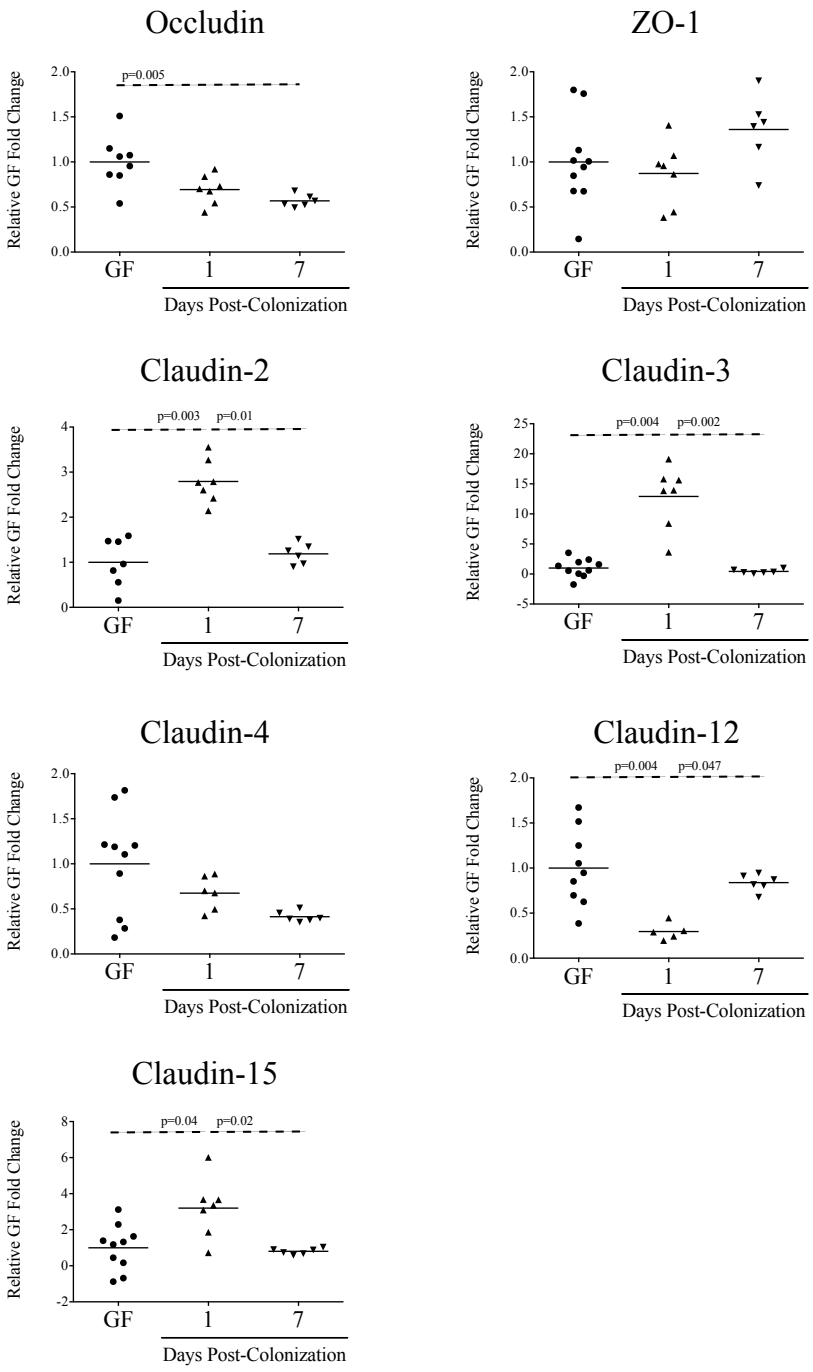


Supplemental Figure 4. No evidence of bacteria-goblet cell associations was observed at day 1 or 7 post-colonization.

Representative images of colon sections co-stained for mucin-2 (by immunofluorescence; green) and the bacterial 16S gene using probe EUB 338 (by fluorescence *in situ* hybridization; red) at days 1 and 7 post-human microbiota colonization. Nuclei stained with DAPI (blue). White arrows indicate goblet cells. Scale bar = 25μm.

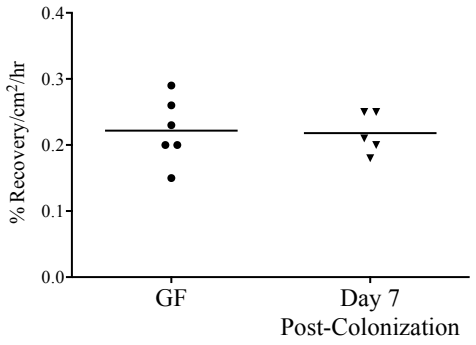


Supplemental Figure 5. Ileum permeability.
Paracellular and transcellular permeability to probes ^{51}Cr-EDTA and HRP, respectively, measured by Ussing chambers. (A) Germ-free (GF) and conventionally raised specific pathogen free (SPF) mice. (B) Days 1, 7 and 21 post-colonization with human commensal microbiota. Statistical significance was assessed by ANOVA with Bonferroni post-hoc test or an unpaired Student's t test, when appropriate.

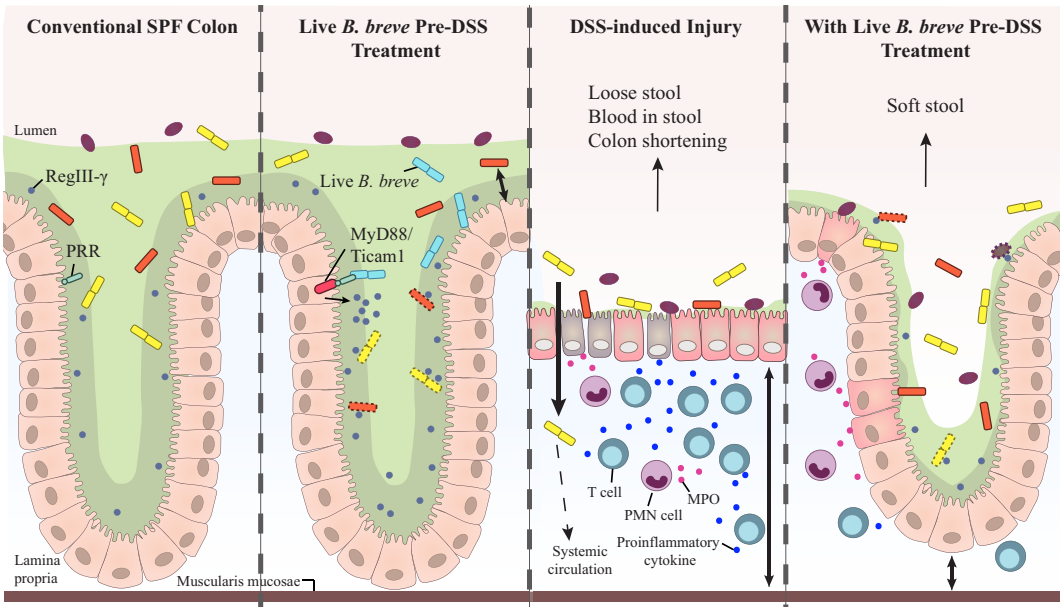


Supplemental Figure 6. Dynamic changes in ileum tight junction protein expression are observed within the first week of colonization.

Germ-free (GF) mice were colonized with human fecal microbiota and expression of ileum tight junction proteins was evaluated by real-time qPCR. Significance was determined by Kruskal-Wallis test with Dunn's post-hoc test.



Suplemental Figure 7. No change in paracellular permeability is observed at day 7 post-colonization with human fecal microbiota in *Nod1*$^{-/-}$;*Nod2*$^{-/-}$ mice.



Supplemental Figure 8. Hypothesized mechanism of *Bifidobacterium breve* NCC2950-promoted resilience to DSS-induced injury and inflammation.

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