UNIVERSIDADE ESTADUAL PAULISTA

"JÚLIO DE MESQUITA FILHO"

FACULDADE DE CIÊNCIAS FARMACÊUTICAS

CAMPUS ARARAQUARA

LARISSA SBAGLIA CELIBERTO

INTESTINAL HOMEOSTASIS AND HOST DEFENSE AS PROMOTED BY COMMENSAL BACTERIA AND THE COLONIC MUCUS LAYER

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Tese apresentada à Faculdade de Ciências Farmacêuticas da Universidade Estadual Paulista "Júlio de Mesquita Filho", para a obtenção do título de Doutora em Alimentos e Nutrição, área Ciência dos Alimentos.

Orientadora: *Prof^a^. Dr^a^. Daniela Cardoso Umbelino Cavallini*
Orientador no exterior: *Prof. Dr. Bruce A. Vallance*
Co-orientador: *Dr. Luis Caetano Martha Antunes*

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Intestinal homeostasis and host defense as promoted by commensal bacteria and the colonic mucus layer

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Abstract

The intestinal tract harbours the largest population of microbes in the human body where they play an important role in promoting the health of their host. If the composition of these microbes is altered, this may lead to dysbiosis that triggers or exacerbates intestinal and extra-intestinal diseases. Probiotics have been investigated as a complementary therapy in dysbiosis-related diseases. However, their effectiveness in treating severe conditions such as Inflammatory Bowel Disease (IBD) is quite variable and have shown controversial results. To address the importance of a personalized probiotic approach to treat intestinal inflammation, we first examined the effect of personalized bacteria using a model of chemical induced colitis. The animals that received commensals isolated from their own feces were more protected against inflammation as they showed reduced signs of colitis, less histological damage and lower levels of inflammatory markers as compared to mice given a commercial probiotic strain. Next, the role of the intestinal mucin Muc2 and the Core-1 enzyme that glycosylates it were explored using the *Citrobacter rodentium* model of infectious colitis. The intestinal mucus layer is the first line of defense in the intestine and is largely composed of the secreted mucin Muc2. Since almost all enteric bacteria must cross the overlying mucus layer to infect the host, the mucus-enteric bacterial interactions provide fundamental knowledge about infectious diseases as well as inflammatory conditions linked to dysbiosis (e.g. IBD). Specifically, we compared *C. rodentium* susceptibility by infecting WT, *Muc2* $^{-/-}$, core 3 (*C3GnT)* $^{-/-}$, core -1 (*C1galt1)* $^{-/-}$, and *C1galt1* $^{f/f}$ mice. While *C3GnT* $^{-/-}$ mice showed a very similar phenotype to WT mice with only mild inflammation, complete absence of Muc2 or just core 1 derived O-glycans resulted in significantly higher histological damage, barrier disruption, and increased pathogen burdens. Interestingly, the supplementation of tributyrin

protected mice against infection resulting in less histological damage and lower *C. rodentium* colonization as compared to control groups. These studies highlight a novel personalized therapy that may be considered relevant to diseases affected by dysbiosis as well as the key role of Muc2 and its core 1 glycosylation in host defense against enteric infections.

Keywords: IBD; colitis, microbiota; microbiota biobank, personalized probiotic, mucus layer

Lay Summary

The human gut harbors several types of bacteria that play an important role in the well-being of their host. The intestinal mucus layer is also important for intestinal health, since it acts as a physical barrier that prevents bacteria and food products from escaping the gut and causing inflammation. Using an animal model of intestinal inflammation, I discovered that beneficial bacteria isolated from the host are more effective in protecting mice against intestinal damage as compared to probiotic bacteria available on the market. Further, using an animal model of a gut bacterial infection, I tested the impact of the mucus layer on protection against intestinal inflammation. I found that the mucin Muc2 and one of its sugar compounds protect mice against intestinal damage caused by enteric bacteria. These findings have implications for both Inflammatory Bowel Disease and people with gut infections– and could help develop new and effective complementary treatments.

Preface

Chapter 2

I designed and conducted the majority of the studies reported in this chapter, analyzed the data, prepared all of the figures and wrote the manuscript under the supervision of Dr. Daniela C.U. Cavallini and Dr. Bruce A. Vallance. Dr. Elizeu A. Rossi provided input to the study design. Ms. Roseli A. Pinto assisted in microbiological tests and performed the antibiotic susceptibility test leading to Supplemental Figure A1.

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Ethics approval was required for this research and was obtained from the Sao Paulo State University Animal Care Committee certificate number 34/2014 and the University of British Columbia Animal Care Committee certificate number A15-0206.

Chapter 3

I conducted the majority of the studies reported in this chapter, analyzed the data, prepared all of the figures and wrote the manuscript under the supervision of Dr. Bruce A. Vallance and Dr. Daniela CU Cavallini. Dr. HT Law, Dr. Genelle Healey, Mr. Justin HY Chan and Ms. Qiaochu Liang assisted with study design, animal experimentation, histopathological scores and provided useful insights regarding the results discussion. Dr. Kiran Bhullar performed experiments with *C3GnT -/-* mice leading to figure 3.4 IEC *C1galt1 -/-* and *C3GnT -/-* mice were generated and kindly provided by Dr. Lijun Xia, University of Oklahoma. A version of this chapter will be submitted for publication.

Ethics approval was required for this research and was obtained from the University of British Columbia Animal Care Committee certificate number A15-0206.

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List of symbols and abbreviations

α	alpha
β	beta
γ	gamma
κ	kappa
μ	micro
°	degree
C	Celsius
<	less than
≤	less than or equal to
>	greater than
+	positive
±	plus or minus
-/-	deficient
5-HT	5-hyroxytyrtamine (serotonin)
ADP	adenosine diphosphate
A/E	attaching and effacing
AMP	antimicrobial peptide
ATP	adenosine triphosphate
CFU	colony forming units
CTRL	control
DAI	disease activity index

DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DNA	deoxyribose nucleic acid
DNBS	2,4-dinitrobenzenesulfonic acid
DSS	dextran sodium sulfate
EHEC	Enterohemorrhagic *Escherichia coli*
e.g.	*exempli gratia* (for example)
ER	endoplasmic reticulum
EPEC	Enteropathogenic *Escherichia coli*
F/B	*Firmicutes/Bacteroidetes*
FITC	fluorescein isothiocyanate
FOS	fructooligosaccharide
g	gram
GI	Gastrointestinal tract
GF	germ free
GL	glycerol
GOS	galactooligosaccharide
GPR	G protein coupled receptor
HIV	Human Immunodeficiency virus
HMP	Human Microbiome Project
IAP	intestinal alkaline phosphatase
IBD	Inflammatory Bowel Disease
IBS	irritable bowel syndrome

i.e.	*id est* (that is)
IEC	intestinal epithelial cell
IEC-C1galt1-/-	IEC specific deletion of core 1-derived O-glycans
IEL	intraepithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ITS	internal transcribed spacer
L	litre
LEE	locus of enterocyte effacement
LGG	*Lactobacillus rhamnosus* GG
LI	large intestine
LPS	lipopolysaccharide
m	meters
M cells	microfold cells
MAPK	mitogen activated protein kinase
MDA	malonaldehyde
MetaHIT	Metagenomics of the Human Intestinal Tract.
MLN	mesenteric lymph nodes
MPO	myeloperoxidase
Muc2	mucin 2
NGS	next generation sequence
NIH	National Institute of Health

NOD	nucleotide-binding oligomerization domain
NF-κB	Nuclear factor kappa-B
pi	post infection
PP	personalized probiotics
PTS	Proline- threonine -serine
qPCR	quantitative PCR
Reg III γ	regenerating islet-derived protein 3 gamma
Relmβ	resistin like molecule beta
RNA	ribonucleic acid
rRNA	ribosomal RNA
ROS	reactive oxygen species
SCFA	short chain fatty acid
SD	standard deviation
SEM	standard error mean
SI	small intestine
S. Typhimurium	*Salmonella* enterica serovar Typhimurium
T3SS	type 3 secretion system
TB	tributyrin
TCR	T cell receptor
TFF3	trefoil like factor 3
TGF β	transforming growth factor beta
Th	T helper
Tir	translocated intimin receptor

TJ	tight junction
TLR	toll like receptor
TNBS	2,4,6-trinitrobenzene sulphonic acid
TNF	tumor necrosis factor
Treg	T regulatory
Wnt	Wingless/Integrated
WT	wildtype
ZO	zonula occludens

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To my mom and dad, for always loving and supporting me.

Chapter 1: Literature review

1.1 The human microbiome

Humans have co-evolved with the trillions of microbes that inhabit their bodies, thereby creating a complex yet symbiotic ecosystem. These microbes include bacteria, viruses, archaea, and eukaryotic microorganisms (1). It is estimated that the human body contain slightly more bacterial cells than human cells (4×10^{13}/ 3×10^{13}) (2), with over 1000 different strains of bacteria. (3,4). Several definitions have recently been used to describe this microbial community that colonizes our bodies. While microbiota refers to a community of microorganisms that are present in a particular habitat, the microbiome comprises both the microorganisms at a specific site as well as their genomes, physiochemical properties and activities (5). Therefore, microbiome is a more refined term that should be used when taking into consideration the genes carried by the microbes, as well as the environment that a particular microbiota inhabits.

Several bacterial and fungal communities have been investigated in the past decades using traditional microbiology techniques such as culturing using selective and differential media. These media usually contain all the necessary nutrients for the growth of certain microorganisms as well as inhibitors to select and identify target microorganisms. Studies using conventional microbiology methods provided significant information regarding microbial environments and are still very useful in terms of classifying different genera and/or determining their antimicrobial susceptibilities. However, considerable disadvantages are involved in culturing techniques - such as poor sensitivity for some sample types (blood, tissues, fecal content), challenges to characterize

microbes to the species level, the long incubation time (especially for fungi and mycobacteria), poor clinical application, and little information provided about community dynamics (6,7). Moreover, with regards to the human microbiota, it is estimated that 20% to 60% of the microbes living within the human body are unculturable (8). This range varies according to the body site but is it already a strong indication of how little was known about the human microbiome and its diversity until recent years.

Over the last two decades, advances in next-generation sequencing (NGS), along with bioinformatic developments, have brought new insights regarding our understanding of the unculturable microbes that inhabit soil, oceans and the human body (9,10). This field of research, called metagenomics, allows the examination of the total genomic DNA of microbial communities sampled from natural environments without the prior need for culturing, thus providing valuable information about the complexity and diversity of the human microbiome – *in situ*. The two main types of sequencing data analysis are marker gene metagenomics and shotgun metagenomics (11,12). Sequencing of the 16S ribosomal RNA (rRNA) gene is restricted to microbes of the bacteria and archaea domains, with the sequencing data compared to a database containing a great number of sequences of this gene fragment (16S libraries). A similar approach can be applied for fungi and eukaryotes, although in this case the preferred marker genes are the internal transcribed spacer (ITS) and the 18S rRNA gene, respectively (11). Through shotgun metagenomics analysis, the complete sequences of all microorganisms present in a sample (previously characterized or novel) are investigated, thus offering extremely useful insights regarding microbial community dynamics (11,12). Besides the knowledge gained about the differences in the microbiome at particular body sites, these molecular techniques help researchers understand how microbial

communities affect health and disease and how it is possible to influence or even manipulate the human microbiome (13). In addition, more than just aiding our understanding of microbiome-host interactions, some of these high-throughput sequencing technologies allow the identification of microbiome changes in a more detailed manner such as to the strain-level, which poses a significant improvement over the detection of species-level differences which may not represent a reliable marker in several health conditions (3).

The Human Microbiome Project (HMP) was created in 2008 by the National Institutes of Health (NIH) in the United States, as an initiative to study the different microorganisms living in association with the human body, and thus potentially involved in human health and disease. The mission of this project is to generate resources that will help scientists in the field understand the makeup of the "normal" human microbiome and if certain medical conditions affect the microbes living in our body (8). The term "normal" rather than "healthy" was chosen by body site-specific experts and was used in the study as a matter of criteria selection for volunteers. According to clinicians' opinions, the recruitment of "healthy" volunteers would lead to several exclusion criteria making the selection a very slow and complicated process (8). The main (and ongoing) goals of the HMP are: to characterize the human microbiome by studying samples from multiple body sites (gastrointestinal tract, mouth, nasal cavity, vagina, and skin) collected from "normal" volunteers; to understand the influence of the microbiome in health and disease by studying their differences in several clinical conditions such as obesity, psoriasis, bacterial vaginosis, and cancer; and finally to provide standardized data resources as well as improved technology to facilitate future studies in this field (8).

The HMP and all the other various studies characterizing the human microbiome play a very important role in better defining the concept of “dysbiosis” and the diseases related to this condition. Presently, there is no specific description of what constitutes a “healthy" microbiome or which group of microorganisms are present in a dysbiotic state. Early research in the field aimed to identify a “core” group of microorganisms that are universally present in healthy individuals but are also absent in those individuals presenting with disease phenotypes (14,15). However, microbiomes regularly show a large degree of interpersonal variation - even in the absence of any overt disease (16,17), therefore the general concept of a “healthy” microbiome is no longer practical as a reference or marker for eubiotic or dysbiotic states. Moreover, besides the variability in the metagenome of the human microbiome, only a third of its constituent genes are found in the majority of healthy individuals (14), making the search for specific taxa a poor reflection of what constitutes the “normal” microbial community.

Although no specific characteristics can be used to define dysbiosis, several authors associate this term with certain conditions. The oral cavity carries hundreds of bacterial species and an imbalance in this microbiota is associated with oral diseases, mainly periodontitis and dental caries (18). In periodontitis, pathogens such as *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia* are described to play a key role in this disease due to their ability to produce biofilms (19). Regarding dental caries, *Streptococcus mutans* seems to be the chief pathogen associated with this condition, although *Lactobacillus* spp., *Prevotella* spp., *Atopobium* spp., *Olsenella* spp. and *Actinomyces* spp. have also been implicated (20). The skin microbiota composition varies according to the body site and its particular properties (dry versus moist versus sebaceous sites), and is mainly colonized by *Corynebacterium* spp., *Propionibacterium* spp., and *Staphylococcus*

spp. (21). *Staphylococcus aureus* is closely related to atopic dermatitis and although most species of *Corynebacterium* do not cause any disease in humans, *Corynebacterium minutissimum* and *Corynebacterium tenuis* have been associated with superficial skin pathology (22). Curiously, rather than showing a lack of microbial diversity - female urogenital diseases (bacterial vaginosis, yeast infection, sexually transmitted infection) and their consequences (e.g. pre-term birth, HIV infection) are often associated with a more diverse vaginal microbiota profile, where there is a shift from predominantly lactic acid-producing bacteria (i.e. *Lactobacillus* spp.) to more strict anaerobes comprising taxa such as *Sneathia* spp., *Prevotella amnii*, *Atopobium* spp. and *Gardnerella vaginalis* (23,24). In gastrointestinal disorders, a reduced diversity of microbes, a lower abundance of obligate anaerobic bacteria, and an expansion of facultative anaerobic bacteria, such as *Escherichia* spp., are thought to indicate the presence of an abnormal/aberrant microbiota. These changes in the microbiota are often seen in combination with intestinal and extra-intestinal disorders such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), obesity, type 2 diabetes, among others (25).

Interestingly, studies investigating the human microbiome in different countries have observed inter-population differences in the composition of what is considered a "healthy microbiome", emphasizing the geographical variation in microbes worldwide (14). This inter-country variation in taxonomic composition was compared using large cohort studies from different continents – MetaHIT (Europe), HMP (America), and Chinese diabetes cohorts – and together with genetics, diet and other environmental factors, one's place of residence joins the list of factors that influence the human microbiome (14). Besides evolution, immigration patterns and modifiable factors that reflect lifestyle (diet, exercise, hygiene habits), the healthcare system of a particular country may

also influence the microbiome composition of that population. Medical procedures like mode of infant delivery and use of antibiotics at an early age are directly associated with differences in microbiota composition, and the frequency of these procedures usually varies between countries. Moreover, climate and sunlight exposure have also been described as factors that impact microbial dysbiosis, by, for example, influencing the biosynthesis of vitamin D (25). Lower sunlight exposure combined with a lack of adequate vitamin D supplementation can result in vitamin D deficiency, pre-disposing to dysbiosis and inflammatory conditions such as IBD (25).

1.2 The human gastrointestinal tract

The mammalian gastrointestinal (GI) tract is a complex organ system that includes anatomically and functionally distinct regions – each with a unique diversity of cell types (26). The GI tract extends from the mouth to the anus and is in essence, a long tubular structure comprising (in order) the oral cavity, esophagus, stomach, small and large intestines, rectum and anus. Each of these segments is strategically separated by sphincters that not only create different compartments in the GI tract but also control the flow of the digestive process (27). The primary functions of the GI tract are to maintain water homeostasis, secrete enzymes to aid the digestion process, sample and absorb essential nutrients and electrolytes, and finally eliminate waste products in the form of feces (26,27). In addition, adjacent glandular organs (e.g. salivary glands, liver, gall bladder, and pancreas), neurons and vasculature are connected to the GI tract, where they help facilitate digestion in an integrated process whereby each organ and all the different cell types play their specialized roles in the gut (26).

The small intestine is the longest segment of the GI tract, measuring 6-9 m long in adults, and consisting of three parts: the duodenum, jejunum, and ileum. The duodenum is the most proximal segment of the small intestine and it plays an important role in the mixing of food products with digestive enzymes from the pancreas as well as with alkaline secretions from duodenal glands, that together are responsible for neutralizing the acidic chyme that is received from the stomach. The bile produced in the liver and stored in the gall bladder also helps the digestive process by emulsifying dietary fat into micelles for absorption (28). Another critical enzyme for digestion, intestinal alkaline phosphatase (IAP), is expressed and secreted by intestinal epithelial cells (IEC) and is found in high concentrations in the duodenum. One of the major functions of IAP is the regulation of bicarbonate secretion and duodenal surface pH, thus contributing to intestinal homeostasis along with other functions such as inactivation of bacteria lipopolysaccharide (LPS) and regulation of the gut microbiome through dephosphorylation of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) in the intestinal lumen. IAP converts these phosphorylated nucleotides to adenosine which acts as a scavenger of oxygen thus influencing bacterial growth and diversity (29). Following the digestion of food, nutrient absorption continues to take place throughout the jejunum and ileum. The main function of the small intestine is thus the digestion and absorption of nutrients, and to do this, the inner surface of the small intestine is covered with villi and microvilli that project into the lumen resulting in a very high surface area of approximately 30 m^2 in humans (27).

The large intestine, also known as the colon, is wider and shorter (~1.5 m) than the small intestine and extends from the distal end of the ileum to the anus (30). This region receives chyme from the

small intestine and is responsible for forming the feces, as well as absorbing fluids and salts that maintain homeostasis in the human body. The colon can be functionally divided into the ascending, transverse and descending colon. The cecum and ascending colon are located on the right side of the abdomen and play a central role in water and electrolyte absorption, in the fermentation of complex carbohydrates (i.e. dietary fiber) as well as in the production of metabolites (i.e. short-chain fatty acid) by resident microbiota (28). The transverse colon crosses the abdomen transversely connecting the right side to the left side, including the descending colon, sigmoid colon and rectum, which are primarily involved in the storage and evacuation of feces (28). The appendix is a tube-like structure found just off the cecum, and acts as a storage compartment for commensal bacteria and immune cells (31).

The wall of the human GI tract consists of four distinct functional layers, which from the outer surface inward include: the serosa, the muscularis externa, the submucosa, and the mucosa. The serosa is the outermost layer formed of connective tissue that contains major blood vessels and nerves. The muscularis externa consists of smooth muscle which is usually arranged as an inner circular layer and an outer longitudinal layer that together coordinate the muscle contractions that mediate peristalsis (32). The submucosa layer is made up of connective tissue that supports the mucosa and contains larger blood vessels, lymphatic vessels and nerves. Together, they help control secretions from the mucosal glands and regulate mucosal movement and blood flow. The mucosa is made up of three sublayers: the muscularis mucosae, which consists of a thin smooth muscle layer that separates the lamina propria and the underlying submucosa; the lamina propria, which resides at the base of the intestinal epithelium and is rich in immune cells; and finally a

single layer of IEC lining the lumen of the GI tract, which defends the host against the varied noxious stimuli that can be found in the luminal environment (32).

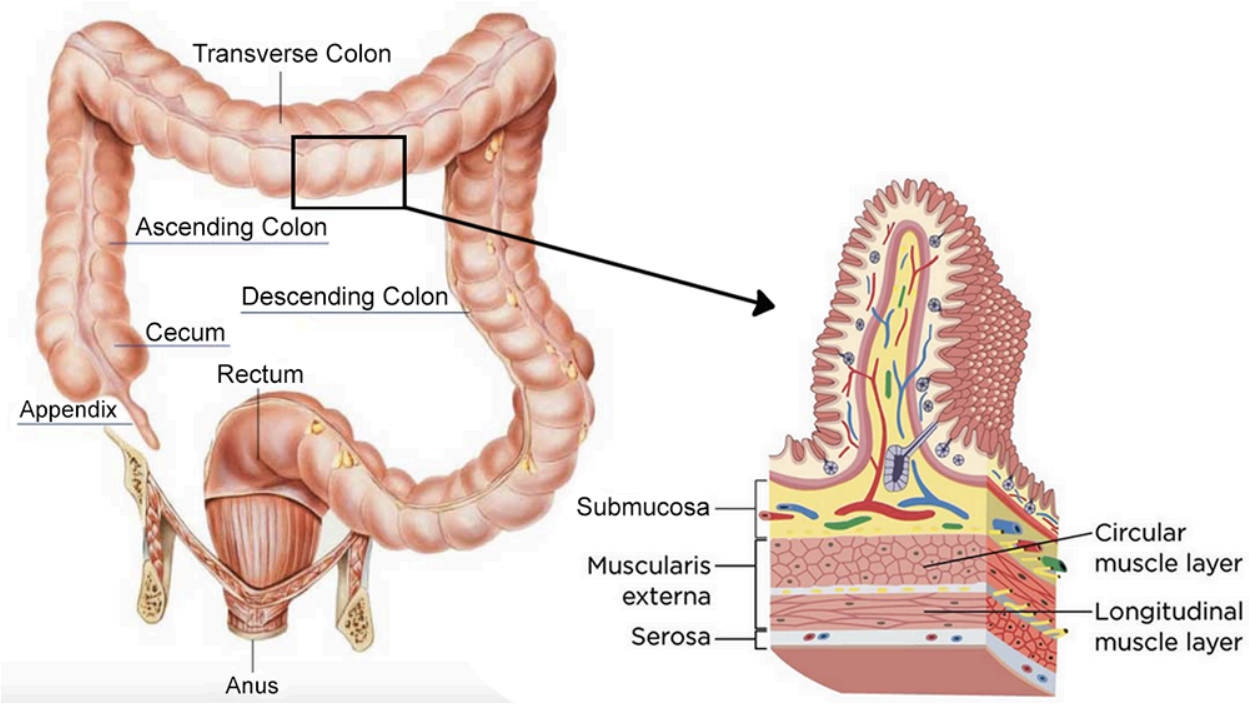


Figure 1.1. Schematic of the human colon showing the full thickness of the intestinal wall. Epithelial cells originate from stem cells at the base of crypts. The crypts are embedded in the lamina propria and are separated from the submucosa by the muscularis mucosae. The mucosa is made up of crypts, the lamina propria and the muscularis mucosae. The surface of the mucosa is scattered with the openings of crypts, which are continuous with the intestinal lumen. Image modified from ref. (33) with permission.

The intestinal epithelium consists of an array of IEC subtypes, and each one carries out different and specialized functions (34). Enterocytes constitute 80% of IEC and are mainly responsible for water absorption and nutrient transport due to their large surface area. Goblet cells (GCs), enteroendocrine cells and Paneth cells are classified as secretory cells and are known to release mucins, hormones and antimicrobial factors, respectively. In addition, intestinal stem cells reside at the base of intestinal crypts and are constantly being sorted into transient proliferating cells or mature cells that travel up the crypt before being released into the lumen. Lastly, there are tuft cells

which are chemosensory cells that can sense luminal helminths, and M cells which overlie Peyer's patches and aid in antigen uptake and sensing (34,35). All together, these various IEC subtypes work together harmoniously to maintain intestinal homeostasis and promote host defense, thus guaranteeing a functional balance within the GI tract (34).

The mucosal surface that coats the GI tract is approximately 200-300 m^2 in humans (36) and it harbors a vast array of microorganisms known as the microbiome (25,37). These microbes include bacteria, viruses, fungi, and eukaryotes and under normal conditions they maintain a healthy symbiotic relationship with the host. Since the GI mucosal surface is the largest area of the body that is in contact with the external environment, the interactions between the different cells in the gut play a key role in regulating normal gut physiology as well as host defense by working to exclude harmful opportunistic microbes. Before particles present in the luminal environment reach the IEC, they first encounter the mucus layer, which is a physical and biochemical protective barrier comprised of mucin glycoproteins that coats the entire GI tract (38). This dynamic barrier is constantly renewed, thus limiting any transient impairments in epithelial barrier integrity (39).

1.3 The intestinal microbiota

The first time a human usually encounters environmental microorganisms occurs at birth. During delivery the newborn comes into contact with the microbes present in the mother's vaginal canal and on her skin, leading to these commensal bacteria colonizing the infant. This community of microorganisms gradually develops into a diverse ecosystem as the host ages and grows, with both microbes and the immune system maturing together in a symbiotic relationship (37). The host

provides a place to reside as well as nutrition for the microbes, while the gut microbiota in turn protects the host against pathogenic bacteria (40,41), helps in food digestion and vitamin biosynthesis (42,43), produces important metabolites such as short chain fatty acids (SCFA) (40,44), and promotes normal immune function (45).

The intestine harbours the largest population of microorganisms within the human body, and the four main intestinal microbial phyla in humans are *Firmicutes*, *Bacteroidetes, Proteobacteria* and *Actinobacteria* (46). *Firmicutes* and *Bacteroidetes* are described as the dominant phyla in the gut (4,17) accounting for more than 90% of the total community (47–49), and although this broad level of classification may not directly indicate a health status association, the *Firmicutes/Bacteroidetes* (F/B) ratio is a common index used as a comparison of gut microbiota composition in certain diseases such as obesity and intestinal disorders. Obese subjects usually show a higher proportion of *Firmicutes* over *Bacteroidetes* as compared to lean people, and the F/B ratio tends to decrease as an individual loses weight (50). Conversely, a higher *Bacteroidetes* population has been associated with intestinal conditions such as IBD (51). At the genera level, it has been suggested that most individuals can be classified into one of three clusters or "enterotypes" according to their prevalent genera (*Bacteroides* spp., *Prevotella* spp. or *Ruminococcus* spp.) (52). These enterotypes seem to be driven primarily by dietary habits and despite being still a wide categorization, they may help us understand underlying mechanisms linking gut microbiota to diseases. The stratification of individuals in these three groups may identify possible correlations with dietary patterns such as the consumption of animal protein versus vegetarianism, fermented food, "Western" diet lifestyle, among others.

As previously discussed, the concept of a "healthy microbiome" is difficult to define, however this definition may be characterized by the microbiome behavior over time, where a personalized "functional core" of metabolic and molecular functions provides benefits to the host and protects it against noxious stimuli (14). Since interpersonal variation in the gut microbiome is high, a better way to predict dysbiosis related diseases would be the stability of the microbiota within an individual through time. It is believed that our microbiota develops during the first few years of life becoming relatively stable in healthy adults (1,42). However, studies have indicated that a decrease in *Bifidobacterium* spp., *Bacteroides* spp., and *Lactobacillus* spp. occurs in elderly population, accompanied by an increase in the number of facultative anaerobes (53–55).

Mode of delivery is considered the first aspect that influences gut microbiota composition, even though recent studies indicate that diet and stress in late pregnancy can also influence the first microbiome colonizers (49,56,57). Infants born by vaginal delivery present a microbial community close to the one found in the vaginal microbiota of their mothers (mainly *Lactobacillus* spp.), while infants delivered by caesarean section display a microbiota dominated by typical skin microbial taxa such as *Staphylococcus* spp. and *Propionibacterium* spp. (58). Breastfeeding is another factor that has been suggested to enrich *Lactobacillus* spp. in comparison with formula feeding in newborns (59). Furthermore, *Bifidobacterium* spp. present in breast milk display bifidogenic activity that protects infants against gastrointestinal infections and acute diarrhea (60).

Antibiotic use is one of the major external perturbations that directly affects the microbiota composition. The aim of antibiotic treatment is to fight against severe - and sometime life-threatening - infections that may cause major complications to the host. However, despite the acute

beneficial effects of eliminating pathogenic bacteria, the use of antibiotics is also linked to long term negative consequences as the microbial community may not always return to its pre-treatment composition. Interestingly, even months after antibiotic treatment - a low bacterial diversity has been described in the microbiota of adults treated with ciprofloxacin (61). Furthermore, studies show that a single course of antibiotics is sufficient to modify the microbial community with this change persisting for years (62–64). Lastly, the overuse of antibiotics is associated with an increase in antibiotic-resistant pathogens, as after the treatment some pathogens have more opportunity to outgrow commensal bacteria and to colonize the microbiota in a permanent way (1).

Dietary changes are also another modifiable factor that directly influences the microbiota composition. Products of digestion that were not absorbed in the upper digestive tract reach the colon where they come in contact with the gut microbiota. As an anaerobic environment, the fermentation process provides both energy and carbon sources for the microorganisms in addition to changing pH and substrate availability in the colon, therefore impacting both host metabolism and health (65). In the last several decades, dietary patterns have shifted dramatically from diverse and nutritious whole foods to what is known as a modern "Western" style. The "Western" diet comprises large amounts of sugar and fat rich, high calorie processed foods combined with a lack of fruits, vegetables, legumes and whole grains (66). Aside from lacking fiber and essential nutrients such as vitamins and minerals, this "Western" dietary lifestyle is associated with inflammatory and detrimental health effects leading to increased rates of obesity and other chronic diseases (67).

Studies using animal models have demonstrated that lean mice have a greater percentage of *Firmicutes* as compared to *Bacteroidetes* (60% and 40% respectively). However, obese mice have an even greater percentage of the same phyla, thus correlating obesity with increased numbers of *Firmicutes* in mice (68–70). Additionally, it has been shown in mice that shifting to a high-fat, high-sugar "Western" diet from a low-fat, plant polysaccharide-rich diet can change the microbiota within a day (71). Moreover, animals fed with a high fat diet showed reduced cecal *Bifidobacterium* spp., increased circulating LPS concentrations (72,73), and lower abundance of *Clostridium* cluster XIVa, including *Roseburia* spp. (74).

The consumption of the typical "Western" style diet is also associated with significant changes in human microbiota composition at the phylum and genus levels, including reductions in both Gram positive (e.g., *Bifidobacterium* spp.) and Gram negative bacteria (e.g., *Bacteroides* spp.) in African and European Americans as compared to native Africans with a diet rich in resistant starch and low in animal products (75). In another study in humans, shifting from a high-fat/low-fiber diet to a low-fat/high-fiber diet caused notable changes in the gut microbiota composition within 24 hours (76). Interestingly, enterotypes are associated with long-term diet, as individuals on a diet high in protein and animal fat have a *Bacteroidetes*-dominated enterotype, whereas a carbohydrate-rich diet is associated with a *Prevotella* dominated enterotype (76).

Regarding the impact of macronutrients in the gut microbiota, most benefits are related to carbohydrates in the form of dietary fibers. Carbohydrates are the major carbon/energy source for colonic microbes and fiber promotes gut health by increasing digesta mass thus facilitating fecal transit thought the colon. Additionally, SCFA (acetate, propionate and butyrate) are the principal

endproducts of carbohydrate fermentation and besides helping lower pH levels within the colon thereby limiting pathogenic bacterial activity, these acids have roles beyond the gut - impacting metabolic and immune system diseases and disorders (77). These by-products of colonic microbiota have received significant attention in the recent years, in particular butyrate, which is produced by bacteria from the *Clostridiale* clusters IV and XIVa. The main butyrate producing species are described to be *Eubacterium rectale* and *Faecalibacterium prausnitzii*, in addition to others in the genera *Coprococcus* and *Roseburia* (78). The presence of butyrate producers in the colon has been shown to be negatively correlated with functional dysbiosis, while reducing the risk of infections with opportunistic pathogens and decreasing oxidative stress, highlighting beneficial synergistic interactions between diet, microbes and host. Butyrate producers can respond to different environmental conditions, such as diet or pH, and engage different fermentation pathways in which the final products are lactate, formate, hydrogen and carbon dioxide. It has been shown that cross- feeding between *Bifidobacterium* spp. and butyrate producers is also possible: *Bifidobacterium* spp. break down polysaccharides and produce lactate and acetate, which are further utilized by butyrate - producers to form butyrate (78). Butyrate is also the main source of energy for colonocytes and it inhibits expression of pro-inflammatory cytokines in the mucosal layer of the intestine, thus playing a key role in maintaining homeostasis of the intestine (79). Moreover, butyrate has a positive effect on the integrity of the mucosal layer by stimulating expression of tight junction proteins, and by inducing the production of mucin and antimicrobial peptides (80).

The use of probiotics and prebiotics as nutritional strategies are also known for promoting general health benefits by improving the gut microbiota composition. A prebiotic is “a substrate that is

selectively utilized by host microorganisms conferring a health benefit" (81). Established prebiotics include inulin-type fructans (i.e. fructo-oligosaccharides [FOS], inulin and oligofructose), galacto-oligosaccharides (GOS) and lactulose. Other fermentable carbohydrates that have shown prebiotic potential include resistant starch, β-glucans, arabinoxylan oligosaccharides, xylo-oligosaccharides, soy bean oligosaccharides, isomalto-oligosaccharides and pectin. Prebiotics are found naturally in foods (i.e. inulin is found in breads and cereals, onions, garlic and artichokes), are added to foods to increase their fiber content (i.e. inulin containing yoghurts), or can be added to the diet in the form of powdered supplements. Prebiotics have the potential to create a new nutritional niche within the human GI tract, providing microbes sufficient nutrients to establish residence. Probiotics, are defined as "microorganisms that confer a health benefit to the host when administered in adequate amounts" (82,83) and will be discussed further in this chapter as a strategy to modulate the gut microbiota.

1.4 Gut microbiome in health and disease

Through its metabolic activity and its direct interactions with the host, the gut microbiota plays an important role in regulating certain metabolic functions such as insulin resistance, lipid and choline metabolism, vitamin biosynthesis, and the breakdown of complex carbohydrates – thus generating SCFA (37,84,85). Another critical role of commensal microorganisms is their ability to promote immune system development and maturation (86), as animals raised under germ-free conditions often display defective T and B cell function, poorly developed lymphoid tissues, lower levels of CD4+T cells and decreased antibody production (87,88). Germ-free animals also have anatomic

alterations such as cecum enlargement, villi hyperplasia and defective crypt cell cycling (89,90), thus emphasizing the importance of microbes in normal gut architecture.

Considering the important role of the gut microbiota and the several benefits described in the literature, it is clear that a breakdown in gut microbiota homeostasis impacts an individual's predisposition to chronic diseases. For example, over the last few decades, intestinal dysbiosis has been linked to several intestinal and extra-intestinal conditions such as IBD (91–93), IBS (94), obesity (50), type 2 diabetes (95), asthma (96), colon cancer (97,98), non-alcoholic fatty liver disease (99,100), and neurological diseases (101,102).

IBDs, including Crohn's Disease (CD) and Ulcerative Colitis (UC) are chronic relapsing diseases characterized by intestinal inflammation and microbial dysbiosis. The gut microbiota of individuals with IBD are characterized by low microbial diversity (103,104), a reduced abundance of *Bifidobacterium* spp. (103,105), *Lactobacillus* spp. (104) and *Faecalibacterium prausnitzii* (103,105,106), and a higher abundance of pathobionts such as AIEC (107,108) and *Clostridium difficile* (109), resulting in lower SCFA concentrations (110) as compared to healthy individuals. The reason why a dysbiotic microbiota is found in so many IBD patients has not been fully elucidated. Several studies have indicated that intestinal dysbiosis might play a causative role in IBD, since inflammation is usually located in the distal ileum or colon, which are also the sites of highest bacterial abundance in the intestine. Moreover, studies using spontaneous and induced animal models of IBD have shown that animals develop little if any inflammation under germ free conditions (111–113). However, an inflamed environment on its own influences oxygen levels in the gut and thereby seems to favour the typical dysbiosis seen in IBD patients, i.e. depletion of

Firmicutes and the expansion of *Enterobacteriaceae*, especially *E. coli* strains (108,114,115). Considering the unique ability of *Enterobacteriaceae* to thrive proximal to inflamed tissues, these examples support the concept that microbial dysbiosis might be a consequence rather than a cause of inflammation in IBD patients (25).

1.5 Animal models of intestinal inflammation

In the past decades, several animal models have been developed to study IBD and intestinal inflammation. These models can be broadly divided into those involving chemically induced colitis, bacterially induced colitis, spontaneous colitis (i.e. congenital and genetically engineered), and adoptive transfer models (116). Each model possesses advantages and disadvantages, and therefore should be carefully chosen according to the research hypothesis. Although animal models cannot fully represent human diseases, they do allow us to study different aspects of GI inflammation thus being extremely helpful in understanding the pathogenesis of IBD and in the development of novel therapeutic approaches. The remainder of this section will focus on chemical and bacterial induced colitis models, since they are directly relevant to this thesis.

Chemically induced mouse models of colitis are commonly used to test potential therapeutic agents such as drugs, peptides, and probiotics (117). The chemical administration varies from rectal to oral delivery and recreates similar histopathological and clinical features to those seen in GI disorders. These models are widely employed to investigate intestinal inflammation based on their simplicity, short duration, practicality and controllability of disease severity (116). Acute and chronic colitis can be induced by rectally injecting a haptenating agent (e.g. trinitrobenzene

sulfonic acid (TNBS), dinitrobenzene sulfonic acid (DNBS) and oxazolone) dissolved in ethanol. The ethanol damages the epithelial barrier in the colon allowing the haptenating substances to trigger an inflammatory response. Moreover, the inflammation causes epithelial and mucus barrier disruption, thereby resulting in an altered and dysbiotic microbial ecosystem (117).

Colitis can also be induced orally by providing rodents with drinking water supplemented with dextran sodium sulphate (DSS) for several days (118). The DSS model is the most commonly used model to test probiotic candidates and multistrain combinations due its simplicity and reproducibility. This was therefore our model of choice for testing the probiotic strategies outlined in chapter 2. DSS is a sulfated polysaccharide, which seems to be toxic to colonic epithelial cells, thereby causing a disruption of the surface epithelium that affects tight junction (TJ) proteins and compromises the mucosal barrier. One cycle of 3-5% DSS in drinking water for 5-7 days results in an acute colitis, characterized by weight loss, loose stools/diarrhea and rectal bleeding (119). Histologically, the DSS-colitis phenotype resembles the clinical course of human UC, with crypt and epithelial cell damage in the distal colon, tissue edema and ulceration, as well as infiltration of granulocytes and mononuclear cells (Figure 1.2) (116,119). Moreover, DSS can be used to study chronic and relapsing intestinal inflammation by optimizing the protocol with different concentrations, frequency or even cycling the administration of this chemical. Regarding the inflammatory response, chronic DSS colitis seems to be T-cell mediated while acute DSS colitis is independent of the adaptive immune system (B and T cells) since immunodeficient mice also develop severe intestinal inflammation when treated with DSS (120). Therefore, the DSS model is useful in studying the role of the innate immune system in colitis as well as several aspects of IBD such as barrier disruption, mucosal healing and bacteria-host interactions at the mucosal

surface. Furthermore, this model can be applied in several mouse backgrounds, thus facilitating the investigation of probiotic effectiveness in different diseases (117,118).

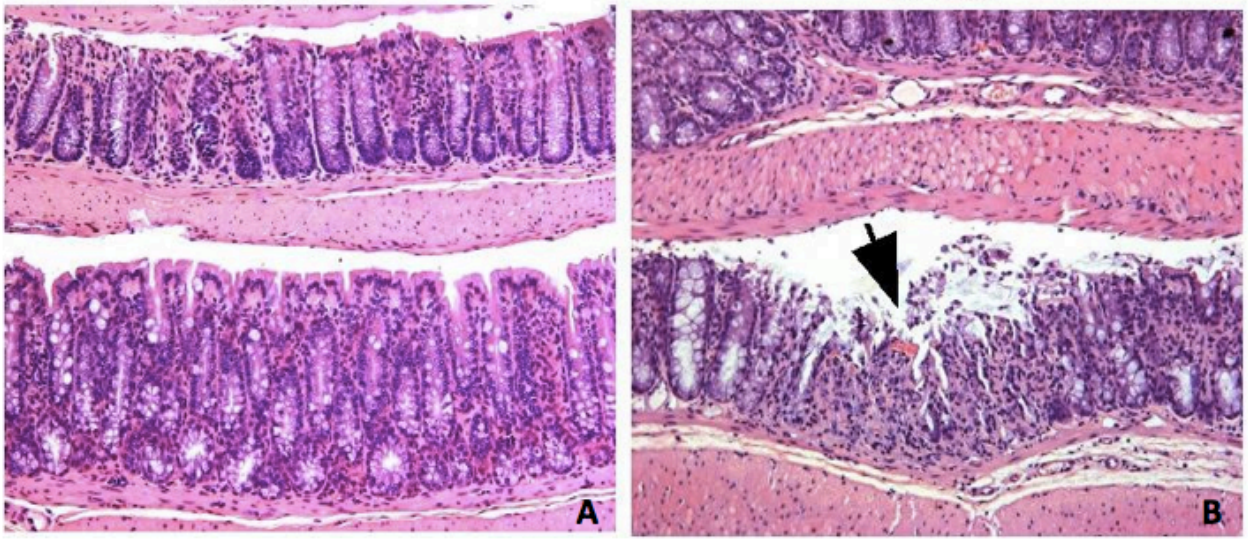


Figure 1.2. Histological analysis of damage caused in colonic tissues by DSS-colitis.
A: Images show healthy distal colon tissue from a mouse that received only water as a control. **B**: inflamed distal colon from mice that received 3% DSS in the drinking water for 4 days, characterized by epithelial damage (arrow), ulceration, crypt damage and severe inflammation. Image reproduced from reference (121) with permission.

Another way to study intestinal disorders is by infecting the host with a pathogen that triggers gut inflammation. There are several enteric bacterial pathogens that are used as animal models of intestinal inflammation: *Salmonella, Vibrio, Shigella, Clostridium difficile, Campylobacter* enterohemorrhagic and enteropathogenic *Escherichia coli* (EHEC and EPEC). These pathogens alter the intestinal ecosystem when infecting their hosts (122). Moreover, how the host responds to and recovers from the GI inflammation and the dysbiosis caused by the virulence factors of these pathogens highlight mechanisms that could be useful for future therapeutic options and clinical applications. Additionally, changes in the microbiota composition post-GI inflammation

such as increased colonization of *E. coli* and *C. difficile* has been linked to a higher risk of developing IBD (123).

Citrobacter rodentium is a Gram-negative bacterium and murine pathogen surrogate to EPEC and EHEC, two human pathogens of significant clinical interest (124,125). Our laboratory has been one of the leading groups using *C. rodentium* as a model of infection to understand the pathogenesis of attaching and effacing (A/E) pathogens, a family of bacteria that attach to the apical cell membrane of IEC – thereby forming a pedestal-like structure (126). Since EPEC and EHEC are unable to infect mice effectively, *C. rodentium* is commonly chosen for studying A/E pathogen-host interactions *in vivo* as it shares 67% of its genes with both EPEC and EHEC (including the locus of enterocyte effacement (LEE) pathogenicity island) thus causing very similar pathological lesions (127). Following administration by oral gavage, *C. rodentium* colonizes the cecum at earlier stages of infection progressing to the distal colon 2 to 3 days later. The peak of infection occurs between day 6 and 10 and clearance is complete between 21 and 28 days post-infection in most mouse strains, including C57BL/6, NIH Swiss and Balb/c. A few mouse strains such as C3H/HeJ and CeHOu/J have been described as being extremely susceptible to this infection, suffering high mortality rates (128). During its peak, *C. rodentium* is usually shed from its host in the stool where it is hyper-infectious and can effectively transmit to new hosts via coprophagy (oral-fecal route). The hallmark pathologies associated with *C. rodentium* infection include dramatic crypt hyperplasia, goblet cell depletion, barrier disruption as well as a strong Th1/Th17 response, resulting in immune cell infiltration into the intestinal mucosa (Figure 1.3) (124,129). As a non-invasive pathogen, *C. rodentium* is an ideal microorganism to investigate A/E bacterial pathogenesis as well as the mucosal host responses generated through different cells and mediators against A/E pathogens (128). Furthermore, it is believed IEC and

epithelial-derived mucin production play a significant role in limiting *C. rodentium* colonization (130–132), which explains our choice of model for the experiments performed in chapter 3.

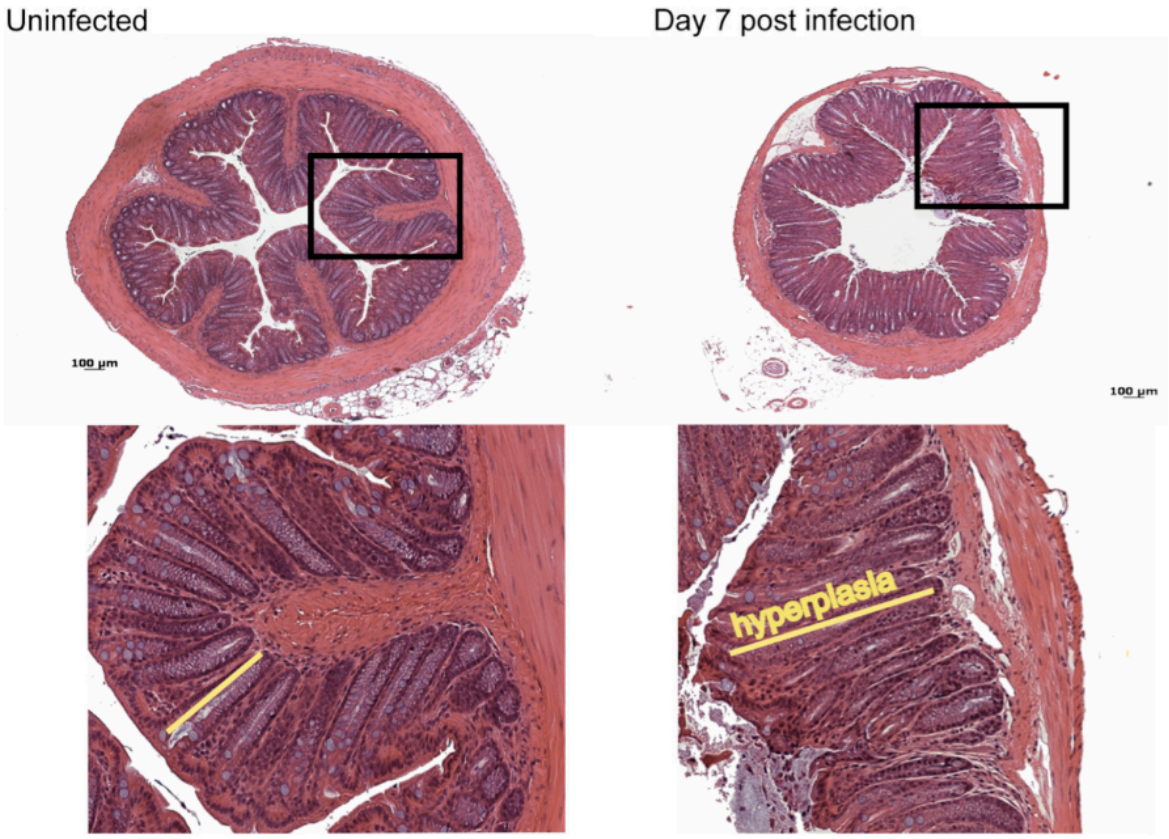


Figure 1.3 Histological damage caused to murine colonic tissues by *C. rodentium* infection.
By day 7 post-infection moderate immune cell infiltration, as well as elongation of crypts, goblet cell depletion and mild edema are observed – as compared to control uninfected tissue. Image reproduced from reference (126) with permission.

1.6 Modulation of the gut microbiome through probiotics

One approach to overcome the microbial dysbiosis seen in IBD patients is through the oral or enema delivery of beneficial gut microbes (probiotics) known to be lacking in IBD patients. Probiotics can be easily incorporated into the diet through the consumption of fermented foods

(i.e. yoghurt, kefir, kimchi, sauerkraut) or, consumed on a daily basis as a probiotic supplement. Studies attribute several health benefits to probiotics including direct effects such as producing SCFAs (e.g. butyrate) and excluding pathogens from the gut by competition for space and nutrients, as well as indirect effects such as enhancing epithelial barrier function and promoting antimicrobial peptide secretory IgA production. Moreover, probiotics have been shown to increase mucin secretion from intestinal goblet cells and to beneficially modulate the host immune system through the stimulation of anti-inflammatory cytokines such as IL-10 and TGF-β as well as stimulate the induction of Tregs (133,134). The exact mechanism(s) by which probiotics exert these positive effects are unclear; however, it is clear that the efficacy of probiotics varies depending on the microbial strain used and the dose administered.

Microorganisms from the genera *Lactobacillus* and *Bifidobacterium* as well as the yeast *Saccharomyces boulardii* are among the most common probiotic candidates. Extensive *in vitro* research has shown that several *Lactobacillus* spp. exhibit anti-inflammatory effects, as primarily assessed by toll-like receptor (TLR) activation (135). For example, *Lactobacillus casei* Shirota treatment restores the normal stimulatory capacity of dendric cells (DC) from UC patients by reducing TLR2 and TLR4 expression (136,137). *Lactobacillus plantarum* CGMCC1258 increases tight junction protein levels and decreases permeability in the intestinal epithelial cell line, IPEC-J2. Moreover, this probiotic reduces IL-8 and TNFα expression in intestinal porcine epithelial cells challenged by *E. coli* K88, possibly via a decrease in TLR expression, nuclear factor kappa B (NFκB) activation, and mitogen-activated protein kinase (MAPK) pathways (138).

In mouse models of intestinal inflammation, *Lactobacillus acidophilus* Bar 13 and *Bifidobacterium longum* Bar 33 promote the expansion of Treg cells and reduce the number of intraepithelial lymphocytes in the 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis (139). In a similar model of murine colitis (2,4-Dinitrobenzene sulfonic acid [DNBS]), *L. casei* DN-114 001 ameliorates disease severity through the induction and expansion of colonic $CD4^{+}FoxP3^{+}$ Treg cells (140). Other studies using mice with dextran sulfate sodium (DSS) colitis show that a combination of eight different probiotic strains (VSL#3) effectively reduces disease activity and colon inflammation including a significant reduction in inflammatory markers such as IL-1β, NFκB, and the neutrophil marker, myeloperoxidase (MPO) (141–143). Similarly, administration of *L. plantarum* 299V prevents spontaneous colitis development in IL-10 deficient ($Il10^{-/-}$) mice (144), and treatment with VSL#3 ameliorates colitis and overall disease activity in $Il10^{-/-}$ mice.

Curiously, despite the broad success of probiotics in animal models of colitis, their effects in clinical IBD trials have been less successful, with only small subsets of treated patients showing beneficial effects (reviewed in (145–147). One reason behind this limited effect in IBD patients may stem from the "one size fits all" approach that has been commonly employed with probiotics. It is strongly believed that as an infant's immune system matures, they develop a mutualistic relationship with the resident microbes in their intestine. This ensures that these resident gut microbes establish an environmental niche, as well as an immunological niche which is recognized by the immune system as a long-term part of the host. In contrast, new microbes encountered after this relationship has developed are typically seen as foreign and are expelled. Thus, providing exogenous probiotic microbes to patients without defining whether there are environmental/

immunological niches for those gut microbes, may mean that the probiotics will be seen as foreign and unable to take up permanent residence in the GI tracts of those patients. Similarly, the inflamed intestines of IBD patients are often\inhospitable to probiotic microbes due to the exaggerated inflammatory and antimicrobial responses seen during disease. These responses clear new microbes, including potentially beneficial bacteria, rapidly from the intestine, often before they have the opportunity to work.

Clearly, new approaches to designing probiotics, and promoting their survival will be key to the future success of this potential therapy. Additionally, engineered probiotics have been developed that produce and release the anti-inflammatory cytokine IL-10 (148,149) or trefoil factor (TFF) as strategies to locally suppress intestinal inflammation and promote healing (150). Moreover, recent insights regarding the makeup of the human microbiome should allow us to identify potential next-generation probiotic species with improved potential for colonizing the human GI tract. Recently, Mandonado-Gómez and colleagues (151) demonstrated that the microbe *B. longum* AH1206 was able to persist in the intestines of a subset of individuals for at least six months after administration without causing side effects or overtly altering the resident microbiota composition. This microbe's extended colonization is attributed to its ability to establish a nutritional niche related to genes involved in carbohydrate utilization. This finding suggests that the establishment of new probiotic microbes will depend on an individual's baseline microbiota as well as on the availability of nutritional resources, thus supporting the critical role of dietary substrates such as fermentable carbohydrates and prebiotics in permitting the long-term persistence of a probiotic strain.

Studies over the past two decades have provided important information identifying person-specific particularities (e.g. allelic gene variations, increased disease predisposition in patients under medication from a pre-existing condition) that could be used to diagnose disease as well as optimize both disease prevention and therapies (152,153). This new strategy represents a shift from a disease-specific approach towards a patient-specific approach, favoring stratifications of subpopulations that consequently improves the accuracy and cost-effectiveness of follow up and treatment. Most progress in precision medicine has been within the oncology field (152,153) (e.g. preventive mastectomy for BRCA1/2 mutation patients (154)), however, this personalized concept has also been discussed by scientists in the microbiome field. It is believed that the gut microbiome may act as a marker for diseases such as obesity (69,155), asthma (156), and rheumatoid arthritis (157), and assessing its composition could be useful in stratifying a patient's disease risk and consequently aid in early disease detection in humans.

In the same line of thought, we believe personalized strategies for the use of probiotics are clearly needed - especially for dysbiotic-related diseases. While commercial and novel probiotics are promising in conditions with a specific target, diseases such as IBD do not appear to benefit from single or even multi-strain probiotics since each patient seems to have a distinct dysbiotic profile. The individual characteristics of a person's microbiota, as well as its changes during their lifetime or in response to environmental stimuli, are the only known factors regarding microbiota composition. Therefore, strategies focusing on personalized probiotics may provide advantages over specific isolates from an exogenous source.

1.7 The intestinal mucus layer and its role in host defense

The GI mucosal barrier comprises epithelial cells and immune cells that together with the resident microbiota form a protective barrier against harmful luminal substances. The IECs are covered by a thick mucus layer that serves as a first line of innate host defense. Besides lubricating the epithelium, the mucus layer also acts as a physical and biochemical barrier preventing noxious luminal substances from reaching the surface of the epithelium (158). The major building blocks of mucus are mucins (mucin (MUC)2 in intestinal mucus), which are large and highly glycosylated proteins with over 80% of their mass comprised of carbohydrates concentrated into mucin domains. These domains are built on a protein core that is rich in the amino acids proline, serine and threonine, called PTS sequences, with the length varying according to each particular mucin. Thus, the PTS sequences have a critical role in MUC2 function by serving as sites of *O*-linked glycosylation (159).

Primarily produced by goblet cells, mucins are packaged into secretory granules within the goblet cell cytoplasm and secreted at their apical membrane (39). Although the mechanisms defining the thickness of the intestinal mucus layer are not completely elucidated, it is known that mucus thickness varies throughout the GI tract. While the small intestine has only one layer of surface mucus, the colon has a two-layered mucus system. The inner layer is very dense, attached to the underlying IECs and relatively sterile whereas the outer mucus layer is loosely attached and filled with commensal bacteria (158,159). Both layers are comprised of the mucin MUC2 (Muc2 in mice), in spite of behaving differently in each part of the intestine (160). Underneath the mucus

layer, a dense network of highly diverse glycoproteins and glycolipids form a thin layer called the glycocalyx which is directly attached to the IEC.

Mucins can be widely classified into secreted or transmembrane mucins. The secretory forms (MUC2, MUC5AC, MUC6) are found throughout the GI tract and can be further subdivided into gel-forming or non-gel forming mucins, based on their ability to form polymers. However, studies have described MUC2 as the major small intestinal and colonic secretory mucin (161,162). Transmembrane mucins (MUC1, MUC4) are membrane-bound glycoproteins, abundantly expressed and found attached to the apical surface of epithelial cells. In addition to hydrating and lubricating the epithelial surface, they provide defense against enteric pathogens (anti-adhesive) and participate in inducing host-signaling pathways such as Wnt, the mitogen-activated protein kinase (MAPK) and the PKB/Akt pathways – all of which are involved in epithelial growth, cell migration and wound healing (131,163,164).

1.7.1 MUC2 structure and synthesis

The MUC2 protein comprises a central PTS domain made up by sequences ordered in tandem known as Variable Number of Tandom Repeats (VNTR). Likewise, MUC2 has cysteine rich N- and C-terminus regions decorating the core protein with four complete von Willebrand D domains (3 at the N-terminus and 1 at the C-terminus) and one incomplete von Willebrand D domain at the N-terminus flanking the central PTS region. These domains are rich in cysteine residues, which can form disulphide bridges with individual mucin proteins that together result in a polymerized network (160,165,166). After synthesis of its main protein core (within goblet cells), MUC2 is

transported to the endoplasmic reticulum (ER) where it forms head-to-head covalently linked dimers via disulfide linkages between the cysteine-knot domains on the C-terminus. Next, the MUC2 dimers pass into the Golgi apparatus, where the *O*-glycans are attached onto the VNTR region through the actions of specific glycosyltransferases (i.e. Polypeptide N-acetylgalactosaminyltransferase, Core 1 β1-3 galactosyltransferase, Core 2 β1-6 N-acetylglucosaminyltransferase, Core 3 β1-3 N-acetylglucosaminyltransferase, Core 2/4 β1-6 N-acetylglucosaminyltransferase), resulting in dimers with molecular masses of approximately 5MDa. In the trans-Golgi, disulfide bridges are formed between the N-terminal D termini to form a net-like MUC2 structure (167). Once ready to transport, MUC2 polymers are packaged into numerous secretory granules and stored in the goblet cell theca creating the apical granule mass that gives the theca its swollen appearance. Once released, MUC2 becomes highly hydrated and instantly and dramatically expands in volume, forming the gel-like structure that comprises the inner and outer mucus layers (39,168).

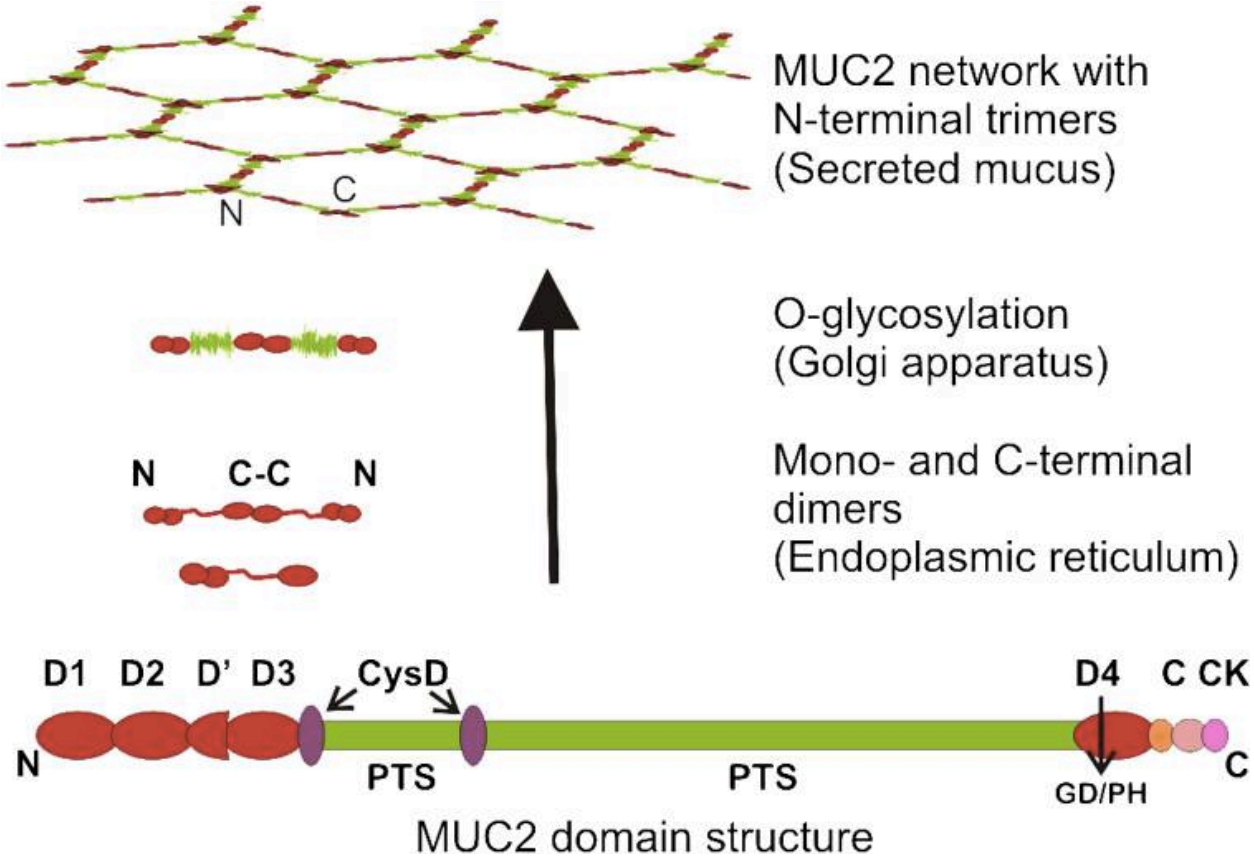


Figure 1.4. Domain structure and biosynthesis of MUC2 mucin.
MUC2 has cysteine-rich N- and C-terminal parts with four complete von Willebrand D domains in total. The central PTS domains are rich in serine, threonine, and proline, and these domains become heavily *O*-glycosylated to generate mucin domains. MUC2 forms dimers in the endoplasmic reticulum by disulfide bonds between the C termini. The dimer is translocated into the Golgi apparatus, where it is *O*-glycosylated, resulting in a size of ≈5 MDa. The MUC2 network is formed by disulfide-linked trimers connecting the N termini. The large polymers are stored in mucin granules in the goblet cells before being secreted. Image reproduced from reference (167) with permission.

1.7.2 MUC2 glycosylation

The MUC2 mucin is a highly glycosylated molecule where 80% of its mass is composed by glycans while only the remaining 20% is the protein mass (160,169). Mucin type *O*-linked glycosylation begins with the addition of N-acetylgalactoseamine (GalNac) on Ser/Thr regions, forming the "Tn antigen" which is further modified by downstream specific glycosyltransferases

to generate a series of core *O*-linked glycans. The major *O*-glycan is Galβ1-3GalNAc, also known as the core-1 *O* glycan, which is generated after the addition of a galactose residue to the Tn antigen by the glycosyltransferase core 1 β1,3-galactosyltransferase (C1galt1, also known as T-synthase). Core 2 *O*-glycans are generated after the addition of N-acetylglucosamine to the core 1 structure, while addition of N-acetylglucosamine to the Tn antigen by core 3 beta1, 3-N-acetylglucosaminyltransferase (C3GnT) forms core 3 *O*-glycans. Further addition of N-acetylglucosamine to the core-3 structure results in formation of core 4 *O*-glycans. The addition of core 2 and core 4 monosaccharaides to the precursor core 1 and core 3 structures respectively is facilitated by tissue specific glycosyltransferases (170,171). There are eight core structures that can be modified by the addition of sugars, however core glycans 1-4 are the most common and important for mucin structure. Moreover, the majority of the oligosaccharides in human colonic MUC2 are based on core 3 and core 4 structures whereas murine colonic Muc2 is predominantly characterized by the presence of core 1 and core 2 based glycans (172,173). Each core structure can further undergo terminal modifications such as fucosylation, sialylation and sulfation. Complex *O*-glycosylation of the mucin structure provides protection against protease degradation. Likewise, *O*-linked glycans are hydrophilic and negatively charged, thus being essential for Muc2 hydration and expansion through the binding of water and salts (172).

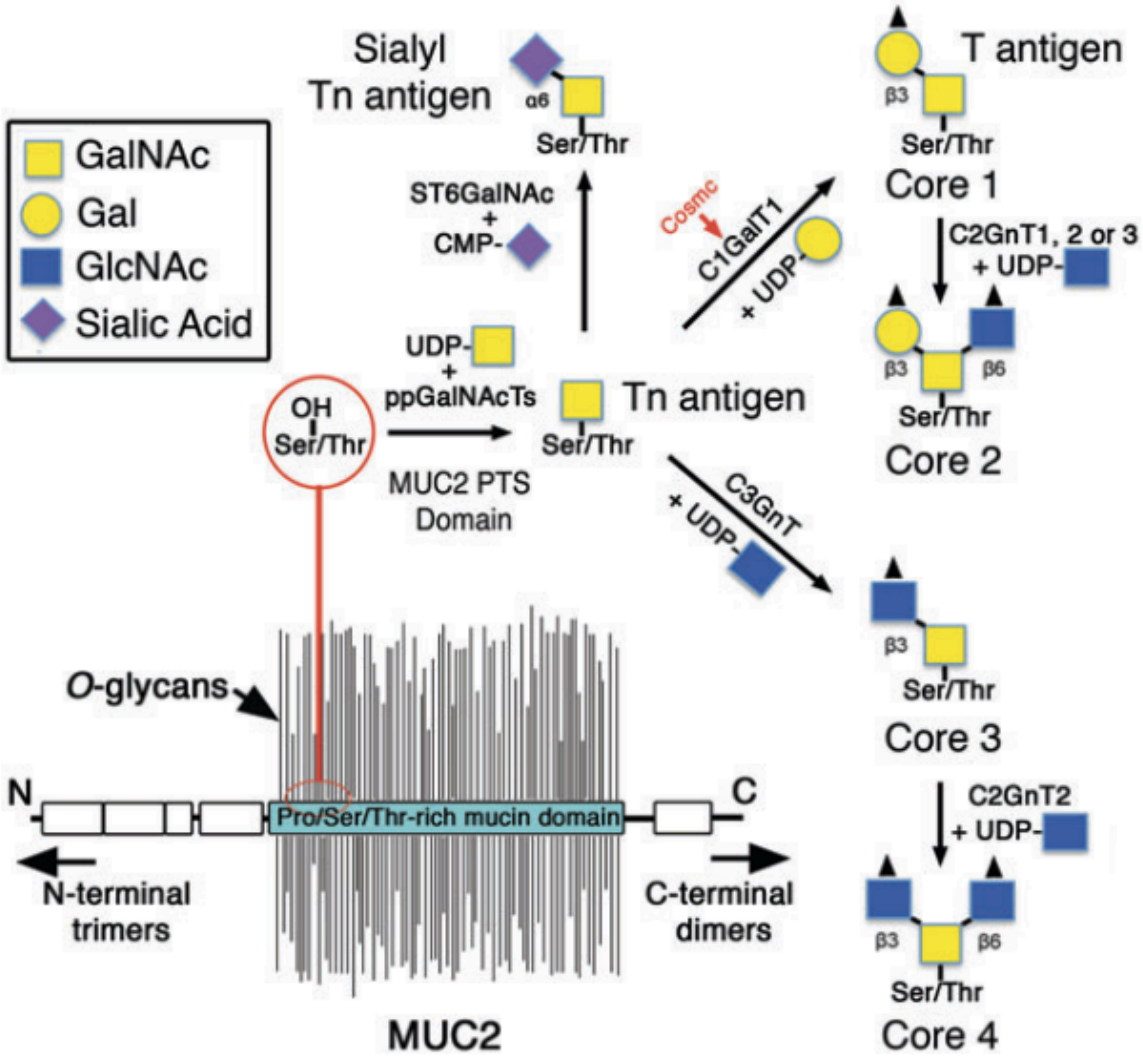


Figure 1.5. The biosynthesis of mucin type O-glycans.
Muc2 glycosylation begins with the addition of GalNac onto the hydroxyl group of PTS (proline, threonine, serine) amino acids, generating a Tn antigen. Next, the Tn antigen can be modified by the addition of galactose by core 1 glycosyltransferase (*C1galt1*) generating core 1 O-glycans. Similarly, addition of a GlcNac residue to the Tn antigen by core 3 glycosyltransferase results in the formation of core 3 structures. Core 1 and core 3 glycans can be further modified to generate Core 2 and Core 4 O-linked glycans, respectively. Addition of sialyl by sialyltransferase forms the sialylated Tn antigen which cannot be modified further. Image reproduced from reference (171) with permission.

1.7.3 Mucus and the gut microbiome

The colonic mucus layer plays an important role in intestinal homeostasis by protecting the underlying epithelial cells from commensal bacteria and food products. In addition, the mucins

present in this barrier seem to influence the composition of the GI tract microbiota. Although the fetal intestine possesses both GCs and mucus, it is only after birth that the complete glycosylation structure is determined with the addition of either sialic acid or fucose residues, thus supporting the role of bacterial colonization in determining the glycosylation patterns of Muc2 (39,174). Moreover, studies using germ free (GF) mice describe their mucus layer as thinner and more penetrable as compared to conventionally housed mice, with their intestinal tissues possessing fewer GCs and only modest levels of *Muc2* as well as the pro-inflammatory resistin-like molecule-β (Relm-β) (175). Likewise, the expression of enzymes responsible for these glycosylations also appear to be reduced in GF mice (176). Correspondingly, it has been hypothesized that these glycan structures influence the makeup of the gut microbiota since they impact the expression of binding sites for specific bacteria thus controlling their ability to adhere to the intestinal mucus (39).

Studies using mice deficient in Muc2 (*Muc2*$^{-/-}$) have shown that they exhibit an increased susceptibility to infection by enteric pathogens. When infected with *C. rodentium*, *Muc2*$^{-/-}$ mice exhibited rapid weight loss, higher pathogen burdens and suffered more histological damage as compared to WT mice (130). Moreover, commensal microbes seem to cluster with *C. rodentium* and adhere to host tissues in *Muc2*$^{-/-}$ mice in the form of multispecies biofilms, suggesting a key role for *Muc2* in regulating the intestinal microbiota as well defending the gut mucosal surface (130). Another recent study demonstrated that *Muc2*$^{-/-}$ mice carry an altered bacterial community within their guts, and this dysbiotic state was attenuated when the deletion of *Muc2*$^{-/-}$ was combined with loss of another GC derived mediator (RELM-β) (177). Analysis suggested that

expression of RELM-β promoted an antimicrobial environment that led to the depletion of specific microbes, such as *Lactobacilli.*

Several bacteria from the *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Verrucomicrobia* phyla have been described as mucin degraders. These bacteria produce specific enzymes (i.e. fucosidases, mucinases, glycosilsulfatases, sialidases, sialate *O*-acetylesterases and N-acetylneuraminatelyses) that are able to degrade glycans found within the mucus layer. Thus, terminal *O*-glycans cleaved/released from the MUC2 protein can serve as a nutrient source for both commensal and pathogenic bacteria in the gut (178). *Bacteroides thetaiotaomicron* is a member of the human intestinal microbiota that relies on mucins and other host glycans for nutrients in the absence of dietary complex carbohydrates (179). It has been described that *B. thetaiotaomicron* produces enzymes that cleave fucose from host glycans, thus increasing the concentration of this terminal sugar within the gut lumen (180). Besides *B. thetaiotaomicron* itself, specific enteric pathogens such as EHEC and *Campylobacter jejuni* are able to sense these newly released glycans, thus resulting in an indirect influence on their virulence and ability to colonize hosts. Regarding potentially beneficial microbes, *Akkermansia muciniphila* is another well studied example of a mucin-degrading bacterium that is present both in within feces and on the gut mucosal surface (181–183). Notably, *A. muciniphila* levels in the stool seem to be reduced in IBD patients thus suggesting an anti-inflammatory role for this bacterium – with some groups suggesting *A. muciniphila* as a potential next generation probiotic (183). The metabolism of certain *Bifidobacterium* spp. is also impacted by the presence of mucin-type *O*-glycans (171) as various strains of this genus possess enzymes to degrade glycans from mucin (i.e. glycoside hydrolases, endo-α-N-acetylgalatosaminidase) (184,185). Members of this genus are Gram-positive obligate

anaerobes and several strains are able to confer beneficial effects to the host thus being classified as probiotics.

Although the mechanisms are not completely elucidated, the mucus layer is considered to be a key ecological niche for many of the bacteria found in the gut. Hence, the relationship between mucin and bacteria – and their products - has a direct impact on health and disease by regulating host functions that are associated with the gut microbiota. Besides serving as a nutrient and carbon source for commensal bacteria within the gut, these mucin-derived glycans also promote the production of important metabolites from bacterial fermentation such as SCFA (186). These metabolites have the ability of diffuse through the mucus layers where the IECs can use them - especially butyrate - as an alternative energy source apart from SCFA derived from dietary fiber (39).

Members of *Clostridia* followed by the *Bacteroides* class are the major butyrate producers within the gut as these bacteria encode the necessary metabolic pathways for butyrate production (i.e. acetyl-CoA, glutarate, lysine, and the 4-aminobutyrate pathways) (187). The oxidation of butyrate to carbon dioxide (CO2) through β-oxidation required by surface colonocytes consumes high amounts of oxygen within the host cells, thereby making the epithelium hypoxic. Consequently, less oxygenation within the epithelium drives an anaerobic environment in the intestinal lumen, hence favoring anaerobic microbes that are usually associated with intestinal homeostasis and thus protecting the host against pathogens via microbiota based colonization resistance (187,188). When there is a depletion of butyrate producers within the gut, this can shift the energy metabolism of colonocytes towards fermentation of glucose, which increases the circulating oxygen within

host cells and further into the intestinal lumen. The resulting increase in oxygen levels in the gut favors facultative anaerobic bacteria such as *E. coli*. This leads to a bloom of *E. coli* which is a marker for intestinal dysbiosis and has been strongly associated with IBD pathogenesis (34).

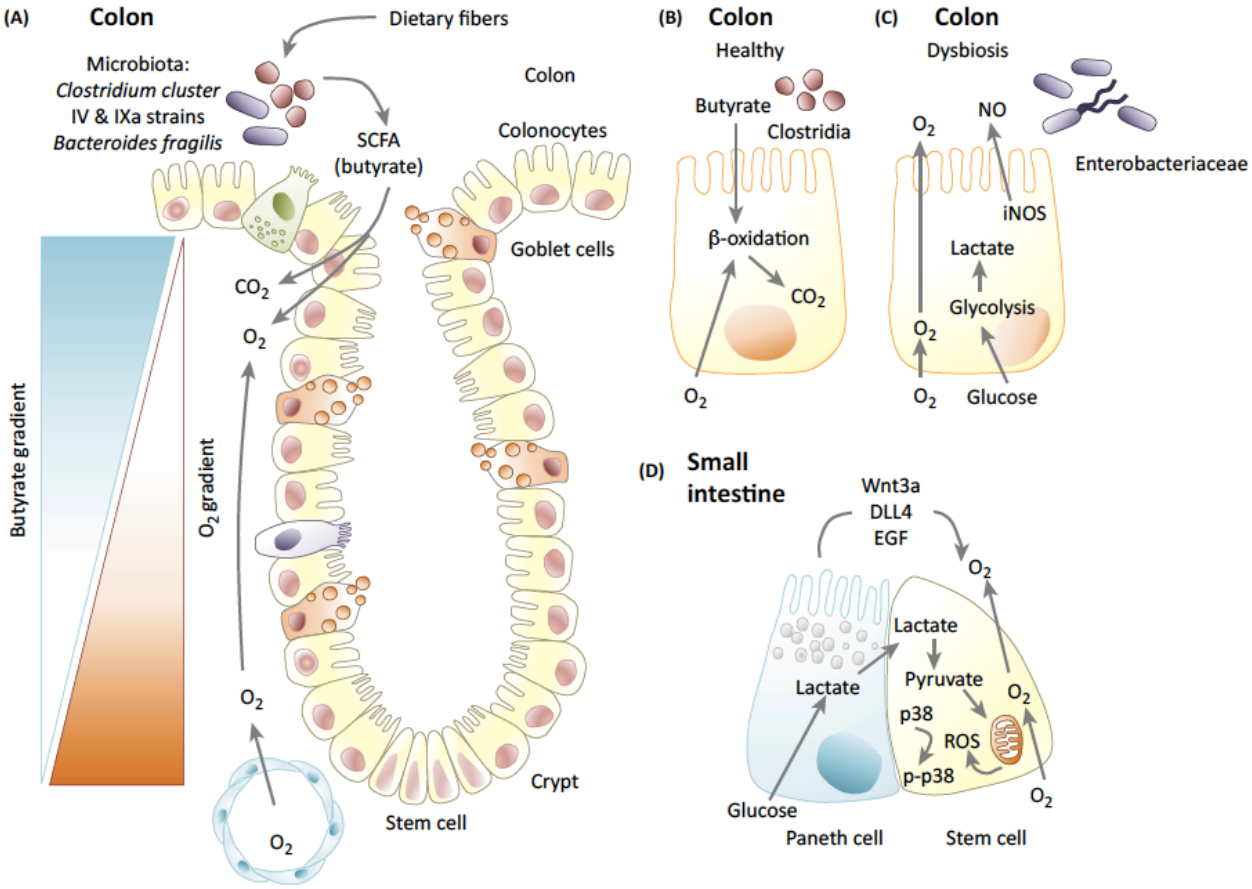


Figure 1.6. Intestinal homeostasis as promoted by SCFA.
A: Butyrate and oxygen gradients are inversely correlated during colonic homeostasis. Members of *Clostridia* and *Bacteroides* class produce butyrate from dietary fibers and colonocytes are able to use this SCFA as a nutrient through β-oxidation that consumes local oxygen within the host cells. **B:** Colonic cells metabolism during intestinal homeostasis consumes oxygen and creates the ideal anaerobic environment for butyrate-producing bacteria. **C:** Colonocyte metabolism during dysbiosis. A shift in the butyrate-oxygen gradient leads to anaerobic glycolysis pathway which favors the proliferation of microaerophilic bacteria (like *E. coli*) over butyrate-producers resulting in a dysbiotic state. **D:** Metabolic interactions between Paneth and stem cells. The lactate produced by Paneth cells via glycolysis is essential for small intestinal homeostasis. Lactate is then converted to pyruvate by LGR5+ stem cells and further leads to reactive oxygen species (ROS) signaling and p53 activation. Image reproduced from reference (34) with permission.

1.8 Research hypothesis and objectives

Taken together, these studies suggest that beneficial microbes such as probiotics help in maintaining an overall bacterial balance within the gut and may be beneficial in preventing dysbiotic-related diseases. However, the effect of probiotics in severe conditions such as IBD still show conflicting results mainly due to strain specific effects, the baseline status of the host, and microbiota resilience in incorporating new microbes in a permanent way. This lack of reproducibility highlights the need for further research in the field and the mechanisms involved need to be better elucidated. Moreover, we wondered whether the mucin Muc2 could potentially impact host defense, since the mucus layer is not only a physical protective barrier but also serves as a nutrient source for several bacteria in the gut, thus impacting gut microbiota composition and bacterial pathogenesis. Therefore, the goals of my PhD thesis were twofold. First - I sought to determine how personalized commensal bacteria isolated from the host could impact inflammatory responses in the GI tract during DSS induced colitis, focusing on intestinal epithelial pathology and gut microbiota modulation. DSS-colitis was chosen based on its wide application when testing novel probiotics and because it replicates closely several phenotypes found in IBD patients including loss of colonic crypts, severe ulceration and infiltration of inflammatory cells. Second - for the experiments investigating the role of mucus layer in host defense, the *C. rodentium* model was chosen as it replicates A/E bacterial pathogenesis as well as certain aspects of IBD, especially epithelial barrier disruption, dysbiosis, and the histological tissue damage. Furthermore, the *C. rodentium* mouse model is well established and allowed us to explore enteric microbe-mucus interactions from both the host and pathogen perspective in a dynamic way. We hypothesize that a personalized probiotic approach represents a better strategy to beneficially modulate the host response against DSS-colitis as endogenous bacteria are already incorporated in the environment

and it is just a matter of quantitative balance to protect the host against noxious stimuli. Additionally, we believe the mucin Muc2 and its core 1 glycosylation play an important role in host defense against enteric pathogens functioning as a physical barrier as well as providing a niche for complex interactions with the intestinal microbiota and its metabolites. The objectives of my thesis are 1) To examine the protective role of endogenous commensal bacteria acting as a personalized probiotic during intestinal inflammation induced by DSS, and 2) To determine how the mucin Muc2 and one of its major glycosylations (core 1) impact colonic homeostasis and susceptibility to infection with *C. rodentium*. Findings from these studies will increase our understanding of the interactions between a balanced gut microbiota and disease susceptibility thus helping define basic mechanisms of intestinal homeostasis and consequently intestinal health. Moreover, my work also highlights the importance of the mucus layer in maintaining host-microbial homeostasis by presenting a possible mechanistic role for intestinal disease caused by enteric pathogens.

Chapter 2: Isolation and characterization of potentially probiotic bacterial strains from mice: proof of concept for personalized probiotics

2.1 Introduction

At birth, the human gastrointestinal (GI) tract becomes colonized by a complex ecological community of microorganisms, referred to as the "gut microbiota" (15,37). Most microbes residing in the gut are harmless or even beneficial to the host, thus resulting in a harmonious and generally symbiotic relationship. However, disruption of the normal balance in bacterial composition, function, and diversity (termed dysbiosis) has recently been associated with several negative health conditions such as Inflammatory Bowel Disease (IBD) (91–93), Irritable Bowel Syndrome (IBS) (94), obesity (50), type 2 diabetes (95), asthma (96), colon cancer (97,98), non-alcoholic fatty liver disease (99,100), and neurological diseases (101,102).

The term "microbial dysbiosis" is still poorly defined, as there is much uncertainty regarding what constitutes a 'healthy' microbiota. Previous studies in the GI tract have claimed that a dysbiotic condition is usually characterized by a reduced diversity of microbes often in combination with a lower abundance of obligate anaerobic bacteria and an expansion of facultative anaerobic bacteria, particularly from the *Proteobacteria* phylum (189–192). Nevertheless, more evidence is needed to better describe the concept of dysbiosis, considering that it may be defined differently at the population and individual levels. For the purpose of this paper, 'dysbiosis' refers to an aberrant

microbiota that is represented by low microbial diversity, loss of beneficial microbes, and an expansion of pathobionts.

Besides genetics, environmental factors, such as diet and the gut microbial community, appear to be involved in IBD development. These modifiable factors have gained more attention over the last few decades since IBD incidence has increased significantly in developing countries that have recently transitioned to a more 'Western' diet style and lifestyle (25). One of the strategies proposed to positively modulate the gut microbiota is the oral administration of beneficial microbes known as probiotics. Studies suggest that probiotics promote several positive host-microbe interactions, by excluding pathogens via their competition for nutrients and space, and by promoting epithelial barrier function and mucus secretion from intestinal goblet cells. Probiotics have also been shown to increase the production of antimicrobial peptides and SCFA, and stimulate the expression of anti-inflammatory cytokines such as IL-10 and TGF-β (117,193–198).

Despite the beneficial effects of probiotics seen in different conditions (reviewed in (25,199–201)), the mechanisms by which they exert these benefits in humans remains uncertain. Moreover, the effects of probiotics are known to be strain- and dose- specific, which may explain in part the divergent results found when using different probiotic strains, even though they are from the same genera or species. Additionally, there are several factors that might influence the results seen using probiotics in clinical trials, including the use of different probiotic strains, either alone or in combination with other therapies, the baseline health status of the host, the resistance of the host microbiota to incorporating new microbes in a permanent way (microbiota resilience), and the environmental niche created (and controlled) by the host immune system during the early stages

of life (25). Furthermore, in certain serious conditions such as IBD, probiotics are usually taken as adjuvants to traditional therapies or during the remission stage of the disease, thus leaving little to no opportunity to evaluate their potential effectiveness during times of acute inflammation and severe intestinal dysbiosis.

Recently, an area of significant discussion has emerged regarding the self-regenerative capacity of the host microbiota (202). The gut microbiota is sensitive to many environmental challenges such as diet, infections, antibiotic use, and hygiene habits (i.e. early-life exposure to environmental microbes, household pets and siblings, city vs. rural living conditions) (49,58). Even so, the changes in microbiota makeup caused by these challenges appear to be limited and/or transient, as microbes are considered both resilient and resistant to change, and thus, an individual's microbiota composition and function are thought to be fairly stable in the face of external perturbations (194,202). While the resilience of the intestinal microbiota clearly protects us from infections by the myriad pathogens we encounter (202–204), it also poses an obstacle to the use of probiotic strains, making it a significant challenge to manipulate/alter the microbiota, even during states of dysbiosis. This phenomenon explains the need for probiotics to be repeatedly delivered to observe their health benefits over the long term (194).

Based on our understanding of how an individual's genetics influences their physiology, a new concept termed "personalized" or "precision" medicine has emerged focusing on diagnostics and treatments that favor the specific characteristics of the host, thus avoiding the "one size fits all" approach, that has often proven ineffective (153). A personalized strategy using probiotics was recently proposed for bacterial vaginosis, an aberrant state of the vaginal microbiota, which may

be attenuated by the administration of *Lactobacillus* spp. isolated from the host (205). The method called "TripleA" involves three consecutive steps described as the **A**cquisition of the patient sample during an infection, the **A**lteration of the microbial composition by sampling and enriching *Lactobacillus* spp. *ex-vivo*, and finally the **A**dministration of the enriched sample in a personalized gel formula (205). This method hypothesizes that *Lactobacillus* spp. isolated in a personalized way would facilitate colonization based on the specific genetic makeup of the host. Although there are no studies yet demonstrating the efficacy of the TripleA method, this strategy presents possible advantages in comparison to the common antibiotic treatment (i.e. extensive side effects and infection recurrences), as well as to commercial probiotics available on the market, because they are adapted to the host microbiota and would (in theory) simply restore a healthy vaginal microbiota.

Taken together, the aim of the current study was to introduce the concept of a personalized probiotic therapy for intestinal diseases, where commensal bacteria isolated from the gut microbiota of healthy hosts could be stored in a 'microbiota biobank' after having their intrinsic characteristics tested, and ultimately used as a therapy for dysbiosis-related diseases.

2.2 Experimental procedures

2.2.1 Mice

Male C57BL/6 mice were purchased from CEMIB (Campinas, SP, Brazil), kept in sterilized, filter-topped cages and fed autoclaved food (Standard Rodent Diet, Presence, Paulínia, SP, Brazil) and water ad libitum under specific-pathogen-free conditions at the Sao Paulo State University. Findings in Brazil were repeated with C57BL/6 mice bred in house at the British Columbia Children's Hospital Research Institute (BCCHRI) (Vancouver, BC, Canada). Sentinel animals were routinely tested for common pathogens at both facilities. The protocols employed were approved by the Sao Paulo State University (34/2014) and by the University of British Columbia's Animal Care Committee (A15-0206), and were in direct accordance with guidelines provided by the Brazilian College of Animal Experimentation and by the Canadian Council on the Use of Laboratory Animals.

2.2.2 Isolation of commensal bacteria strains

Several commensal bacteria strains were isolated from the stool of healthy C57BL/6 mice (n=10). In brief, fresh stool pellets were homogenized in 1.0 mL phosphate buffered saline (pH 7.2), plated on Man Rogosa Sharpe agar (MRS – Acumedia, Lansing, MI, USA) and *Bifidobacterium* iodoacetate medium 25 agar (BIM-25 - Acumedia, Lansing, MI, USA) for *Lactobacillus* spp. and *Bifidobacterium* spp. selection, respectively. The plates were incubated under anaerobic conditions at 37 °C for 48 h to 72 h. Five colonies with distinct morphologies were selected from *Lactobacillus* spp. and *Bifidobacterium* spp. genera from each mouse sample. The selected colonies were further purified by streak plating in the same media. The isolated colonies were

transferred to MRS broth (*Lactobacillus* spp.) or MRS broth with the addition of 0.5% L-cysteine (InLab, Brazil) (*Bifidobacterium* spp.), and then incubated under anaerobic conditions at 37 °C for 48 h to obtain a liquid culture of each individual isolate.

2.2.3 Preliminary identification

The isolated colonies were stained using Gram's method (206) and then classified into Gram-positive and Gram-negative bacteria based on their cell wall properties and the resulting color (pink or purple). The slides were analyzed using a trinocular microscope with a camera (E200 Nikon, Tokyo, Japan). Additionally, a loop containing a liquid culture of each isolated bacterium was tested for the expression of the catalase enzyme using 3% hydrogen peroxide (H_2O_2). A positive result for catalase was confirmed with the rapid evolution of oxygen (5-10 s) as evidenced by bubbling (206)

2.2.4 Genera confirmation

All selected strains underwent a combination of colony-PCR and randomly-amplified polymorphic DNA–polymerase chain reaction (RAPD-PCR) to confirm the target genera and as a preliminary form of species identification. In brief, single colonies from each isolated strain were used directly as the template, without any DNA extraction and purification prior to PCR. Next, a set of two short arbitrary (10bp) primers was used in the PCR reaction: *Lactobacillus* spp. (Lab-0159: 5'- GGA AAC AG (A/G) TGC TAA TAC CG-3'; Lab-0677: 5'- CAC CGC TAC ACA TGG AG -3') (207), *Bifidobacterium* spp. (OPA-02: 5'-TGC CGA GCT G -3'; OPA-07: 5'- AGG CGG GAA C -3') (208). The RAPD-PCR was performed by adding 1x Taq DNA buffer, 1.5 mM of $MgCL_2$, 0.2 mM of each deoxynucleotide, 1 μM of each *primer*, 2 ng of genomic DNA and 2U of Taq DNA

polymerase enzyme, in a final volume of 25 μL. All reactions were assembled in duplicate and the amplification was carried out using a Veriti Thermo Cycler (Thermo Fisher Scientific, Waltham, MA, USA) under the following conditions: initial denaturation at 94°C for 10 min, followed by 35 cycles at 94°C for 20 s, 55°C for 20 s, 72°C for 30 s and then a final extension at 72°C for 5 min. Distinct species of *Lactobacillus* spp. and *Bifidobacterium* spp. were identified by analyzing the PCR products through agarose gel electrophoresis (1% in TAE buffer– 40 nmol/L Tris, 11% glacial acetic acid, 1 mmol/L EDTA) using a 100 bp ladder (Invitrogen, Carlsbad, CA, USA) as a molecular size marker. The gels were stained with SYBR Safe (Invitrogen, Carlsbad, CA, USA) and the images acquired under UV illumination using a Gel Doc XR System (Bio-Rad, Hercules, CA, USA). The similarity of the RAPD profiles was compared within the isolates to distinguish possible different species, while commercially-purchased strains of *Lactobacillus* spp. and *Bifidobacterium* spp. were used as controls.

2.2.5 Evaluation of survival in simulated gastrointestinal conditions

The gastrointestinal resistance of the isolated strains was tested according to the approach of Liresse and colleagues (209) and Buriti and colleagues (210), with minor modifications. Aliquots of each bacterial culture suspension (10^8CFU/mL) were added to an acid solution (NaCl 0.85%, 1N HCl, 3g/L of pepsin from porcine stomach mucosa (Sigma® Aldrich Co., St. Louis, MO, USA), and 0.9mg/L of lipase from porcine pancreas (Sigma® Aldrich Co., St. Louis, MO, USA) to reach a pH of 2.4. Samples were incubated at 37 °C (150 rpm) (Incubator shaker, Tecnal, Piracicaba, SP, Brazil) for 2 h, leading to the simulated gastric phase. Next, the pH was increased to 5.0 using an alkaline solution (150 mL of 1 N NaOH, 10.77 g of $PO_4H_2Na.2H_2O$ and distilled water up to 1 L) and biliary salts (Oxgall Powder, Sigma® Aldrich Co., St. Louis, MO, USA) and

porcine pancreatin (Sigma® Aldrich Co., St. Louis, MO, USA) were added to reach a concentration of 10 g/L and 1 g/L, respectively. Samples were incubated again at 37 °C for 2 h under agitation (150 rpm), leading to the simulated enteric phase 1. Finally, the pH was increased to 7.0 using the same alkaline solution. Biliary salts and pancreatin were adjusted to maintain their concentrations at 10 g/L and 1 g/L, respectively, and the samples were incubated again at 37 °C for 2 h under agitation, leading to the simulated enteric phase 2, thus completing the 6 h of assay. Enumeration of *Lactobacillus spp.* and *Bifidobacterium spp.* was performed in aliquots collected in duplicate after 2 h, 4 h, and 6 h. Aliquots of 1 mL were pour-plated in MRS or BIM-25 for colony forming unites (CFU) of *Lactobacillus spp.* and *Bifidobacterium spp.*, respectively.

2.2.6 Antibiotic susceptibility test

Each isolated strain was tested for its susceptibility against ten different antibiotics (ceftriaxone 30 µg, imipenem 10 µg, aztreonam 30 µg, erythromycin 15 µg, vancomycin 30 µg, chloramphenicol 30 µg, tetracycline 30 µg, nitrofurantoin 300 µg, norfloxacin 10 µg, and ciprofloxacin 5 µg) using the disk diffusion assay (211,212). Fresh bacterial cultures were diluted to a suitable turbidity equivalent to 0.5 McFarland Units (10^8CFU/mL). Five antibiotic disks were placed in MRS or BIM-25 agar plates containing 100 µL of each bacterial culture, and the plates were incubated under anaerobic conditions at 37 ^{o}C for 16 h to 18 h. The tests were conducted in duplicate, and the plates were not inverted during the incubation period. The zone diameter around each disk was measured as the inhibited bacterial growth areas, and the strains were categorized as sensitive (≥20mm), intermediate (15-19mm), or resistant (≤14mm), according to criteria established by the *Clinical and Laboratory Standards Institut*e (211).

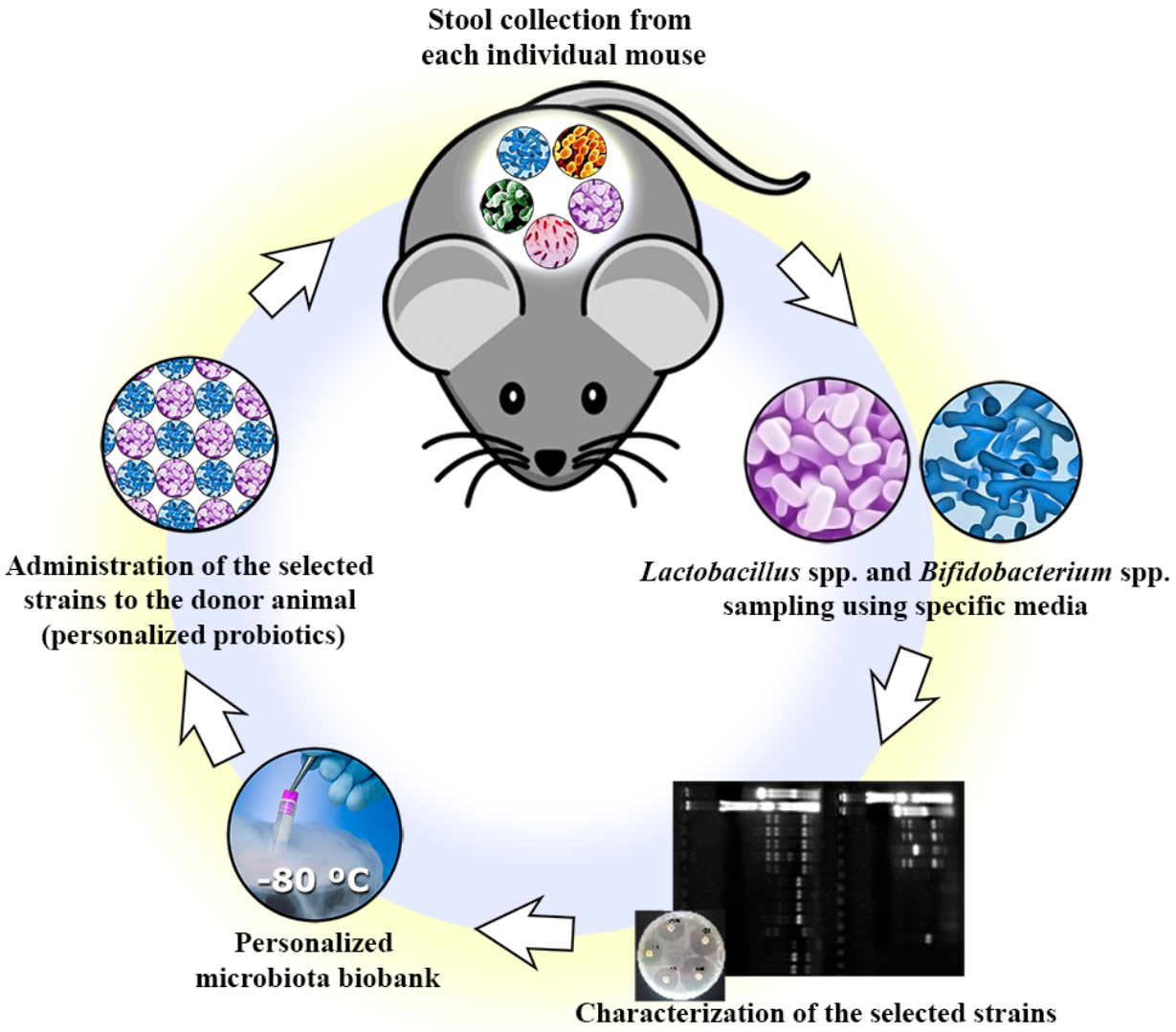


Figure 2.1. Schematic image describing the steps for isolation and characterization of potentially probiotic bacterial strains.

The first step in the personalized probiotic procedure involves the collection of a stool sample from a healthy mouse. For human application in the future, the sample could be collected in disease predisposed individuals or during the remission stage of certain disorders (i.e. IBD, IBS). Several strains of *Lactobacillus* spp. and *Bifidobacterium* spp. are selected using specific media and growth conditions. The selected strains undergo characterization tests to investigate their potential to be classified as a probiotic. Next, the most promising strains in the screening step are frozen at -80^{o}C in a personalized probiotic biobank, allowing long-term storage and potential application in several dysbiotic-related diseases. Finally, the personalized strains are administered to the host (donor animal or patient) during the disease as a personalized probiotic treatment.

2.2.7 Dextran sodium sulfate (DSS)-induced colitis experiment

The bacterial isolates were tested in the DSS-induced colitis model to assess their potential to protect mice against colitis, and thus, to function as a potential probiotic. Male 8-week-old C57BL/6 mice were provided with either the personalized combination of probiotics (PP) or the commercially obtained probiotic *Lactobacillus rhamnosus* GG (LGG), which was used as a comparison based on its wide application in gastrointestinal disorders (213,214). Healthy mice and DSS-only treated mice were used as negative and positive controls, respectively, totaling four study groups (n=10) as described in Figure 2.2.

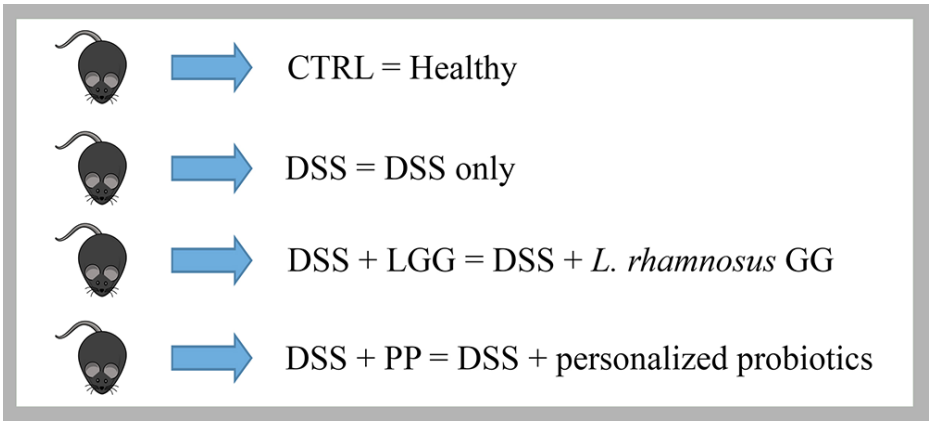


Figure 2.2. Description of the groups used in the DSS experiment.
CTRL: healthy mice with no intervention - negative control; DSS: mice challenged with 3% DSS with no intervention - positive control; DSS + LGG: mice challenged with 3% DSS and treated with *Lactobacillus rhamnosus* GG prior and during DSS – commercial probiotic control; DSS + PP: mice challenged with 3% DSS and treated with a personalized pool of commensal bacteria isolated from their own microbiota.

After the isolates were selected and characterized, three strains were chosen based on their distinct species profiles obtained by RAPD-PCR. These isolates were grown separately in their specific media under the conditions described in section 2.2.2 and then combined into a personalized pool of bacteria for each individual mouse in the PP group. The final personalized pool of probiotics consisted of an equal mixture (1:1:1) of each of the three isolates. Both treatments, i.e. the

personalized pool of probiotics and the LGG, were administered daily by oral gavage (0.1mL = approximately 2.5×10^9 CFU) starting seven days prior to DSS, as well as throughout the course of the DSS exposure (14 days total) (Figure 2.3). Animals from the control groups received the same volume of sterile water by oral gavage to avoid any differences in handling during the experiment. Colitis was induced by adding DSS (36.000-55.000 Da, MP Biomedicals, Santa Ana, CA, USA) to sterile drinking water at a concentration of 3% (w/v). Animals were treated with DSS for seven days and then euthanized by cervical dislocation following prior anesthesia with isoflurane. Over the course of the experiment, mice were weighed daily and monitored for any signs of distress. During the DSS treatment, the severity of the colitis was determined daily using the disease activity index (DAI), which takes into account three parameters: weight loss, stool consistency, and occult bleeding in the feces, as tested using the commercial Hemoccult kit (Beckman Coulter, Pasadena, CA, USA).

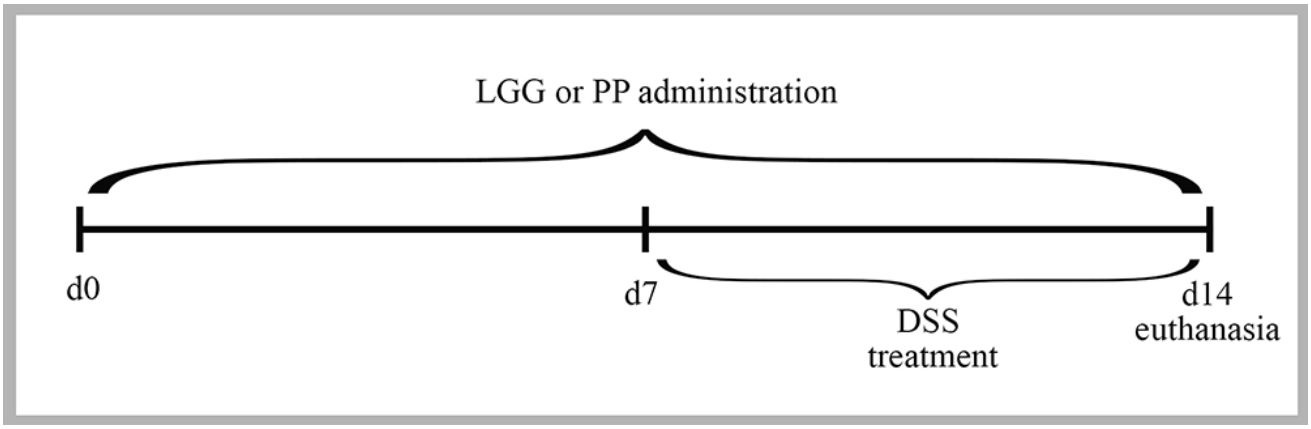


Figure 2.3. DSS experiment timeline.
Male C57BL/6 mice were given either LGG or PP via oral gavage starting 7 days prior to receiving 3% dextran sodium sulfate (DSS) and then every day during DSS exposure until they were euthanized (total = 14 days). DSS was given in drinking water for 7 days. d0: day 0 of experiment - baseline condition; d7: day 7 of experiment – mice received their respective product or sterile water as vehicle; d14: day 14 of experiment – mice received their respective product or vehicle and 3% DSS.

2.2.8 Tissue collection

After euthanasia, mouse colonic tissues were collected for histological analysis. Colon tissues were opened longitudinally, stool was gently removed, and distal colon sections were placed in histological cassettes. The cassettes were immediately placed in 10% neutral buffered formalin (Fisher, Hampton, NH, USA) (24 h, R/T) for histological processing.

2.2.9 Histopathological scoring

Colonic pathology was scored using a previously-adapted scoring system (215). In brief, paraffin-embedded colonic tissue sections (5 μm) were stained with hematoxylin and eosin (HE), and were examined by three blind observers. The tissue sections were assessed for immune cell infiltration (0 = occasional immune cell in LP; 1 = granulocytes in LP; 2 =infiltration into the submucosa; 3 = extensive transmural infiltration), severity of crypt damage (0 = all crypts intact; 1 = loss of basal side of crypts; 2 = some crypt structure can be identified 3 = crypt structure is lost with surface epithelium still intact; 4 = Crypt structure is lost with epithelial surface erosion), edema (0= no edema 1= mild/occasional edema 2=moderate edema 3=severe edema/over long stretches), and amount of tissue affected (0 = 0%; 1 = 5-25%; 2 = 25-50%; 3 = 50-75% 4 =75-100%). The maximum score that could be obtained with this system was 14 points.

2.2.10 RNA extractions and quantitative real-time PCR

Following euthanization of the mice, distal colonic tissues were immediately placed in RNA-later (Qiagen, Hilden, Germany) and stored at -80 °C. Total RNA was extracted using the Qiagen RNeasy Mini Kit according to manufacture's instructions, and then quantified using a Nanodrop spectrophotometer (ND1000 – Thermo Fisher Scientific, Massachusetts, USA). Complementary

DNA (cDNA) was synthesized using 1 μg of RNA with Omniscript RT kit (Qiagen, Hilden, Germany), followed by quantitative real-time PCR techniques. The qPCR reaction had a final volume of 20 μL, where 5 μL of cDNA was added to 15 μL of a PCR mix containing 10 μL of BioRad SsoFast EvaGreen and 5 μL comprised of RNase- and DNase-free water and primers to a final concentration of 0.6 μM. Primer sequences and annealing temperatures were as follows: IL-10 (forward: 5′-GTT GCC AAG CCT TAT CGG AA-3′; reverse: 5′-CCA GGG AAT TCA AAT GCT CCT-3′; annealing 55 °C) (216); TGF-β (forward: 5′-GAC TCT CCA CCT GCA AGA CCA T-3′; reverse: 5′-GGG ACT GGC GAG CCT TAG TT-3’; annealing 59 °C) (216); IL-6 (forward: 5′-GAG GAT ACC ACT CCC AAC AGA CC-3′; reverse: 5′-AAG TGC ATC ATC GTT GTT CAT-3′; annealing 59 °C) (217); IL-1β (forward: 5′-CAG GAT GAG GAC ATG AGC ACC-3′; reverse: 5′-CTC TGC AGA CTC AAA CTC CAC-3′; annealing 59 °C) (217); *Tbp* (reference gene) (forward: 5’-ACC GTG AAT CTT GGC TGT AAA-3’; reverse 5’-GCA GCA AAT CGC TTG GGA TTA-3’; annealing 59 °C) (218). qPCR was carried out on a CFX Connect Real-Time PCR system (BioRad, Hercules, CA, USA) for 40 cycles using the following conditions: denaturation at 95 °C for 5 s, annealing at 57 °C or 59 °C for 10 s, and elongation at 72 °C for 20 s. The data was analyzed using CFX Manager Software (BioRad, Hercules, CA, USA). The mRNA expression was determined by the average quantification cycle (Cq) values from duplicate measurements using *Tbp* as a reference gene (218), and was normalized with the average Cq value of the control group (healthy mice) (219).

2.2.11 Myeloperoxidase (MPO) and malondialdehyde (MDA) activity

Colonic tissue homogenates were used to measure MPO and MDA activity. MPO activity was measured using a colorimetric activity assay kit (Sigma-Aldrich, St. Louis, MO, USA). In brief,

the tissues were homogenized in the buffer solution provided with the kit, and the assay was performed according to the manufacturer's instructions. Colorimetric change was measured on a microplate reader (BioRad, Hercules, CA, USA) using 412nm absorbance, and the results were used to calculate the MPO concentration in each sample as per the manufacturer's instructions. Similarly, the lipid peroxidation in colonic tissue was investigated using a commercial kit (Abcam, Cambridge, UK) that assesses MDA levels though a method dependent on thiobarbituric acid (TBA). The assay was performed according to the manufacturer's protocol where TBA was added to the homogenate supernatant, and the samples were boiled for 60 min at 95 °C before the absorbance was assessed at 532nm using a spectrophotometer (BioRad, Hercules, CA, USA).

2.2.12 Statistical analysis

All results presented in this study are expressed as the mean value ± Standard Deviation (SD). Statistical analysis was performed using the GraphPad Prism Software Version 7.0 (GraphPad Software, San Diego, California). One-way analysis of variance (ANOVA) and Tukey's or Dunnett's multiple-comparison test were used to analyze the results. Significance was declared when $p<0.05$.

2.3 Results

2.3.1 Isolation and genera confirmation of the strains

From the different stool samples of each mouse plated either in MRS or BIM-25 media, colonies displaying typical *Lactobacillus* spp. and *Bifidobacterium* spp. characteristics (220,221) were selected based on their distinct morphology. Initially, ten strains were isolated from each mouse in the experiment, where 50% were *Lactobacillus* spp. and 50% were *Bifidobacterium* spp. All samples presented typical colony morphologies of these two genera, confirming that they were part of the murine gut microbiome as described in previous studies (222). Figure 2.4 shows the PCR products from each strain isolated per mouse, revealing two different band profiles for *Bifidobacterium* spp. and only one profile for *Lactobacillus* spp. The similar species profile found for these mice likely reflects their inbred nature and their co-housing, resulting in similar gut microbiota. Based on the profiles found in the agarose gel, three different bacterial strains per mouse (two *Bifidobacterium* spp. and one *Lactobacillus* spp.) were chosen to proceed for further analysis totaling in 30 host-selected strains (n=10). In summary, a personalized combination of three different commensal bacteria was prepared for each mouse after growing the strains separately in their specific media and conditions.

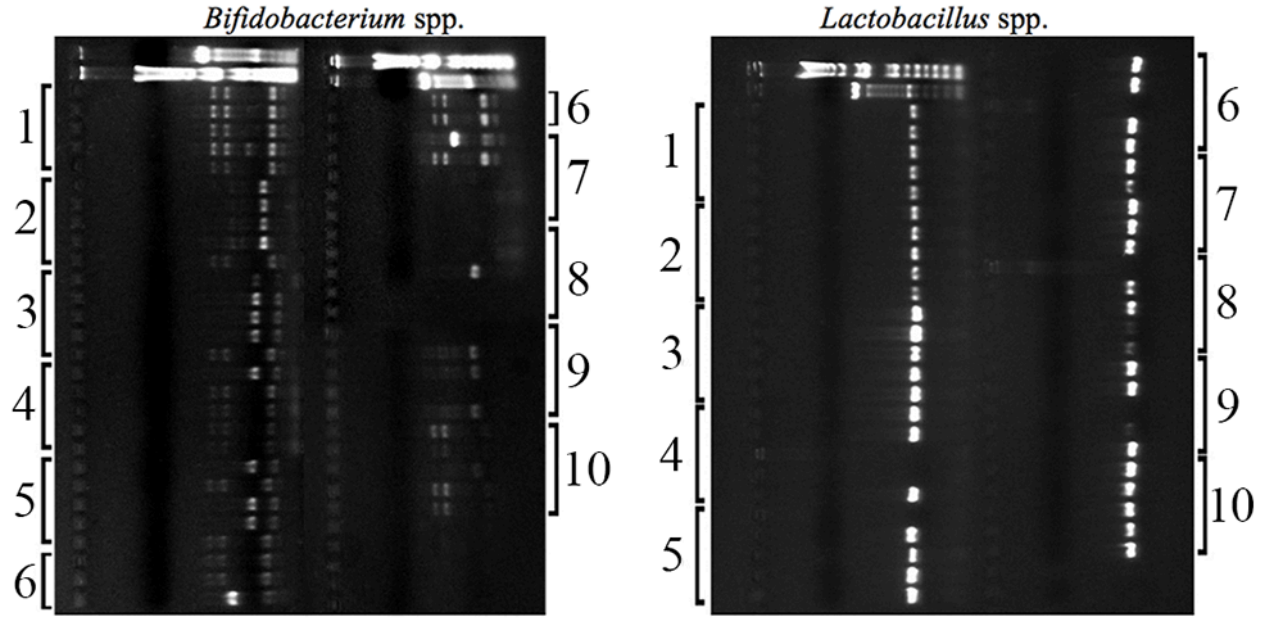


Figure 2.4. PCR products of isolated commensal bacteria.
PCR products (run on 1% agarose gels) obtained from *Bifidobacterium* spp. (left) and *Lactobacillus* spp. (right) colonies isolated from the stool of healthy mice. The numbers correspond to each mouse in the experimental group (n=10). The first 2 lines are 100bp and 1kb ladders, respectively.

2.3.2 *In vitro* tests demonstrate a potential probiotic effect of the isolated strains

Prior to testing the effects of the strains isolated *ex vivo*, we first sought to verify their probiotic potential using established *in vitro* tests such as their resistance to adverse gastrointestinal conditions (i.e. low pH, presence of pepsin and pancreatin enzymes, and tolerance to bile salts), and their antibiotic susceptibility. All the 30 selected strains in the previous analysis were classified as Gram-positive bacteria and catalase-negative, confirming the usual characteristics of both target bacteria genera. Furthermore, we observed that all strains showed high tolerance to the adverse conditions of the in vitro test (Table 2.1). More specifically, the selected strains were able to resist low pH levels, the presence of digestive enzymes, and bile acids, with a maximum reduction of 1.4 $\log_{10}$ CFU/mL in their population. Moreover, all strains maintained their population above 8 $\log_{10}$ CFU/mL after the incubation period in the gastric and intestinal simulated solutions.

Table 2.1. Population of the strains exposed to simulated gastrointestinal solutions

Animal	Strain	log_{10}CFU/mL				
		0h	2h	6h	Gram	Catalase
	Bifidobacterium spp.	8.85±0.02	8.76±0.04	8.10±0.10	+	−
1	*Bifidobacterium* spp.	8.97±0.01	8.44±0.02	8.12±0.11	+	−
	Lactobacillus spp.	9.96±0.01	9.35±0.04	9.19±0.08	+	−
	Bifidobacterium spp.	9.18±0.08	8.95±0.03	8.18±0.15	+	−
2	*Bifidobacterium* spp.	9.99±0.01	8.64±0.12	8.60±0.05	+	−
	Lactobacillus spp.	10.17±0.08	9.62±0.02	9.35±0.04	+	−
	Bifidobacterium spp.	9.35±0.04	9.11±0.02	8.55±0.06	+	−
3	*Bifidobacterium* spp.	8.96±0.03	8.55±0.06	8.12±0.11	+	−
	Lactobacillus spp.	9.62±0.02	9.40±0.06	8.91±0.04	+	−
	Bifidobacterium spp.	8.96±0.03	8.72±0.02	8.38±0.09	+	−
4	*Bifidobacterium* spp.	9.65±0.03	9.34±0.09	8.85±0.02	+	−
	Lactobacillus spp.	10.43±0.06	9.97±0.01	9.85±0.05	+	−
	Bifidobacterium spp.	8.89±0.03	8.10±0.10	8.07±0.03	+	−
5	*Bifidobacterium* spp.	9.96±0.02	9.12±0.01	8.95±0.01	+	−
	Lactobacillus spp.	9.48±0.06	8.89±0.03	8.06±0.07	+	−
	Bifidobacterium spp.	9.17±0.09	8.95±0.01	8.55±0.06	+	−
6	*Bifidobacterium* spp.	9.84±0.04	9.40±0.06	9.34±0.09	+	−
	Lactobacillus spp.	9.34±0.09	8.91±0.04	8.55±0.06	+	−
	Bifidobacterium spp.	8.76±0.07	8.38±0.09	8.07±0.03	+	−
7	*Bifidobacterium* spp.	9.66±0.01	9.36±0.08	8.85±0.02	+	−
	Lactobacillus spp.	9.96±0.02	9.68±0.06	8.77±0.05	+	−
	Bifidobacterium spp.	8.85±0.02	8.44±0.02	8.24±0.08	+	−
8	*Bifidobacterium* spp.	9.41±0.04	9.11±0.01	8.89±0.03	+	−
	Lactobacillus spp.	9.12±0.01	8.60±0.05	8.10±0.10	+	−
	Bifidobacterium spp.	8.97±0.01	8.64±0.12	8.31±0.07	+	−
9	*Bifidobacterium* spp.	8.34±0.04	8.21±0.06	8.06±0.02	+	−
	Lactobacillus spp.	8.89±0.03	8.55±0.06	8.06±0.07	+	−
	Bifidobacterium spp.	9.41±0.04	8.98±0.01	8.74±0.03	+	−
10	*Bifidobacterium* spp.	8.91±0.04	8.72±0.02	8.60±0.05	+	−
	Lactobacillus spp.	9.65±0.02	9.61±0.08	8.63±0.02	+	−

0h= baseline CFU counts before the *in vitro* gastrointestinal test; 2h= after the gastric phase; 6h= after the intestinal phase. +: gram-positive bacteria; −: catalase negative. Data for survival rates are presented as means ± SD of triplicates.

Ten antibiotics from different classes and with diverse mechanisms of action were tested to assure the safety of the commensals isolated from the mice, thus guaranteeing they could be eliminated by antibiotic use if necessary. The antibiotic susceptibility of each strain is presented in Table 2.2 as the zone diameter values for each antibiotic investigated. The results show that all the evaluated strains were resistant to the antibiotic aztreonam (30 μg). In contrast, all evaluated strains proved susceptible to the other antibiotics tested, as evidenced by the inhibition halo formed around each disk (Supplemental Figure A1).

Table 2.2. Zone diameter values to indicate susceptible, intermediate, and resistance breakpoints of each strain.

Animal	Strains	CRO	IPM	ATM	ERI	VAN	CLO	TET	NIT	NOR	CIP
	Bifidobacterium spp.	29.0	27.0	9.0	32.0	23.0	32.0	37.0	25.0	23.0	26.0
1	*Bifidobacterium* spp.	20.0	29.0	0.0	35.0	25.0	34.0	39.0	25.0	26.0	27.0
	Lactobacillus spp.	25.0	25.0	0.0	33.0	27.0	32.0	34.0	29.0	24.0	25.0
	Bifidobacterium spp.	27.0	31.0	0.0	35.0	25.0	30.0	35.0	26.0	29.0	30.0
2	*Bifidobacterium* spp.	26.0	30.0	0.0	31.0	23.0	32.0	32.0	20.0	21.0	23.0
	Lactobacillus spp.	25.0	34.0	0.0	32.0	29.0	30.0	32.0	28.0	25.0	28.0
	Bifidobacterium spp.	29.0	32.0	0.0	34.0	22.0	32.0	35.0	25.0	23.0	24.0
3	*Bifidobacterium* spp.	28.0	33.0	0.0	33.0	26.0	32.0	40.0	22.0	24.0	26.0
	Lactobacillus spp.	27.0	29.0	0.0	30.0	24.0	30.0	35.0	27.0	29.0	21.0
	Bifidobacterium spp.	25.0	29.0	11.0	35.0	25.0	31.0	27.0	24.0	27.0	27.0
4	*Bifidobacterium* spp.	18.0	28.0	0.0	36.0	27.0	38.0	40.0	24.0	26.0	29.0
	Lactobacillus spp.	23.0	25.0	0.0	31.0	26.0	31.0	31.0	25.0	28.0	23.0
	Bifidobacterium spp.	28.0	28.0	2.0	33.0	25.0	35.0	39.0	25.0	25.0	28.0
5	*Bifidobacterium* spp.	28.0	32.0	0.0	36.0	25.0	32.0	35.0	25.0	27.0	30.0
	Lactobacillus spp.	30.0	30.0	0.0	34.0	24.0	32.0	32.0	25.0	26.0	24.0
	Bifidobacterium spp.	26.0	27.0	0.0	39.0	26.0	31.0	35.0	25.0	25.0	29.0
6	*Bifidobacterium* spp.	28.0	32.0	0.0	35.0	25.0	32.0	36.0	24.0	27.0	30.0
	Lactobacillus spp.	31.0	26.0	0.0	36.0	24.0	30.0	34.0	24.0	24.0	32.0
	Bifidobacterium spp.	20.0	30.0	0.0	36.0	26.0	37.0	40.0	26.0	26.0	30.0
7	*Bifidobacterium* spp.	26.0	30.0	1.0	37.0	26.0	32.0	36.0	23.0	27.0	30.0
	Lactobacillus spp.	25.0	25.0	0.0	37.0	23.0	30.0	35.0	23.0	29.0	30.0
	Bifidobacterium spp.	29.0	30.0	0.0	34.0	27.0	39.0	36.0	23.0	26.0	29.0
8	*Bifidobacterium* spp.	26.0	31.0	1.0	37.0	27.0	31.0	37.0	28.0	26.0	29.0
	Lactobacillus spp.	27.0	32.0	0.0	37.0	25.0	30.0	39.0	27.0	27.0	32.0
	Bifidobacterium spp.	30.0	36.0	0.0	37.0	26.0	31.0	36.0	24.0	26.0	30.0
9	*Bifidobacterium* spp.	16.0	30.0	0.0	39.0	27.0	35.0	41.0	21.0	26.0	29.0
	Lactobacillus spp.	29.0	26.0	0.0	36.0	25.0	33.0	39.0	23.0	23.0	31.0
	Bifidobacterium spp.	25.0	28.0	8.0	36.0	25.0	31.0	37.0	25.0	27.0	30.0
10	*Bifidobacterium* spp.	25.0	29.0	9.0	37.0	27.0	30.0	34.0	25.0	26.0	29.0
	Lactobacillus spp.	27.0	25.0	0.0	35.0	26.0	34.0	33.0	28.0	24.0	31.0

CRO=ceftriaxone 30 µg, IPM=imipenem 10 µg, ATM=aztreonam 30 µg, ERI=erythromycin 15 µg, VAN=vancomycin 30 µg, CLO=chloramphenicol 30 µg, TET=tetracycline 30 µg, NIT=nitrofurantoin 300 µg, NOR=norfloxacin 10 µg e CIP=ciprofloxacin 5 µg. All values are expressed in millimeters (mm).

2.3.3 Personalized commensal strains protect mice against acute dextran sodium sulfate-induced colitis

To determine if the isolated strains had the potential to be considered probiotic bacteria, we conducted animal experiments using a well-characterized mouse model of intestinal inflammation (DSS-induced colitis). This model was chosen for its wide application in testing new probiotic strains for use in the GI tract (116,117,223). Fresh cultures of the isolated strains, as well as the probiotic LGG, were prepared every two days in their respective media, and an aliquot was plated to perform CFU counts. All the cultures remained above 9 $\log_{10}$CFU throughout the experimental protocols (Supplemental Table A1). The weekly average population of the commercial strain *L. rhamnosus* GG was 9.34 $\log_{10}$CFU and 9.74 $\log_{10}$CFU in the first and the second week of study, respectively (Supplemental Figure A2).

Mice were given equal quantities of either LGG, the PP, or sterile water via oral gavage 7 days prior to being challenged with 3% DSS in their drinking water, and then every day thereafter until they were euthanized at day 7. As expected, DSS-colitic mice given just water lost significant levels of weight (15%), whereas mice treated with LGG or PP lost much less weight (5%) during DSS exposure. Notably, PP mice showed a significantly lower disease activity index (DAI) as compared to healthy controls, beginning at the 5^{th} day of DSS challenge and lasting until mice were euthanized, indicating a better outcome with PP regarding the parameters of stool consistency and blood in the stool (Figure 2.5). Besides weight loss and the DAI score, mice treated with the PP presented fewer clinical signs of morbidity (i.e. hunched posture, no activity, riffled fur) and appeared active and healthy throughout the experiment. These results suggest that the PP treatment was more effective than LGG at reducing the susceptibility of mice to DSS colitis. As expected,

healthy mice from the control group continued to gain weight and remained healthy throughout the entire experimental protocol.

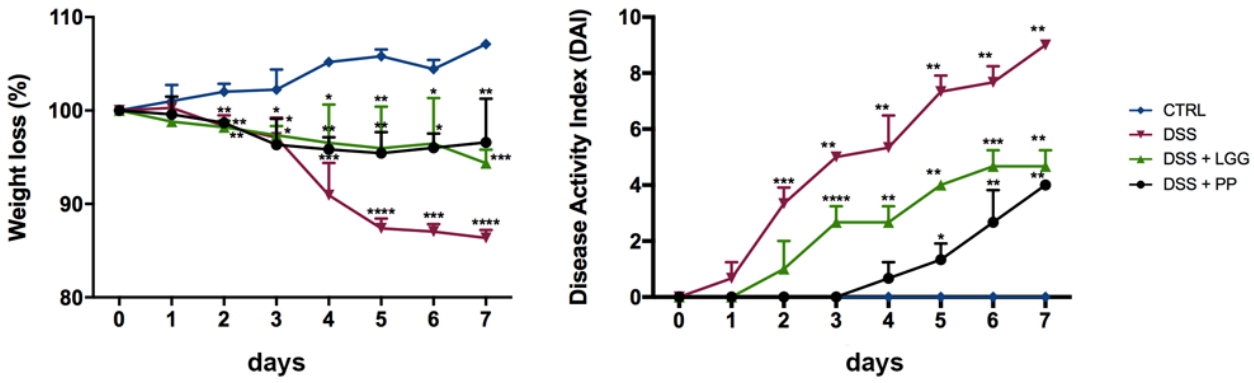


Figure 2.5. Disease activity index (DAI) during the course of DSS-induced colitis.
CTRL: healthy mice; DSS: 3% DSS with no intervention; DSS + LGG: 3% DSS + LGG prior and during DSS; DSS + PP: 3% DSS + PP prior and during DSS. Significant differences as compared to CTRL group were identified using ANOVA and Dunnett's as a post-hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Histologically, DSS treated mice (positive control) developed severe mucosal damage in their distal colons, characterized by the widespread loss of crypts, severe ulceration, and infiltration of inflammatory cells (Figure 2.6). In contrast, the distal colons of PP mice showed only minimal signs of tissue damage with histopathological scoring revealing well-preserved crypt structures, decreased numbers of inflammatory cells, and significantly less tissue damage in comparison with the vehicle-treated mice, scoring 2.8 ± 0.2 versus 10.0 ± 0.5 ($p < 0.05$; n=10 per group) (Figure 7). Mice that received the commercial probiotic LGG also demonstrated less histological damage in comparison with non-treated colitic mice; however, there were still large numbers of inflammatory cells in their colons, indicating a greater severity in their colitis and less protection as compared to the personalized probiotic strains (pathology score 6.0 ± 0.3 versus 2.8 ± 0.2 ($p < 0.05$; n=6-7 per

group) (Figure 2.7). Taken together, our assessments confirm that the PP effectively protects the mammalian GI tract during DSS colitis and to a greater extent than that seen with the commercial probiotic LGG.

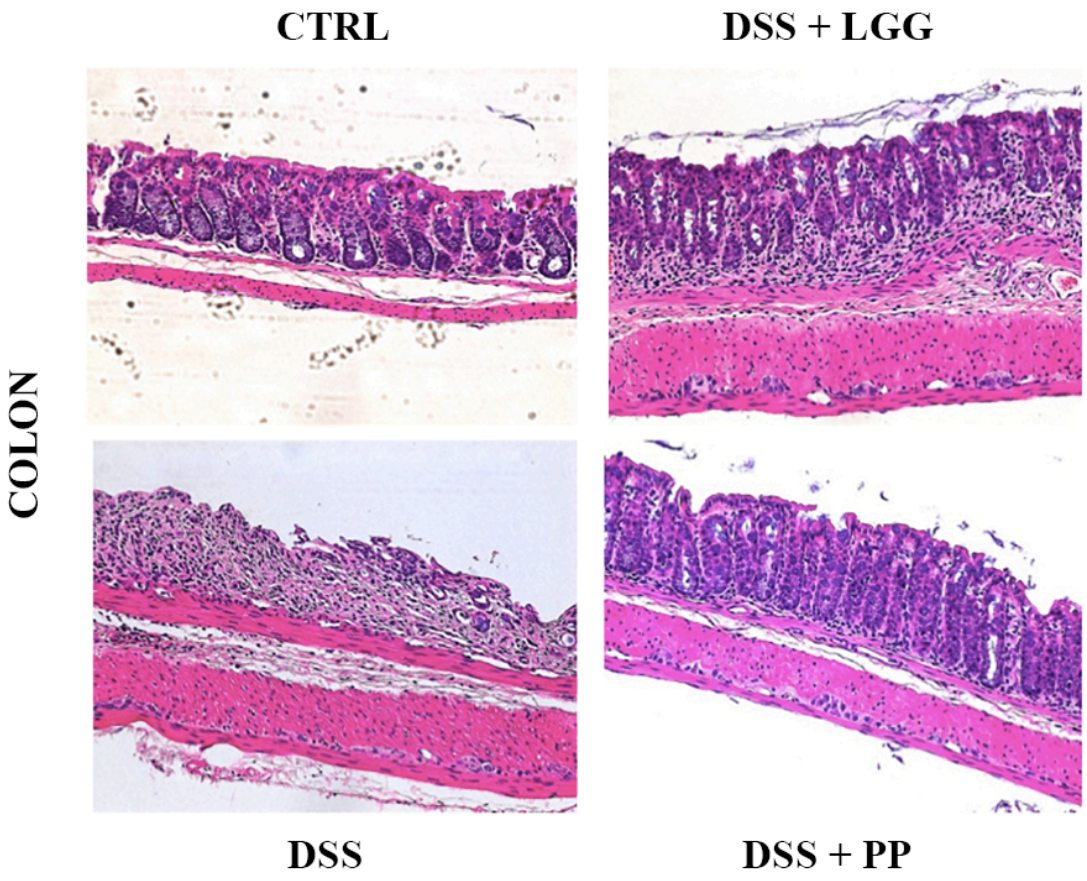


Figure 2.6. Representative photomicrographs of mouse distal colon sections stained with haematoxylin and eosin.
CTRL: healthy mice; DSS: 3% DSS with no intervention; DSS + LGG: 3% DSS + LGG prior and during DSS; DSS + PP: 3% DSS + PP prior and during DSS. (200x magnification).

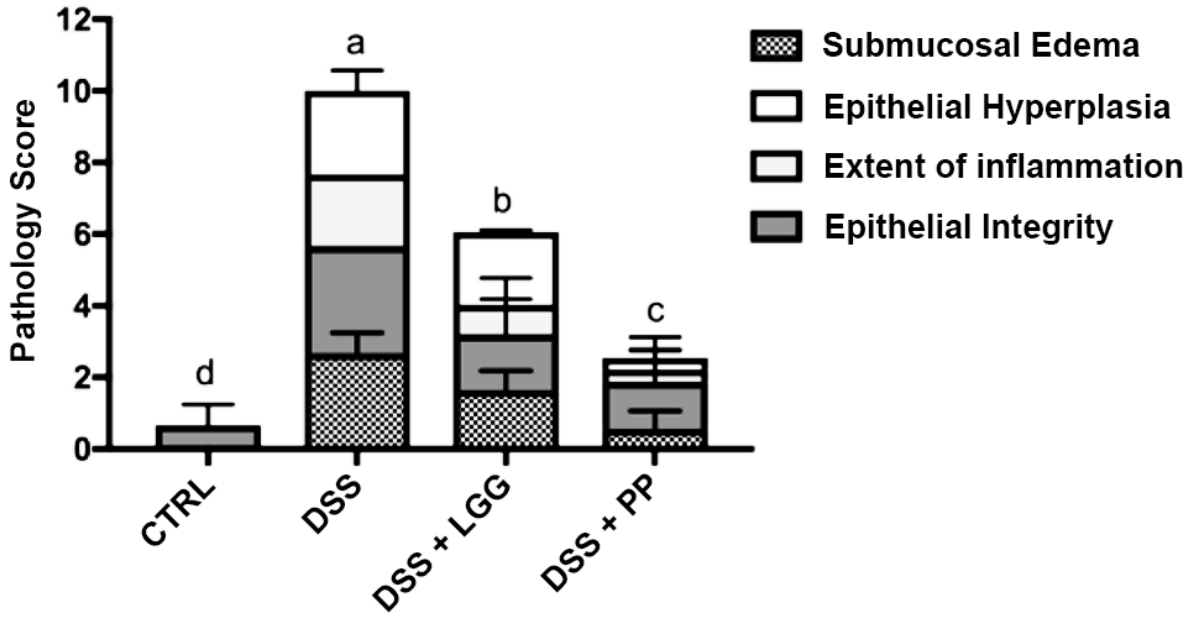


Figure 2.7. Histopathology score.
Histopathological scores as assessed by 3 individuals blinded to the identity of the groups in the study. Data are presented as mean ± SD. Different letters indicate statistical difference between groups using ANOVA and Tukey as a post-test ($p<0.05$).

2.3.4 Personalized probiotic therapy positively modulates the host immune response during DSS-colitis

The transcription of different cytokines was investigated to evaluate if the personalized probiotic therapy was more effective in suppressing inflammatory responses, thereby contributing to tissue homeostasis. The cytokine mRNA levels were measured in the distal colon since the histological damage was limited to this tissue area in all groups. Figure 2.8 shows the relative expression of the pro-inflammatory cytokines (*Il-1β* and *Il-6*), as well as the anti-inflammatory cytokines (*Il-10* and *TGF-β*) after seven days of DSS-colitis. All results are expressed as the fold-change over the gene expression in the control group. As expected, the transcript levels of *Il-6* and *Il-1β* were

higher in the DSS-vehicle treated group, confirming the more severe inflammation found through histological analysis. Moreover, both groups treated with probiotics showed evidence of attenuated inflammation, as their tissues showed lower transcript levels of *Il-6* and *Il-1β* and higher levels of *Il-10* and *TGF-β* as compared to vehicle treated mice. However, the most striking result from data in Figure 8 was the cytokine mRNA expression comparing the two probiotics treated groups (LGG and PP). The PP treatment was clearly more effective in increasing anti-inflammatory responses and in suppressing the elevated expression of pro-inflammatory cytokines as compared to the commercial probiotic LGG ($p<0.05$).

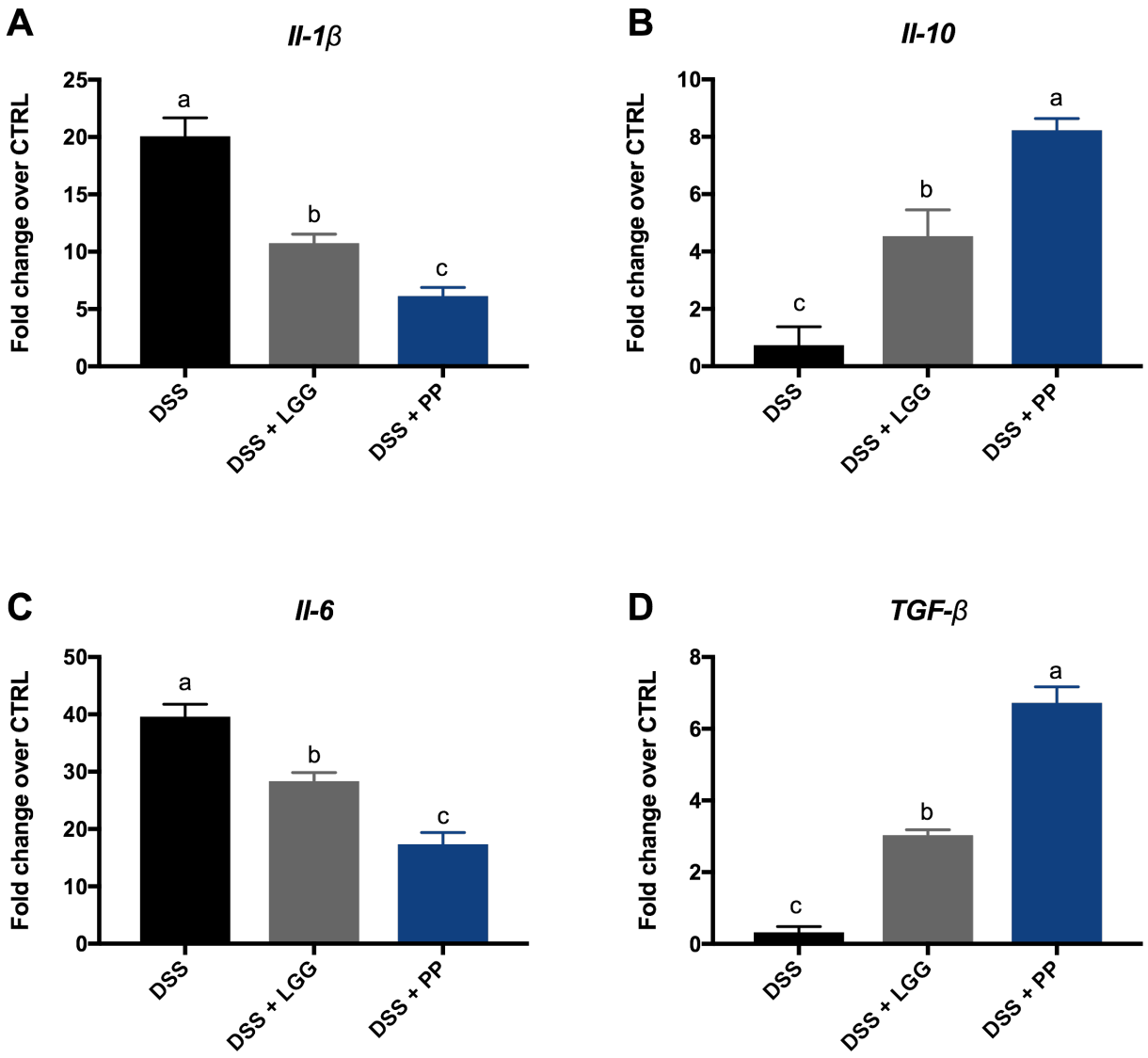


Figure 2.8. Pro-inflammatory and anti-inflammatory cytokines.
Effect of LGG and PP on the mRNA levels of pro-inflammatory and anti-inflammatory cytokines in mice with DSS-colitis. A) Expression of *Il-1β* in the distal colon after 7 days of DSS treatment. DSS+PP group showed the lowest levels of *Il-1β,* which were significantly different from both the DSS and DSS+LGG groups. B) Expression of *Il-10* in the distal colon after 7 days of DSS treatment. DSS+PP group showed the highest levels of *Il-10,* which were significantly different from both the DSS and DSS+LGG groups. C): Expression of *Il-6* in distal colon after 7 days of DSS treatment. DSS+PP group showed the lowest levels of *Il-6,* which were significantly different from both DSS and DSS+LGG groups. C) Expression of *TGF-β* in the distal colon after 7 days of DSS treatment. DSS+PP group showed the highest levels of *TGF-β,* which were significantly different from both the DSS and DSS+LGG groups. DSS: 3% DSS with no intervention; DSS + LGG: 3% DSS + LGG prior and during DSS; DSS + PP: 3% DSS + PP prior and during DSS. Different letters indicate statistical difference using ANOVA and Tukey as a post-test ($p<0.05$).

Figure 2.9 shows the expression of MPO and MDA in distal colon tissues after seven days of DSS-colitis, with all results normalized to the control group data (as the baseline). As shown in Figure 9, the lowest levels of MPO in colitic mice were observed in the group that received the PP therapy. Although the LGG group presented lower absolute levels of MPO, their levels did not reach statistical significance as compared to the DSS-vehicle treated group ($p<0.05$). Regarding MDA level in colonic tissues, there was no significant different within the groups, indicating that neither probiotic tested influenced this byproduct of oxidative stress.

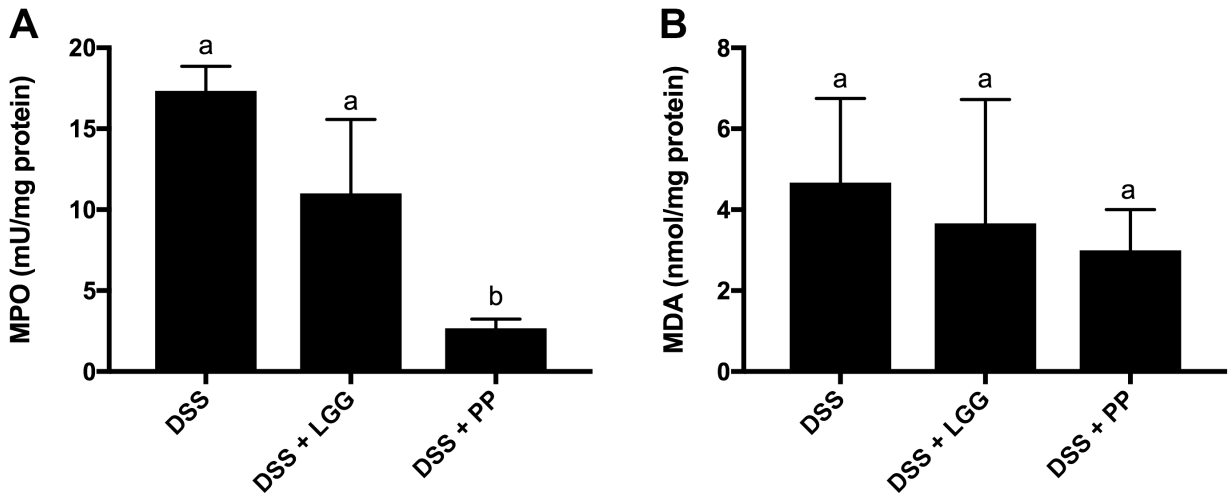


Figure 2.9. Colonic expression of MPO and MDA.
A) MPO levels in colonic tissues after 7 days of DSS treatment. PP group showed levels of MPO that were significantly lower than the DSS and DSS+LGG groups. B) MDA levels in colonic tissues after 7 days of DSS. No significant difference between groups for MDA levels. CTRL: healthy mice; DSS: 3% DSS with no intervention; DSS + LGG: 3% DSS + LGG prior and during DSS; DSS + PP: 3% DSS + PP prior and during DSS. Different letters indicate statistical difference using ANOVA and Tukey as a post-test ($p<0.05$).

2.4 Discussion

At present, it remains unclear if the microbial dysbiosis seen in IBD patients plays a role in their disease pathogenesis, or if it is simply secondary to the inflammation that develops in these conditions. A potential causative role for dysbiosis is supported by studies showing that spontaneous mouse models of IBD often show no disease development when the mice are raised under germ-free conditions. Moreover, the predominant inflamed sites in IBD patients are also the sites that hold the highest abundance of microbes in the intestine (distal ileum and colon). In this case, IBD could be explained as an impaired immune response to gut microbes in genetically-predisposed individuals. However, an inflammatory environment itself seems to favor an expansion of *Enterobacteriaceae* and a depletion of *Firmicutes* bacteria, which curiously are the typical features of the microbial dysbiosis observed in IBD patients (25).

Although the microbiota imbalance seen in the stool of IBD patients has yet to be defined as either the cause or a consequence in IBD, it almost certainly plays a role in these conditions, as IBD patients usually show a significant decrease in commensal bacteria diversity. A healthy and diverse microbiota is important for several intestinal physiological processes, such as protection against pathogens and pathobionts, food digestion, and vitamin biosynthesis, as well as the production of key metabolites and anti-inflammatory mediators such as transforming growth factor-β (TGF-β), retinoic acid, and thymic stromal lymphopoietin (TSLP) (25). Importantly, in the absence of a noxious stimulus, a balanced and diverse microbiota keeps the immune system in a hypo-responsive state, thus influencing both host metabolism and immunity. Therefore, developing new

approaches to positively modulate the gut microbiota and maintain bacterial diversity offer the potential to act as therapies for dysbiosis-related diseases.

Our work demonstrates that a personalized probiotic therapy can protect mice against DSS-induced colitis to a greater extent than that seen with the commercial probiotic LGG, and this better outcome – if replicated in clinical studies – could make a tremendous difference to the treatment of severe diseases such as IBD, IBS, type 2 diabetes, among others. Although probiotic bacteria are thought to benefit hosts through several mechanisms of actions, the ability to persistently colonize the gut may be critical for the ability of probiotics to effectively treat dysbiosis-related diseases. Because the human microbiome is a complex and dynamic ecosystem, and dysbiosis is linked to diverse diseases, we believe that beneficial bacteria need to be present in the gut for an extended period, or preferably in a permanent way, to avoid the bacterial imbalance that triggers dysbiosis. However, the majority of commercially-available probiotics appear unable to accomplish this task. Recent findings outline that a combination of 11 probiotic strains (Superherb Bio-25) varies considerably in their ability to colonize the human gut, depending on the host's indigenous microbiome, as well as on host factors and site-specific immune responses (224), thus highlighting the need for new, personalized approaches. Thus, delivering large numbers of endogenous commensal bacteria to the gut after their propagation *ex vivo* could represent a more promising strategy for serious microbiota-related diseases, since these commensals are already incorporated in the host's microbiota, and will thus more readily colonize the host gut where they can – for example - help promote colonization resistance against invading pathogens/pathobionts.

In our study, we first confirmed that the selected strains isolated from each individual mouse could be considered potentially probiotic due to their performance in preliminary screens using in vitro tests. RAPD-PCR is a practical method that provides both sensitive and rapid results. For this reason, it has been widely used in the differentiation of lactic acid bacteria (225–228). It is worth noting that this step was performed to confirm the bacterial genera, as well as select different species from *Lactobacillus* spp. and *Bifidobacterium* spp. to be administered prior to - and during DSS challenge. However, the exact identification of each particular strain was not the focus of our study, since our current aim was to develop a personalized approach for probiotic administration focusing on the healthy microbiota composition prior to inflammation, rather than isolating particular strains to be commercialized for general health benefits. The results from this part of the study indicated that three commensals (two of *Bifidobacterium* spp. and one of *Lactobacillus* spp.), isolated from each animal in the PP group had the potential to be used as probiotic microorganisms as they were able to survive in adverse conditions, and therefore, they were administered to the donor animal in the second part of the study (animal experiment).

High survival rates during gastrointestinal transit is one of the key requirements to classify a commensal microbe as a potential probiotic bacterium (83,199), as the probiotic needs to be acid and bile resistant to exert its beneficial effects in the colon, when dealing with microbial dysbiosis-related diseases (25). Another important feature when selecting new probiotic strains is to evaluate their susceptibility to the antibiotics commonly used in antimicrobial therapy. Although negative events following the administration of commensal microbial strains are extremely uncommon, antibiotic resistance of each strain is important to guarantee that the bacterium could be easily removed in case of bacterial translocation, and consequently – in extremely rare circumstances –

systemic infection. Considering that we focused on endogenous bacteria, there are fewer risks of complications and negative effects than with commercial microbes. However knowledge about the antimicrobial susceptibility of the isolates also helps our characterization of the personalized strains, as the classification could be used in the future for stratification purposes. Resistance of bacteria to antibiotics may be intrinsic or acquired as a result of a chromosomal mutation or by horizontal gene transfer (229). *Lactobacillus* spp. are usually susceptible to β-lactam antibiotics (greater sensitivity to penicillins and less to cephalosporins), protein synthesis inhibitors (chloramphenicol, macrolides and tetracycline), and more resistant to vancomycin (extrinsic resistance). Strains from *Bifidobacterium* spp. are usually intrinsically resistant to mupirocin (an antibiotic used in selective media for *Bifidobacterium* spp.) and high concentrations of aminoglycosides. On the other hand, they are sensitive to macrolides (erythromycin, aztreonam), chloramphenicol, β-lactams, vancomycin, streptomycin, and rifampicin (230,231). All the strains selected to be administered in the PP group were resistant to the antibiotic aztreonam; however, this does not represent a safety issue in itself, considering that intrinsic antibiotic resistance in probiotic strains could actually be useful for restoring the diverse microbiome after antibiotic treatment (229).

In the second part of this study, we compared the ability of the PP isolated from the host against the ability of the commercial probiotic LGG to reduce the inflammatory characteristics of DSS-induced colitis. The DSS-colitis model is one of the most-widely used mouse models of intestinal inflammation due its simplicity, reproducibility, and controllability (232,233). DSS is a sulfated polysaccharide that cause colitis by disrupting the colonic epithelium and allowing the passage of luminal bacteria and associated antigens into the mucosa. This activates an inflammatory process

in the underlying tissues, resulting in clinical and histopathological features similar to those seen in IBD patients. These include weight loss, diarrhea, occult blood in stools, mucin depletion, loss of epithelial crypt architecture, and neutrophil infiltration (232). In our study, the groups that received DSS in their drinking water showed typical signs of colitis characterized by body weight loss, diarrhea, occult blood in their stools, and piloerection. However, probiotic administration before and during DSS exposure was able to reduce these signs, especially in the group receiving the PP. Furthermore, histological changes were attenuated in the PP group as compared to the mice receiving the commercial probiotic LGG, suggesting that the proposed personalized strategy was more effective in decreasing DSS-colitis. We strongly believe that the provision of endogenous commensal bacteria protected the host against the inflammation caused by DSS, and helped maintain gut homeostasis by replenishing the numbers of endogenous commensal bacteria in the gut after their ex vivo propagation.

Under homeostatic conditions, the intestinal mucosa is able to maintain the balance between pro-inflammatory and anti-inflammatory cytokines. However, IBD patients often display increased intestinal permeability and impaired epithelial barrier function (234–237), leading to elevated levels of pro-inflammatory cytokines such TNF-α, IFN-γ, interleukin (IL)-1, IL-6, and IL-12 (238). Similar increases in pro-inflammatory mediators are also observed in the DSS-colitis model correlating well with clinical parameters (232). Consistent with the known anti-inflammatory effects of some probiotic strains (239–242), we found that both probiotic strategies (PP and LGG) significantly reduced mRNA levels of *Il-1β* and *Il-6* in the colons of treated mice, which were accompanied by increased anti-inflammatory cytokine expression (*Il-10* and *TGF-β*). Interestingly, the personalized probiotic group showed a more effective immune modulation as

compared to the commercial probiotic LGG, suggesting that resident microbes of the gut may yield stronger anti-inflammatory effects since they already possess a mutualistic anti-inflammatory relationship with the host's immune system.

Certain biochemical markers, such as myeloperoxidase (MPO) and malonaldehyde (MDA) activity, have also been investigated in IBD and in animal models of intestinal inflammation as parameters of intestinal damage. MPO is commonly used as a neutrophil marker, while MDA has been studied as a oxidative stress marker associated with the pathogenesis of IBD (243–245). As expected based on previous studies (246,247), we found higher levels of MPO in all groups challenged with DSS in comparison with healthy mice. However, MPO levels in the DSS + PP group were significantly reduced as compared to both the non-treated group and the group that received the commercial probiotic LGG. These results support the idea of personalized probiotics as better candidates to protect against DSS-colitis, at least in part due to their ability to reduce neutrophil infiltration. MDA levels were not significantly different between the groups that received DSS, suggesting that neither probiotic was able to attenuate the typical oxidative stress caused by inflammation.

Based on our results, commensal bacteria isolated from the host microbiota were more effective at preventing the symptoms and pathological changes seen in the DSS-colitis model. The improved efficacy of the personalized treatment in comparison with the commercial probiotic LGG suggests the benefits of using microorganisms that are already incorporated into the host microbiota to reduce the typical dysbiosis seen in DSS treatment, as well as other forms of colitis, since the personalized strategy is based on the maintenance of the initial healthy microbiota by constantly

replenishing the host with these beneficial microbes. We believe that administration of these personalized probiotics has the potential to treat dysbiotic-related and multifactorial diseases based mainly on maintaining the quantitative balance of beneficial versus pathobiont and/pathogenic microbes. Moreover, we recognize that commercial probiotics, as well as novel engineered bacteria expressing specific transgenes, could prove effective in diseases where the etiology and the mechanisms of disease are better elucidated. In additional, specific populations (e.g. infants, athletes) and their particular needs would also benefit from designed probiotics aiming at a specific beneficial effect.

The current evidence supporting personalized therapies are more focused on strategies to treat specific groups of subjects and avoid the generalist approach considered outdated for many disease treatments. Although we recognize the importance of developing novel probiotics for specific populations, we believe that certain diseases linked to microbial dysbiosis could benefit more from the use of personalized probiotics, which we have named “autoprobiotics”. The novelty of our approach includes not only selecting strains from the host in a personalized way, but also by selecting these strains before disease begins, or alternatively, in the remission phase in the case of IBD. As described in Figure 2.1, the prior isolation and selection of the strains leaves the opportunity to store the isolates and use them to treat any disease where microbial dysbiosis is thought to play a central role.

In summary, these results show that personalized probiotics are a potent and promising approach to healthcare, and as such, may protect against dysbiosis-related symptoms during clinical diseases. However, considering the novelty of the proposal, it is clear that additional studies need

to be performed using different models of intestinal and extra-intestinal diseases as well as different comparisons made with commercially available probiotics, before these personalized probiotics are ultimately tested in clinical trials. We emphasize that this strategy has the potential to assist in the treatment of several diseases associated with microbial dysbiosis.

Chapter 3: Role of the mucin (Muc)2 and its glycosylation in controlling susceptibility to *Citrobacter rodentium* infection

3.1 Introduction

Enteric bacterial pathogens are a major cause of diarrheal disease in developed as well as developing countries (248,249). Infection by enteric pathogens is known to promote GI inflammation along with intestinal pathology and pathophysiology, including not only diarrhea but also intestinal epithelial barrier dysfunction (126). Additionally, infections by enteric pathogens as well as impaired intestinal barrier function have been associated with IBD, although it remains unclear if the microbial community in the gut plays a causative or an aggravating role in IBD (25).

To infect their host, most enteric pathogens need to directly infect the intestinal epithelium. However, to do so, they must cross the overlying intestinal mucus layer. As previously discussed in Chapter 1, intestinal mucus is predominantly comprised of the mucin Muc2, a highly *O*-glycosylated protein with core 1 and core 3 derived *O*-glycans (in mice) as its primary constituents. We previously showed that mice lacking *Muc2* are highly susceptible to infection by *Citrobacter rodentium* (130), a mouse specific relative of the bacterial pathogen enterohemorrhagic *E. coli* (EHEC), with *Muc2* deficient ($^{-/-}$) mice carrying very high pathogen burdens as well as suffering severe intestinal inflammation and epithelial damage. Moreover, *Muc2*$^{-/-}$ mice are also more susceptible to *Salmonella* Typhimurium infection as compared to wide-type mice, indicating that the protective role of the mucin Muc2 is not limited to a singular virulence mechanism (217).

Interestingly, IBD patients often display a relatively thin mucus layer in affected regions of their intestines, as well as overt GC depletion in inflamed intestinal tissues (250). At present, it is not completely elucidated if the impaired mucus barrier is a primary abnormality in IBD patients or if it is simply a consequence of the intestinal inflammation in addition to the microbial dysbiosis typically found in the disease. Studies have shown that mice deficient in *Muc2* develop spontaneous colitis as they age (~12-16 weeks), correlating with increased interactions between gut bacteria and the intestinal mucosal surface, thus supporting a causative role for mucin dysfunction in IBD. Moreover, it has been described that some IBD patients and animal models of IBD develop overt defects in their mucus layer even prior to overt inflammation (251,252).

At present, despite the essential role of Muc2 in promoting intestinal homeostasis and mucosal protection, it is still unclear whether this protection reflects the actions of the Muc2 protein itself, its glycosylation, or both. The mucin-type *O*-glycans are localized around the mucin protein domain thus being intimately associated with the external environment. Therefore, based on the evidence that spontaneous colitis in *Muc2*$^{-/-}$ mice is directly associated with increased contact between microbes and the mucosal surface, the mucin-type *O*-glycans seem to influence in host diseases susceptibility as they are constantly interacting with the microorganisms present in the environment (171). Additionally, a similar spontaneous colitis phenotype to that seen in *Muc2*$^{-/-}$ mice is also described in mice lacking the core 1 glycosylation specifically in their IEC (IEC *C1galt1*$^{-/-}$) (171).

Core 1 and core 3-derived *O*-glycans are important components of colonic mucin glycosylation, whereas core 1-derived *O*-glycans are the most predominant *O*-glycans expressed in mouse tissues (171,253). IEC *C1galt1*$^{-/-}$ mice display a thinner inner mucus layer than normal and they suffer overt breaches in its structure as compared to IEC *C1galt1*$^{+/+}$ mice. The spontaneous colitis displayed at an older age by IEC *C1galt1*$^{-/-}$ mice resembles human UC (253), characterized by the infiltration of myeloid cells and the development of crypt abscesses especially in the distal colon and rectum. Although not developing spontaneous disease, mice deficient in core 3 derived *O*-glycans (C3GnT$^{-/-}$) showed higher susceptibility to DSS-colitis due their impaired mucosal barrier integrity (254).

The outer mucus layer contains an array of intestinal bacteria and the glycoproteins that comprise the mucus layer can be considered a potential carbon source for bacteria , although just a few bacteria display the mucolytic properties necessary to break down the complex mucus glycans (255). Besides the impaired mucus barrier, previous studies have shown that mice lacking the mucin *Muc2* also carry an aberrant gut microbiome (256), showing reduced levels of *Lactobacillus* spp. (177). Mechanistically, it remains uncertain how this dysbiotic microbiota influences disease susceptibility in *Muc2*$^{-/-}$ mice, but the SCFA butyrate appears to be involved as it is a major energy source for colonocytes and it may promotes mucosal healing (177). Moreover, butyrate seems to play a role in modulating mucin synthesis and release (257,258) as well as in modifying MUC gene expression in intestinal GC deprived of glucose (259).

The aim of this study was to explore the role of Muc2 glycosylation in providing host defense in mice challenged with the bacterial pathogen *C. rodentium*, by comparing the susceptibility of mice

lacking *Muc2* and *Core* 1 glycosylation with their respective littermate controls. We also tested tributyrin supplementation in these knockout mice to investigate its effect in the *C. rodentium* model, as a means to see if the susceptibility of the Muc2 -/- and IEC *C1galt1*$^{-/-}$ mice was due to an impairment in the ability of their commensal microbes to produce butyrate. We hypothesized that core 1–derived *O*-glycans play a key nutritional role within the GI tract, and are thus essential in preventing dysbiotic changes in the gut microbiome. Therefore, alterations in the Muc2 glycosylation pattern may be closely associated with the pathogenesis of common intestinal diseases, such as IBD and enteric infections.

3.2 Experimental procedures

3.2.1 Mice

Six to eight week old C57BL/6 and *Muc2*$^{-/-}$ mice were bred in-house at the BC Children's Hospital Research Institute (BCCHRI). Mice deficient in core 3 derived *O*-glycans (C3GnT$^{-/-}$) and intestinal epithelial cell (IEC) specific knockout mice IEC *C1galt1*$^{-/-}$ (core 1 β1,3-galactosyltransferase) (on the C57BL/6 and 129 genetic background, respectively) were generated in Dr. Lijun Xia's laboratory (University of Oklahoma) as previously described (253) and bred in the BCCHRI animal facility. Briefly, IEC *C1galt*$^{-/-}$ mice (lacking *C1galt1*$^{-/-}$ specifically in their IEC) were generated by crossing mice with loxP sites flanking *C1galt1* with an intestinal epithelium-specific Cre-expressing transgenic line (VillinCre mice). IEC *C1galt1*$^{+/+}$ mice were used as littermate controls in all experiments. Mice were kept in sterilized, filter-topped cages and fed autoclaved food and water while being routinely monitored and tested for common pathogens. The protocol employed in the experiments was approved by the University of British Columbia's Animal Care Committee (A15-0206) and was in direct accordance with guidelines provided by the Canadian Council on the Use of Laboratory Animals.

3.2.2 Bacterial strains, *Citrobacter rodentium* infection and tributyrin supplementation

For *C. rodentium* infection, mice were orally gavaged with 100 µL (~2.5 × 10^8 CFU) of wild-type *C. rodentium* DBS100. The culture was grown overnight with shaking at 200 rpm in Luria-Bertani (LB) broth at 37 °C. For tributyrin supplementation experiments, mice received 100 µL (~5g/kg) of tributyrin (97% FG) (Sigma-Aldrich, St. Louis, USA) or only glycerol as a control by oral gavage every other day starting on day 1 post *C. rodentium* infection. Mice from other groups were

mock treated with glycerol at the same timepoints. Tributyrin is a triglyceride composed of butyric acid and glycerol, that presents some advantages over butyrate supplementation as it is odourless and it is rapidly absorbed and chemically stable (260).

3.2.3 Tissue collection

Mice were anesthetized with isoflurane and euthanized by cervical dislocation at 6 days post-infection or after losing approximately 20% of their initial bodyweight and showing signs of significant morbidity (piloerection, hunching and/or less activity). For bacterial enumeration, gut and systemic tissues (colon, cecum, spleen, liver and mesenteric lymph nodes – MLN) as well as luminal contents (stool) were collected in pre-weighed 2 mL tubes containing 1 mL of phosphate buffered saline (PBS), pH 7.2, and metal beads (Qiagen, Hilden, Germany). Next, samples were homogenized in a Retch MM400 1/30 Htz homogenizer for 6 min and CFU counts were determined by serial dilutions on specific LB agar plates supplemented with 100 μg/mL streptomycin. Plates were incubated overnight at 37 °C and colony counts were normalized by the weight of the respective tissue to obtain CFU/gram results. For histology, colon and cecum tissues were fixed in 10% neutral buffered formalin (Fisher, USA) (24 h, R/T) and then transferred to 70% ethanol. Fixed tissues were embedded in paraffin, cut into 5 μm sections and routinely processed according to standard techniques by the Histology Core Facility at BCCHRI. Additionally, colon and cecum tissues were also stored in RNA later (Qiagen, Hilden, Germany) at -80 °C for further host response analysis by quantitative PCR.

3.2.4 Histopathological scoring

Colonic and cecal pathology were scored using a previously adapted scoring system (216). In brief,

hematoxylin and eosin (HE) stained slides were examined by three blinded observers. Tissue sections were assessed for submucosal edema (0 = no change; 1 = mild; 2 = moderate; and 3 = profound), epithelial hyperplasia (scored based on percentage above the height of the control where 0 = no change; 1 = 1–50%; 2 = 51–75%; and 3 = >75%), epithelial integrity (0 = no change; 1 = <10 epithelial cells shedding per lesion; 2 = 11–20 epithelial cells shedding per lesion; 3 = epithelial ulceration; and 4 = epithelial ulceration with severe crypt destruction) and neutrophil and mononuclear cell infiltration (0 = none; 1 = mild; 2 = moderate; and 3 = severe). The maximum score that could be obtained with this system was 15 points.

3.2.5 Short chain fatty acid analysis

Using gas chromatography, the fecal concentration of the three major SCFAs – acetate, propionate, and butyrate – was analyzed as previously described (261). Briefly, fresh stool pellets were collected from healthy mice before infection and then immediately frozen at −20°C until analysis. Fecal samples were homogenized in distilled water resulting in a 17% (w/w) suspension, which was further acidified with 5M HCl until pH 2-3 was reached. The suspension was centrifuged for 20 min at 5000 rpm and a 2-ethylbutyric acid solution was spiked into the supernatant at a final concentration of 1 mm. The supernatant was injected into a Thermo TG-WAXMS A GC column (Thermo Trace 1310, Fisher Scientific, Waltham, MA, USA), coupled to a flame ionization detector (Thermo Fisher Scientific, Waltham, MA, USA). The results were expressed as mmol of acetate, propionate and butyrate per kilogram of feces.

3.2.6 Statistical analysis

All results presented in this study are expressed as the mean value ± Standard Deviation (SD). Statistical analysis was performed using a two-tailed Student t test, with assistance from GraphPad Prism Software Version 7.0 (GraphPad Software, San Diego, California). Significance was declared when $p<0.05$.

3.3 Results

3.3.1 *Muc2* $^{-/-}$ mice show increased susceptibility to *C. rodentium* infection

As outlined previously, the mucin *Muc2* that makes up the intestinal mucus layer is known to protect the host against infectious and noxious stimuli. We confirmed this observation, finding that mice lacking *Muc2* exhibited heightened susceptibility to *C. rodentium* infection as compared to WT mice, demonstrating increased body weight loss (~15%) (Figure 3.1A) and increased pathogen burdens by day 6 PI (Figure 3.1B). Moreover, *Muc2*$^{-/-}$ mice displayed more severe clinical signs of morbidity such as hunched posture, piloerection and inactivity as compared to WT mice.

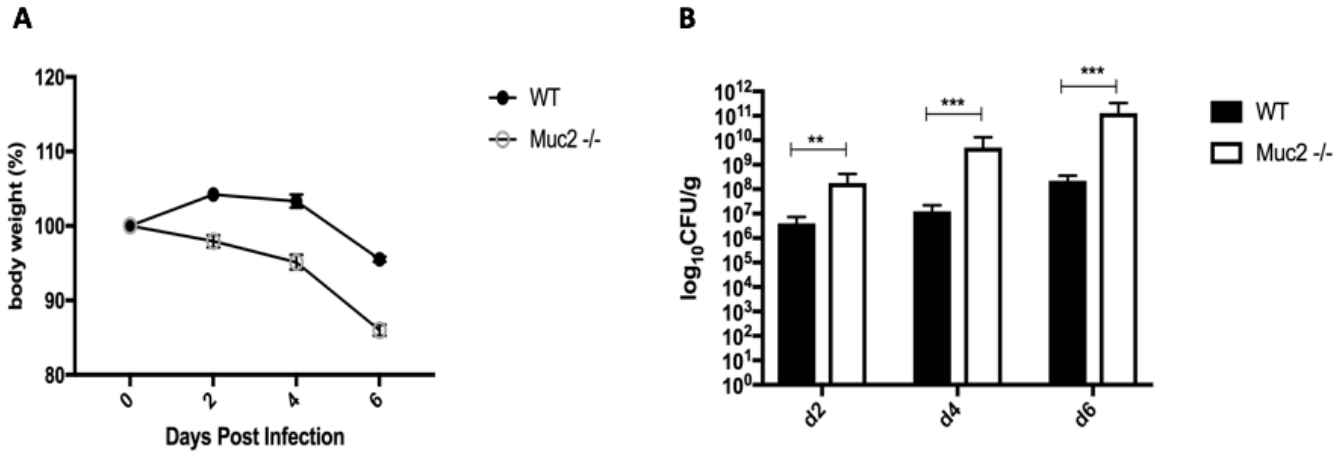


Figure 3.1 Muc2$^{-/-}$ mice exhibit dramatic susceptibility to *C. rodentium*-induced morbidity and mortality.
A. Body weights following *C. rodentium* infection of WT and *Muc2*$^{-/-}$ (n=12) mice. *Muc2*$^{-/-}$ mice rapidly lost weight following *C. rodentium* infection. Results are representative of 3 independent experiments.
B. Enumeration of *C. rodentium* in stool at various times post-infection. Bars represent the average of counts (n=12). Results are pooled from 3 separate infections. (2 DPI, **p<0.01; ***p<0.001, t-student test).

We also confirmed previous findings by our research group that together with higher bacterial burdens, *Muc2*$^{-/-}$ mice showed more severe colitis when infected with *C. rodentium* as compared

to WT mice. Over the course of infection, *Muc2*$^{-/-}$ mice developed progressive diarrhea and after euthanization, their colons were found to be thickened and reddish in color, containing loose stool rather than formed stool pellets. The macroscopic analysis also showed that the ceca of *Muc2*$^{-/-}$ mice were severely shrunken with 50% of them exhibiting focal ulcerations (Figure 3.2).

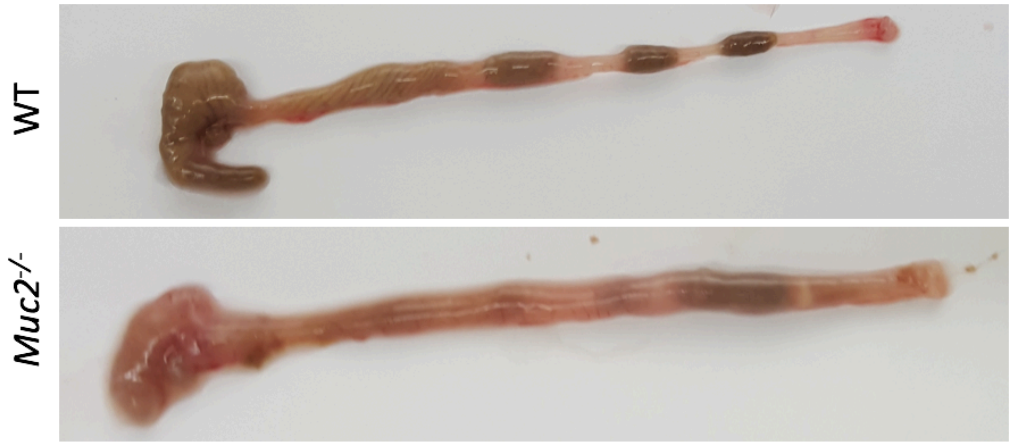


Figure 3.2. Macroscopic image of heightened mucosal damage suffered by *Muc2*$^{-/-}$ mice as compared to WT mice.
Resected large intestines and ceca of infected WT and *Muc2*$^{-/-}$ mice at 6 DPI. The cecum of the *Muc2*$^{-/-}$ mice is shrunken and severe inflamed, showing ulcerations and thickening of colonic tissue.

3.3.2 C3GnT$^{-/-}$ mice show modest susceptibility to *C. rodentium* similar to WT mice

Regarding the major glycosylations of the mucin *Muc2* and their protective role against infections, we found that mice lacking core 3 glycosylation were roughly similar to WT mice -showing only modest susceptibility to *C. rodentium* infection (Figure 3.3). Both their colons and ceca showed only mild histological damage with no significant difference in histopathological scores as compared to WT mice (Figure 3.4).

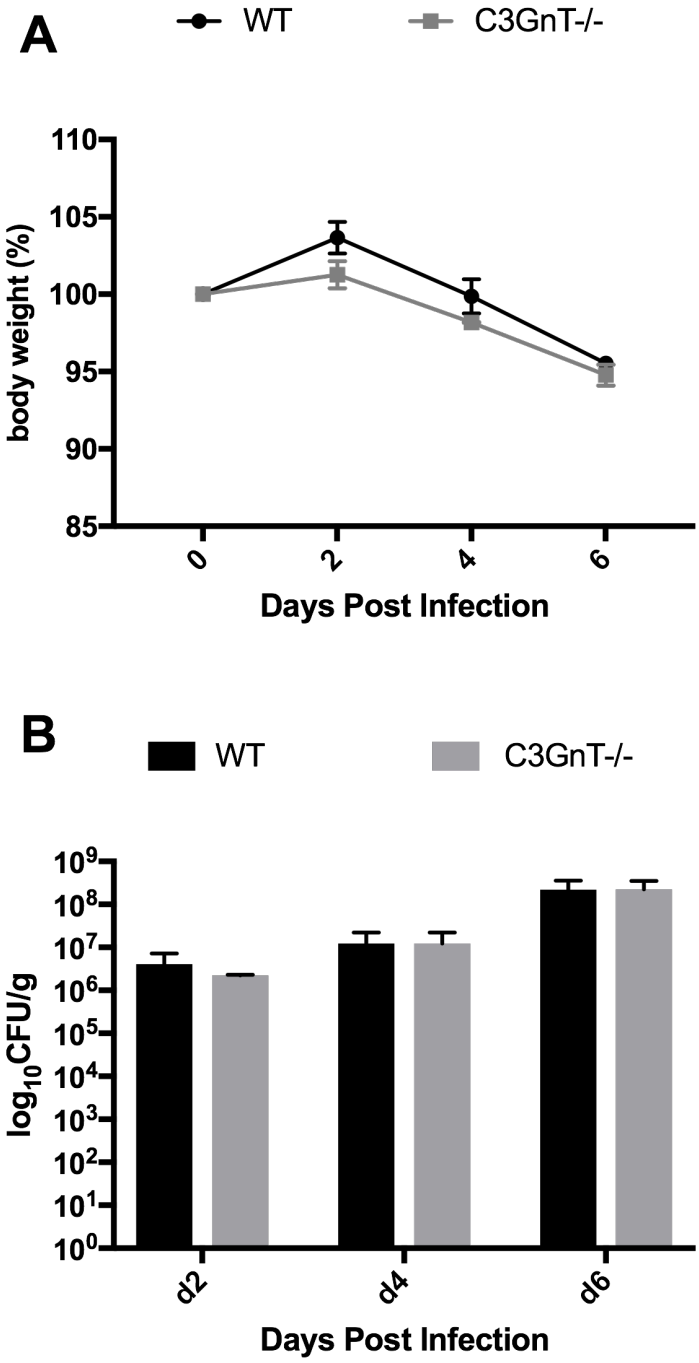


Figure 3.3. C3GnT$^{-/-}$ mice exhibit similar susceptibility to *C. rodentium* infection as WT mice.
A: C3GnT$^{-/-}$ and WT mice show similar weight loss (~5%) throughout the course of infection. **B**: C3GnT$^{-/-}$ and WT mice display similar *C. rodentium* colonization over the course of infection with no significant difference between the groups using t-student test. Bars represent the average of counts (n=12). Results are pooled from 3 separate infections.

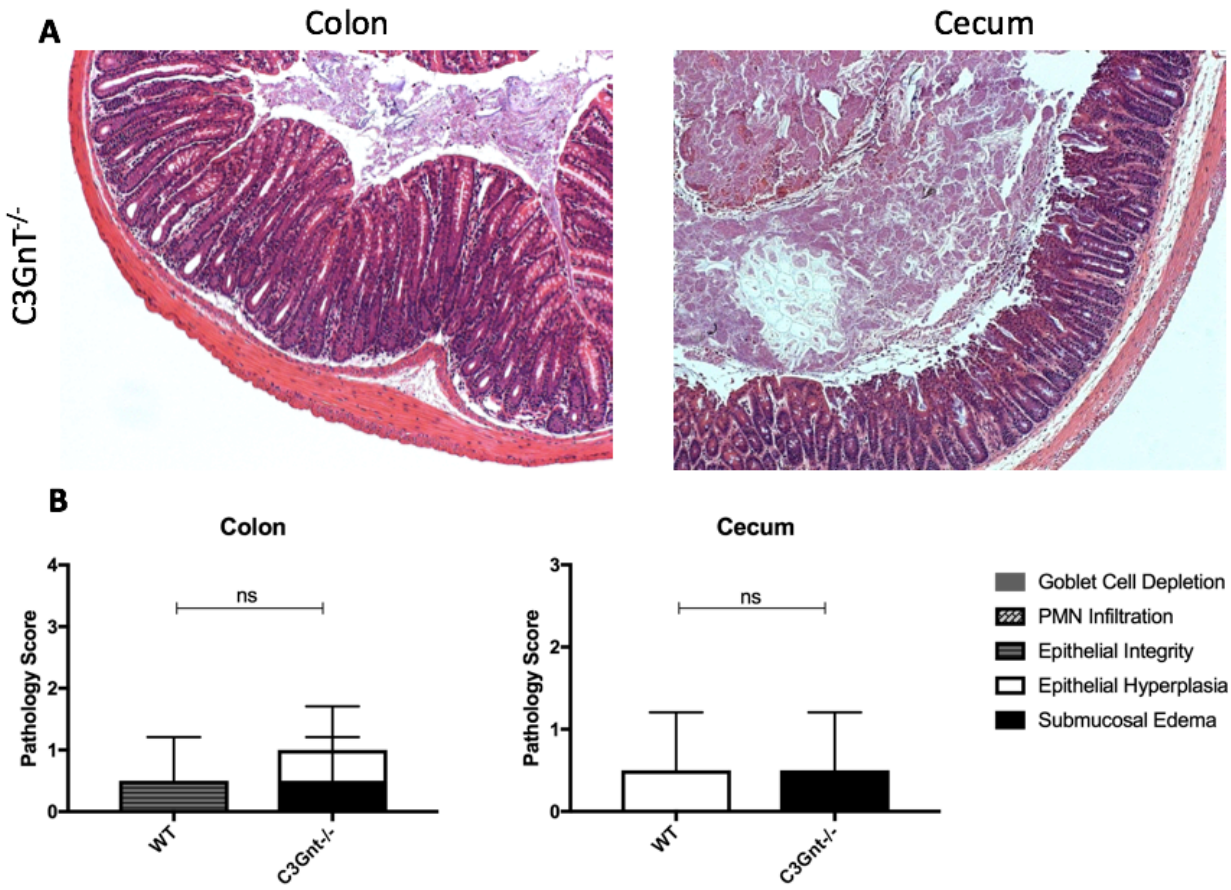


Figure 3.4. C3GnT$^{-/-}$ mice exhibit modest tissue damage during infection, similar to WT mice.
A: H&E stained sections of distal colon and cecum from infected C3GnT$^{-/-}$ mice at 6 DPI. Both colon and cecum tissues showed only modest inflammation with mild epithelial hyperplasia and disruption of epithelial integrity in the cecum (200x magnification). **B**: Pathology scores showed no significant difference between C3GnT$^{-/-}$ and WT type at day 6 PI. Scores were determined by two independent observers under blinded conditions. Bars represent the average of counts (n=12). Results are pooled from 3 separate infections. (ns: no significant difference using t-student test).

3.3.3 *C1galt1 (IEC) -/-* mice develop exaggerated colitis during *C. rodentium* infection

In contrast, mice lacking core 1 glycosylation (*C1galt*$^{-/-}$) in their IEC were similar to *Muc2*$^{-/-}$ mice in terms of showing higher susceptibility to *C. rodentium* as characterized by significant weight loss (~15%) (Figure 3.5A) and increased pathogen burdens starting at 4 DPI (Figure 3.5B).

To assess the role of core 1 glycosylation during *C. rodentium* infection and compare the findings with mice completely deficient in the mucin Muc2, we infected Muc2 -/-, and *C1galt1 -/-* mice with *C. rodentium* and monitored their body weights over a 6 day infection period. The results were compared to WT mice as well as mice with *loxP* sites flanking *C1galt1* (*C1galt1*$^{f/f}$). While WT and *C1galt1*$^{f/f}$ mice showed only modest body weight loss, *C1galt1 -/-* mice displayed significant weight loss during *C. rodentium* infection. By day 6 PI, the *C1galt1 -/-* mice had lost ~15% of their initial/starting body weight (Figure 3.5A) and they also displayed clinical signs of morbidity such as hunched posture, piloerection and inactivity as compared to *C1galt1*$^{f/f}$ mice. Additionally, we enumerated *C. rodentium* burdens in the stool samples of these mice over the course of infection. While there were no significant differences in *C. rodentium* burdens between *Muc2* -/- and *C1galt1 -/-* mice, both knockout mouse strains showed significantly greater pathogen burdens than control mice (10-100 fold greater) starting at day 4 and maintaining this trend until the end of infection (Figure 3.5B). Overall, these findings suggested that both *Muc2-/-* and *C1galt1 -/-* mice were highly susceptible to *C. rodentium* infection, shedding much higher *C. rodentium* burdens than other mouse strains during infection.

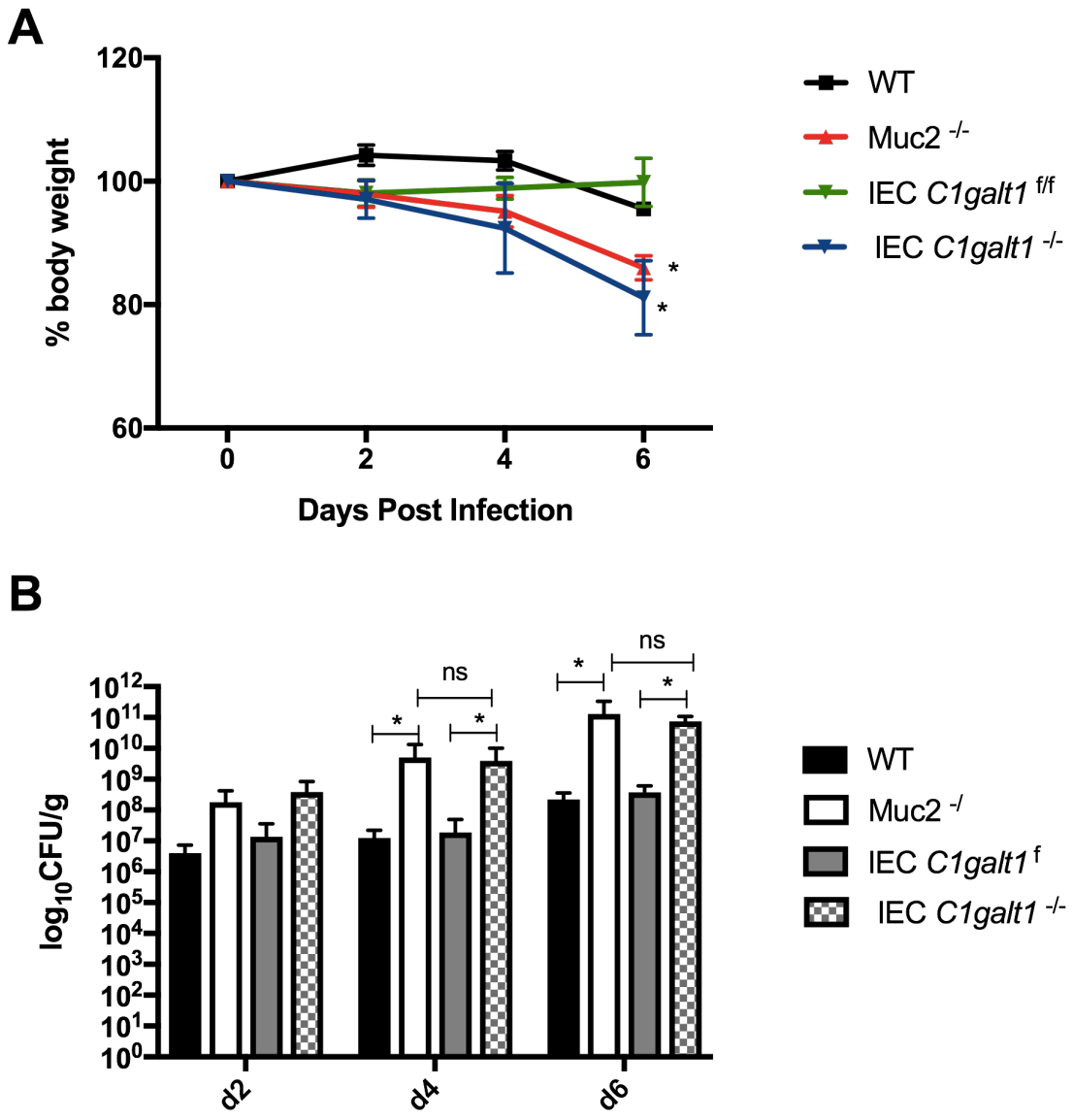


Figure 3.5. *Muc2*$^{-/-}$ and *C1galt*$^{-/}$ mice exhibit similar high susceptibility to *C. rodentium*.
A: *Muc2*$^{-/-}$ and *C1galt*$^{-/-}$ mice show significant weight loss (~15%) at 6 DPI as compared to their respective controls. **B**: *Muc2*$^{-/-}$ and *C1galt*$^{-/}$ mice display similar *C. rodentium* colonization over the course of infection with no significant difference between the groups. Bars represent the average of counts (n=12). Results are pooled from 3 separate infections. (*, $P < 0.05$ by the t-student test).

3.3.4 *C1galt1* $^{-/-}$ mice carry high *C. rodentium* intestinal burdens similar to *Muc2*$^{-/-}$

Next, we enumerated *C. rodentium* burdens within the GI tract and at systemic sites. *C. rodentium* CFU counts from *Muc2-/-* and *C1galt1 -/-* mice was significantly greater (10-1000 fold higher) than in control mice, at all intestinal sites (colon and cecum), with these microbes considered to be adherent (or directly infecting) these tissues. Luminal content burdens, representing non-adherent *C. rodentium* collected from *Muc2-/-* and *C1galt1 -/-* mice were also significantly greater as compared to WT and *C1galt1*$^{f/f}$ mice (Figure 3.6). Likewise, *C. rodentium* burdens in systemic tissues also reflected the trend found within the GI tract, with higher *C. rodentium* burdens found in the *Muc2-/-* and *C1galt1 -/-* mice as compared to their respective controls. Interestingly, no differences were found in CFU counts when comparing *Muc2-/-* and *C1galt1 -/-* mice, thus emphasizing the impact of core 1 glycosylation in host defense against *C. rodentium*, with its loss producing a similar phenotype to the loss of the entire Muc2 protein.

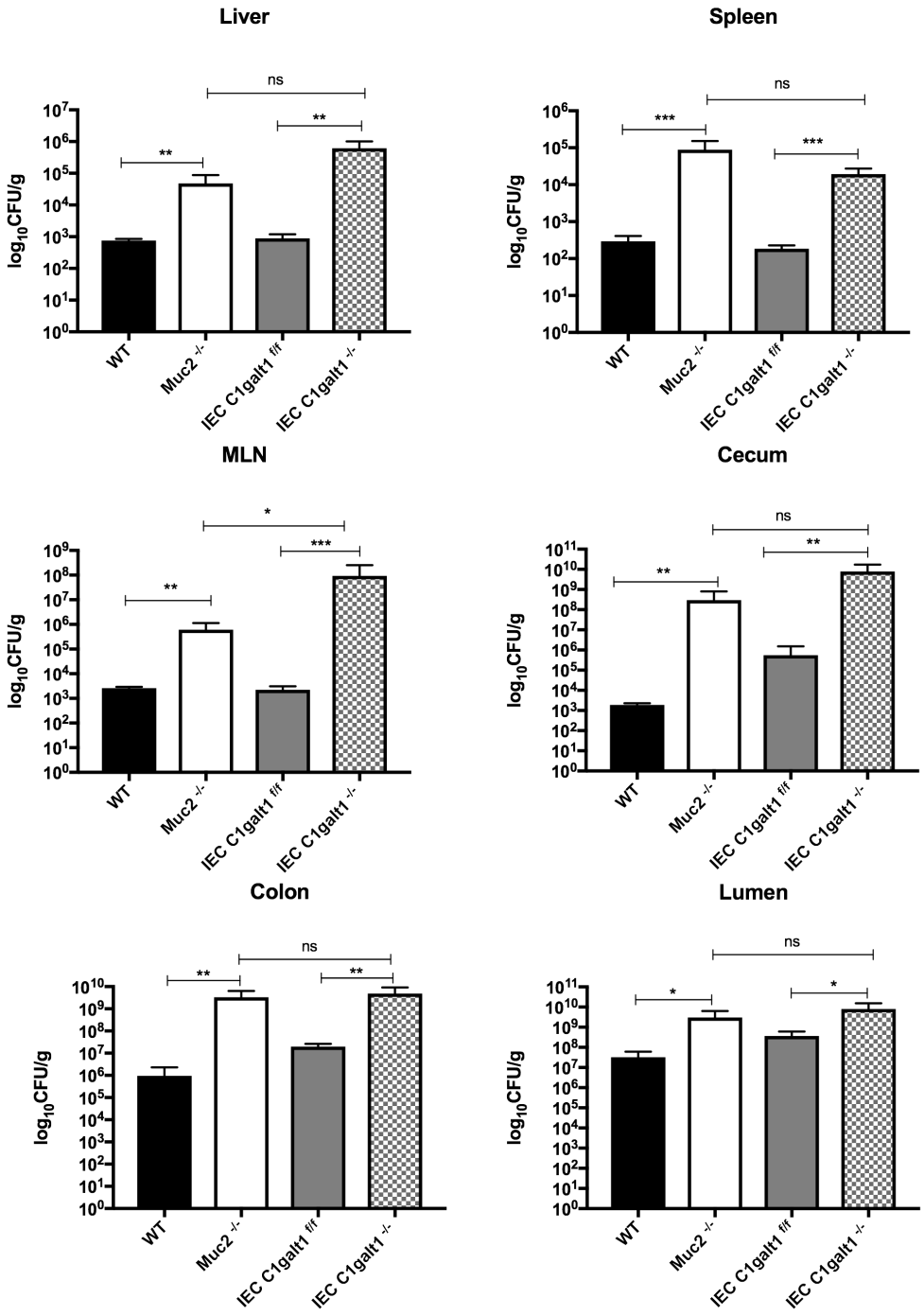
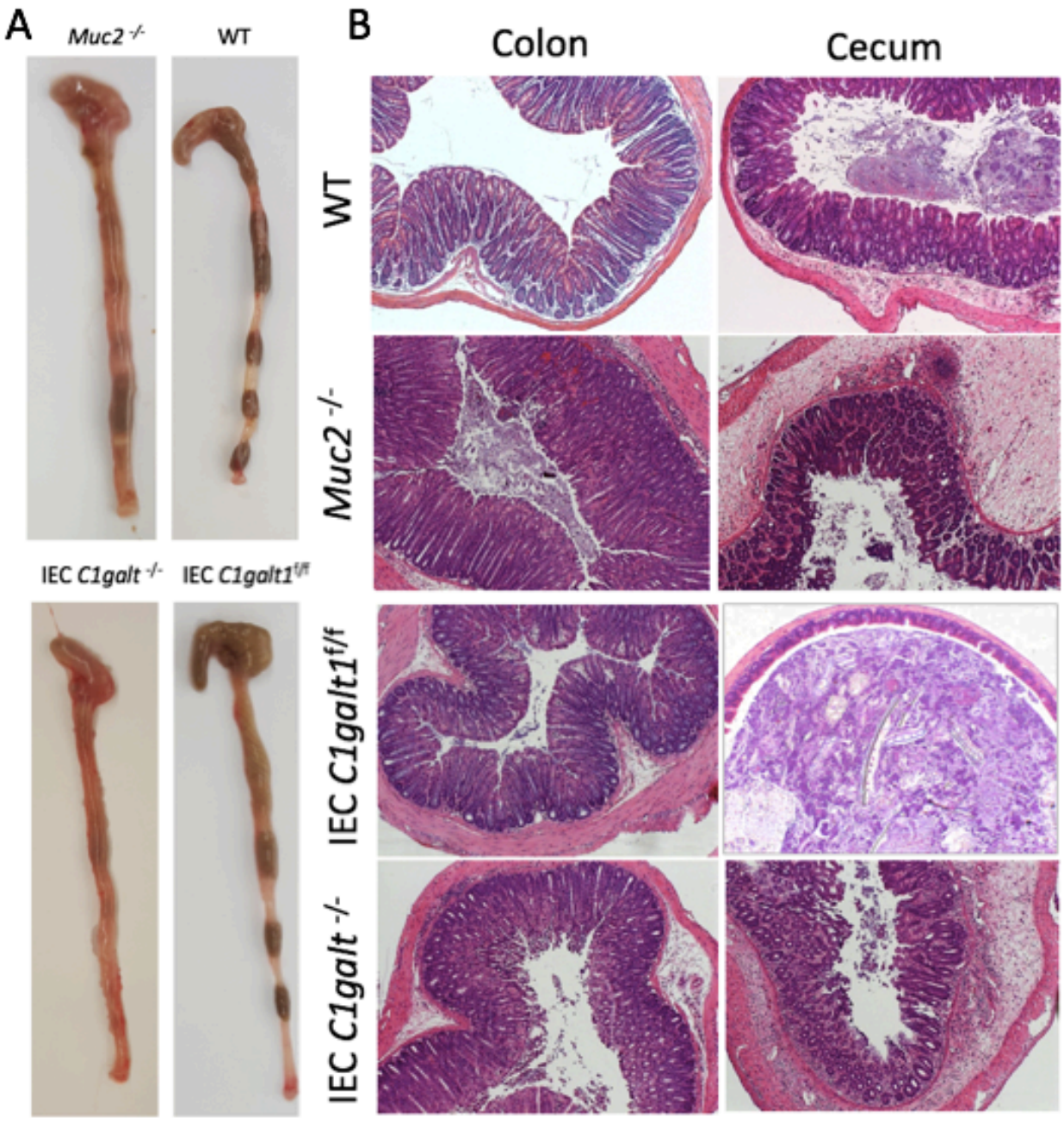


Figure 3.6. *Muc2-/-* and *C1galt1 -/-* mice carry heavier intestinal pathogen burdens.
C. rodentium burdens enumerated from the colon, cecum, luminal content, liver, spleen and mesenteric lymph nodes (MLN) at day 6 PI. *Muc2 -/-* and *C1galt1 -/-* carried significantly higher pathogen burdens than WT and *C1galt1*$^{f/f}$ mice. Note that *Muc2 -/-* mice had comparable pathogen burdens to *C1galt1 -/-* mice, with no significant differences except for the MLN. Bars represent the average of counts (n=12). Results are pooled from 3 separate infections. (*, $P < 0.05$; **, $P < 0.01$ by the t-student test).

Consistent with their increased pathogen burdens, *Muc2 -/-* and *C1galt1 -/-* mice showed greater macroscopic intestinal damage, characterized by shrunken ceca, thickening of the colon, ulcerations and absence of solid stool contents. No such phenotype was observed in either WT or *C1galt1*$^{f/f}$ mice infected with *C. rodentium* (Figure 3.7A). To further examine and characterize the intestinal tissue pathology, H&E stained tissues (distal colon and ceca) were examined histologically (Figure 3.7B). Histological samples from *C1galt* $^{-/-}$ and *Muc2*$^{-/-}$ mice showed significantly higher pathology scores in comparison with WT and *C1galt1*$^{f/f}$ – as demonstrated by severe submucosal edema, loss of epithelial integrity, crypt hyperplasia (increased colonic crypt heights), increased PMN infiltration and goblet cell depletion (Figures 3.7B and C). Once again, no significant difference was observed when comparing *C1galt* $^{-/-}$ with *Muc2*$^{-/-}$ mice, thus reinforcing the similar phenotype and the fundamental role of core 1 glycosylation in the phenotype found during *C. rodentium* infection.


A
Muc2 -/-
WT
IEC C1galt -/-
IEC C1galt1f/f
B
Colon
Cecum
WT
Muc2 -/-
IEC C1galt1f/f
IEC C1galt -/-

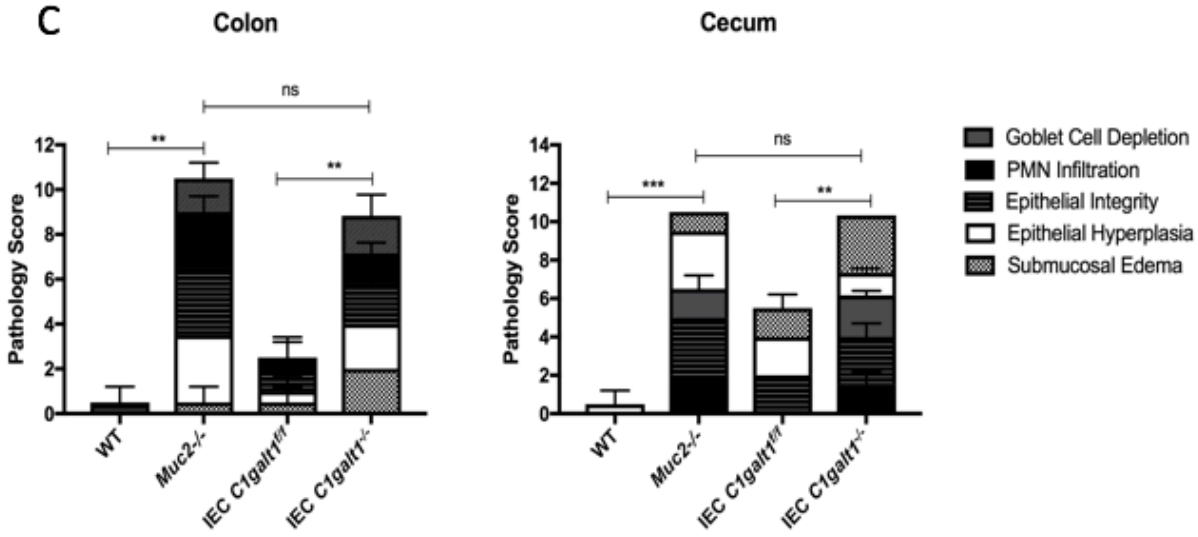


Figure 3.7. *Muc2*$^{-/-}$ and *C1galt1*$^{-/-}$ mice display similar susceptibility to *C. rodentium* infection.
A: Resected large intestines and ceca of infected WT, *Muc2*$^{-/-}$, *C1galt*$^{-/-}$, *and C1galt*$^{f/f-}$ mice at 6 DPI. Ceca of *Muc2*$^{-/-}$ and *C1galt*$^{-/-}$ mice showed similar signs of inflammation with shrunken cecum and thicker colons in comparison with their respective controls. **B**: H&E stained sections of distal colon and cecum from infected WT, *Muc2*$^{-/-}$, *C1galt*$^{-/-}$, *and C1galt*$^{f/f-}$ mice at 6 DPI. Both colon and cecum from *Muc2*$^{-/-}$ and *C1galt* $^{-/-}$mice exhibited severe inflammation characterized by crypt hyperplasia (Muc2$^{-/-}$) and loss of epithelial integrity in colonic tissue (*C1galt* $^{-/-}$*),* together with severe edema and immune cell infiltration in cecal tissues (200x magnification). **C**: Pathology scores showed significant differences between *Muc2*$^{-/-}$ and WT mice as well as between *C1galt* $^{-/-}$ *and C1galt*$^{f/f}$ at day 6 PI. However, no significance was declared when *Muc2*$^{-/-}$and *C1galt* $^{-/-}$mice were compared. Scores were determined by two independent observers under blinded conditions. Bars represent the average of counts (n=12). Results are pooled from 3 separate infections. (ns: no significant difference; **p<0.01; ***p<0.001; t-student test).

3.3.5 *Muc2-/-* mice exhibit lower stool concentrations of propionic and butyric acids under baseline condition

As previously discussed, mucus may serve as a key nutrient source in the gut, thereby directly influencing intestinal microbial composition and their metabolites such as SCFA. As increased intestinal pathology could be associated with intestinal dysbiosis and unbalanced oxygen and butyrate levels in the gut, we next examined the concentrations of the three major SCFA (i.e. acetic acid, propionic acid, and butyric acid) in stool pellets from uninfected WT, *Muc2* $^{-/-}$*, C1galt1* $^{-/-}$

and C1galt1 $^{f/f}$ mice. Interestingly, we found that *Muc2* $^{-/-}$ mice showed significant lower levels of propionic acid as well as butyric acid as compared to WT mice under baseline conditions (Figure 3.8 B and C). While *C1galt1* $^{-/-}$ mice levels did not reach significance as compared to their counterpart for all SCFA investigated, we did observe a trend towards decreased levels of butyric acid before the infection (Figure 3.8C).

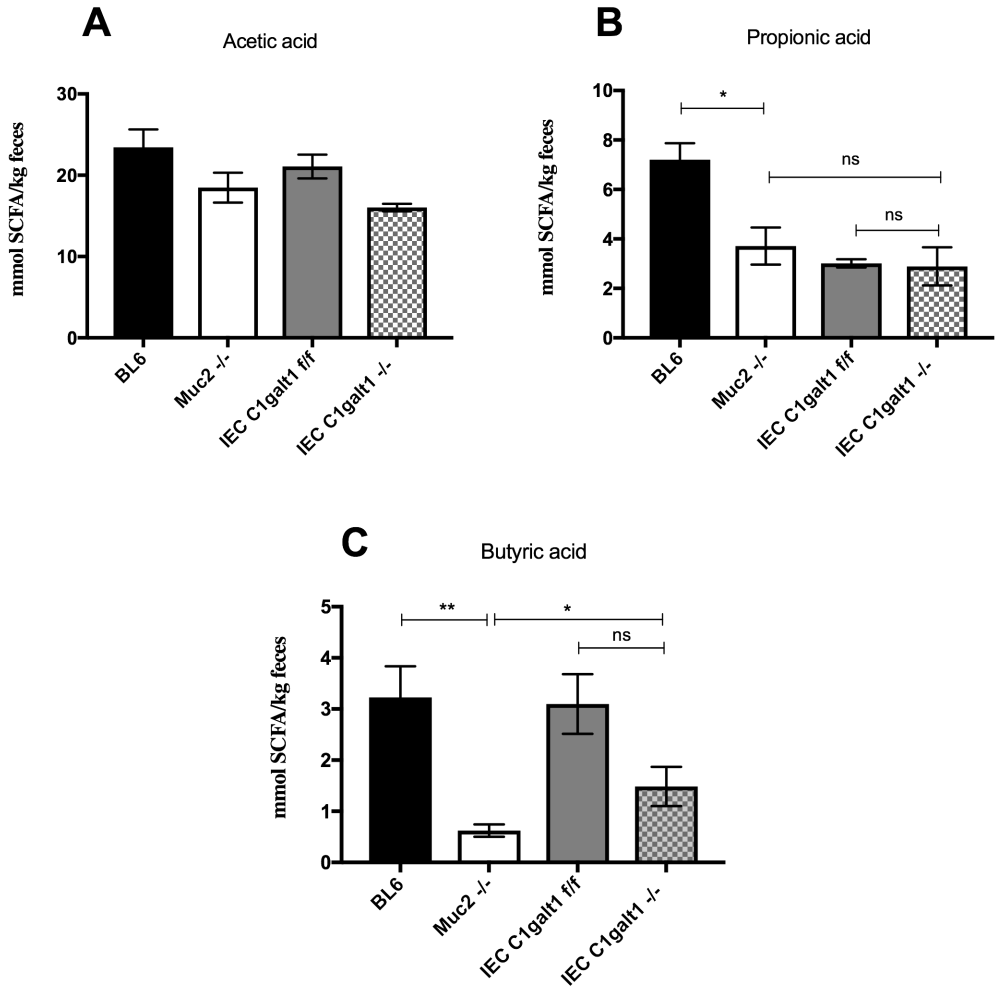


Figure 3.8. *Muc2-/-* mice display lower levels of propionic and butyric acids under baseline condition. **A**: No significant difference in acetic acid levels between groups for all mouse strains analyzed. **B**: *Muc2 -/-* exhibited lower concentrations of propionic acid in comparison with WT mice. **C**: *Muc2 -/-* exhibited lower concentrations of butyric acid in comparison with WT mice as well as in comparison with *C1galt1-/-* mice. Bars represent the average of counts (n=8). Results are pooled from 2 separate infections. (ns: no significant difference; *p<0.05; **p<0.01; t-student test).

3.3.6 Tributyrin supplementation ameliorates damage caused by *C. rodentium* infection

Considering the lower levels of butyric acid under baseline conditions, we tested if supplementation with tributyrin (TB) a triglyceride composed of three butyric acids - would induce any significant alterations in the infection susceptibility of *Muc2-/-* and *C1galt1 -/-* mice. Mice from control groups received only glycerol (GL) by oral gavage to reach a similar caloric intake as the treated groups thus avoiding weight variation due to TB supplementation. Interestingly, *Muc2-/-* mice lost less weight upon receiving the TB as compared to the control group (5% vs. 12%), suggesting TB was protective during *C. rodentium* infection. Similarly, *C1galt1 -/-* mice displayed a strikingly positive outcome when supplemented with TB - showing practically no weight loss over the 6 days of infection while mice receiving solely glycerol lost 8% of their body weight (Figure 3.9). Likewise, both *Muc2-/-* and *C1galt1 -/-* mice exhibited fewer clinical signs of colitis with TB supplementation, characterized by normal posture and activity as well as stool that was more normal in consistency. TB supplementation also impacted *C. rodentium* colonization in *Muc2-/-* and *C1galt1 -/-* mice, as enumeration of this pathogen was found to be significantly lower in groups that received TB, as seen from as early as 2 DPI – (Figure 3.9). These reduced pathogen burdens remained low until the end of the infection (6 DPI) suggesting that TB protects against the typical *C. rodentium* expansion.

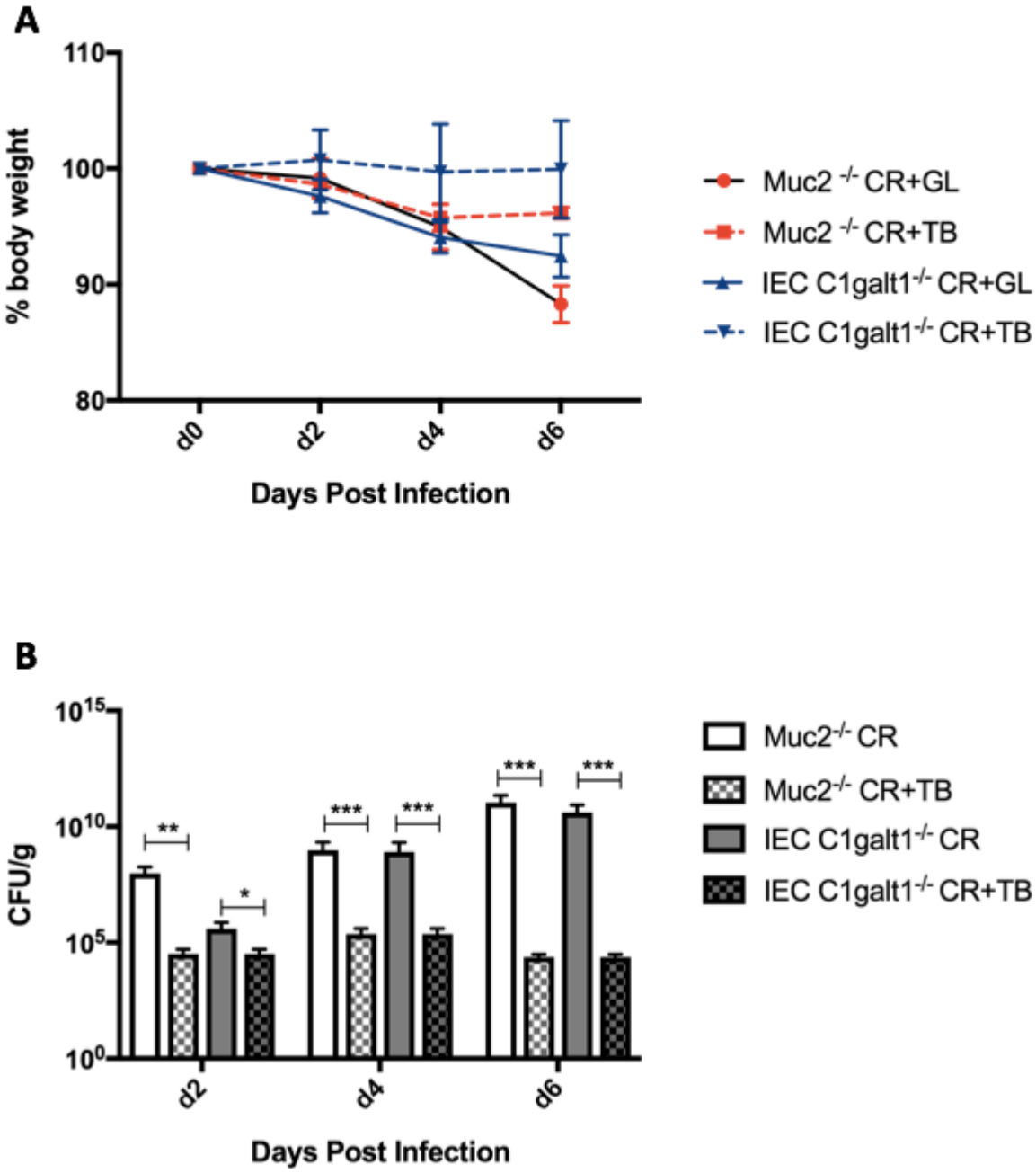


Figure 3.9. *Muc2*$^{-/-}$ and *C1galt*$^{-/-}$ mice supplemented with TB showed reduced susceptibility to *C. rodentium*.

A: *Muc2*$^{-/-}$ and *C1galt*$^{-/-}$ mice show none to mild (5%) weight loss at 6 DPI when supplemented with TB as compared to their respective controls (8% and 12%). **B**: *Muc2*$^{-/-}$ and *C1galt*$^{-/-}$ mice displayed lower *C. rodentium* shedding/colonization over the course of infection when supplemented with TB as compared to GL supplementation. Bars represent the average of counts (n=12). Results are pooled from 3 separate infections. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by the t-student test). CR+GL: *C. rodentium* + glycerol; CR+TB: *C. rodentium* + tributyrin)

As previously described, *C. rodentium* colonization is usually 10-100 fold higher in *Muc2 -/-* and *C1galt 1 -/-* mice as compared to their respective controls. Notably, this trend of higher numbers of *C. rodentium* was attenuated by tributyrin administration in both susceptible mouse strains (*Muc2 -/-* and *C1galt 1 -/-* mice) (Figure 3.10). Correspondently, along with less severe colitis, TB was able to protect mice against higher pathogen burdens in tissues within the GI tract (distal colon and cecum) as well as within the luminal stool content combined from both the colon and cecum.

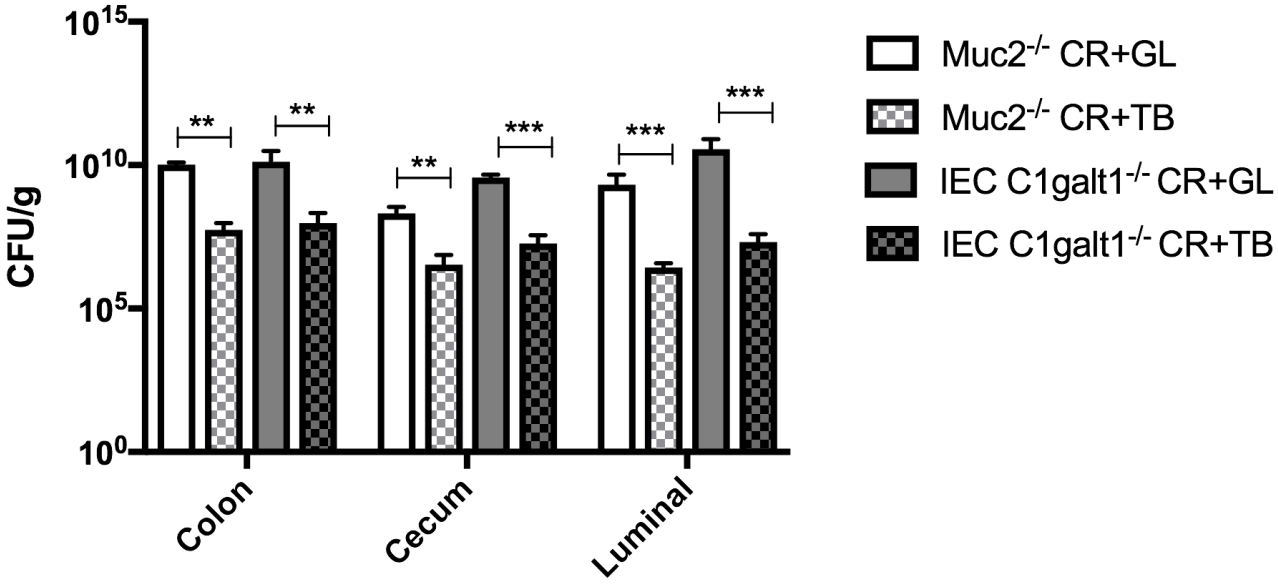


Figure 3.10. *C1galt1 -/-* mice carry heavier intestinal pathogen burdens.
C. rodentium burdens enumerated from the colon, cecum and luminal contents at 6 DPI. *Muc2-/-* and *C1galt1-/-* mice carried significantly lower pathogen burdens (100-1000 fold) when supplemented with TB than groups supplemented with GL alone - for all intestinal samples collected (distal colon, cecum and luminal contents). Bars represent the average of counts (n=12). Results are pooled from 3 separate infections. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by the t-student test). CR+GL: *C. rodentium* + glycerol; CR+TB: *C. rodentium* + tributyrin)

Consistent with fewer signs of colitis and lower pathogen burdens, *Muc2-/-* and *C1galt1 -/-* mice also suffered less histological damage in colonic tissue when supplemented with TB in comparison with mice supplemented only with GL. Tissues from mice that received TB were characterized by

less edema and immune cell infiltration, in addition to have their crypt organization and architecture almost intact and displaying few signs of crypt hyperplasia (Figure 3.11).

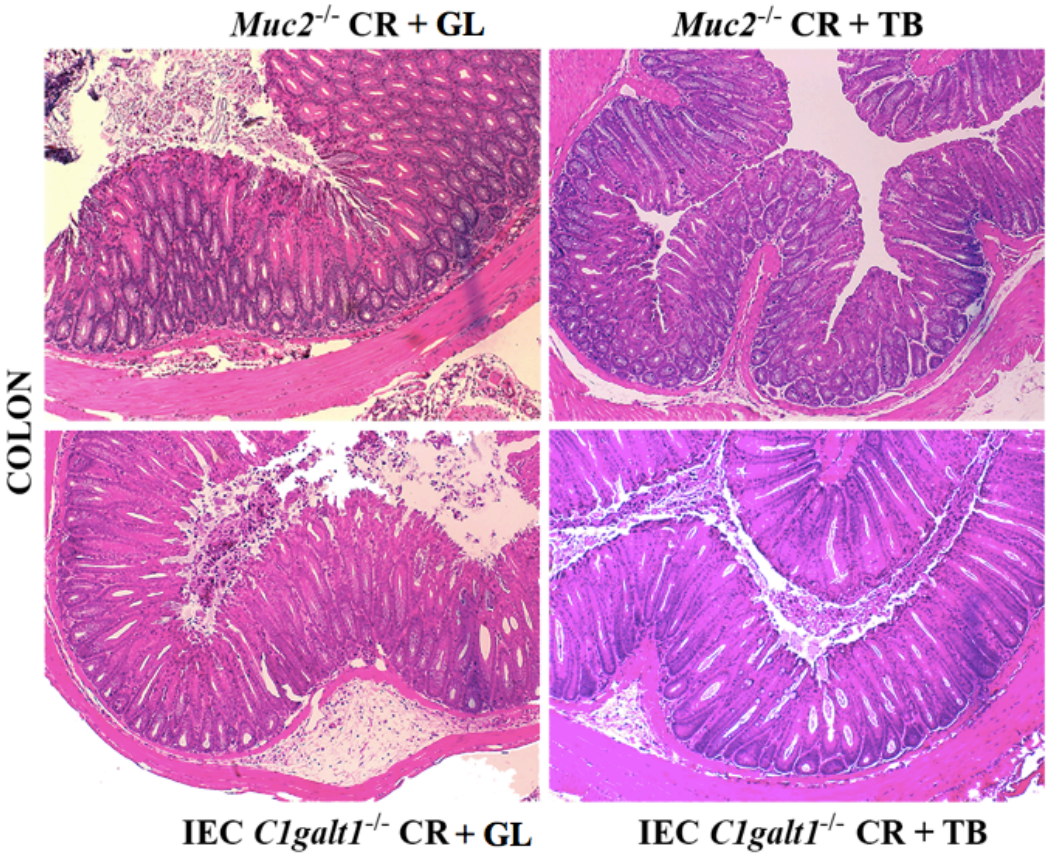


Figure 3.11. *Muc2-/-* and *C1galt1 -/-* display less severe colitis with TB supplementation.
Representative H&E stained colonic tissues of *C. rodentium* infected *Muc2 -/-* and *C1galt 1 -/-* mice. Groups that received TB supplementation develop less exaggerated damage to their mucosal surface and displayed normal crypt heights as well as maintenance of epithelial integrity (200x magnification).

3.4 Discussion

The intestinal mucus layer is considered the first barrier of protection against noxious luminal stimuli such as pathogens and some food/bacterial products. Studies have shown that patients with intestinal disorders such as IBD and colon cancer often have a defective intestinal mucus layer, which seems to be thinner and more penetrable than that seen in healthy subjects (160,262–264). As most bacteria in the gut are localized to the lumen and the outer mucus layer, an impaired mucus barrier may facilitate these microorganisms and their end products crossing this "normally" protective barrier resulting in undesired interactions with the underlying immune cells. The Muc2 mucin is the predominant mucin found within the distal GI tract, and previous studies by our research group have shown that Muc2 promotes host defense against enteric pathogens such as *C. rodentium* (130) and *S.* Typhimurium (217). Although we have already demonstrated that the Muc2 mucin plays an important role in host defense, the exact mechanisms behind its protective role have not been completely elucidated. 80% of the mass of the Muc2 mucin is comprised of carbohydrates, and in fact Muc2 is decorated by several different terminal glycans that are able to interact with bacterial products and dietary fibers (265). Besides providing a physical and biochemical barrier against luminal products, our understanding of the role and the mechanism of action of Muc2 glycosylation in providing protection in the gut is limited.

Considering the importance of the mucin MUC2/Muc2 in IBD patients as well as in enteric infections, our focus was to investigate which part of the Muc2 molecule is key to promoting host defense – the protein core, the glycosylation of the protein, or both. In this study, we provide evidence that the loss of core 1 derived O- glycans dramatically increases host susceptibility to the

A/E pathogen *C. rodentium*, with the *C1galt1 -/-* mice showing a very similar infectious phenotype, to that seen in mice suffering the complete loss of the mucin Muc2. An impaired mucus barrier also increased intestinal pathogen burdens along with systemic translocation of *C. rodentium* in both *Muc2-/-* and *C1galt1 -/-* mice, resulting in dramatically increased morbidity and pathology suffered by these mice during infection. Overall these findings suggest that core 1 derived-O glycans, rather core 3 derived-O glycans, play a significant role in controlling luminal and mucosal pathogen burdens in mice. Additional host innate and adaptive immune responses may also influence *C. rodentium* colonization and burdens during infection (266,267). Moreover, interactions of the intestinal microbiota with components of the intestinal mucus seem to regulate pathogen growth and colonization, since the mucus barrier also prevents microbes from reaching and adhering to the intestinal epithelial surfaces and thus play a key role in innate defense. However, for the purpose of this study, our focus was to investigate the role of the mucin Muc2 and its glycosylation in host defense as well as the relationship between the mucus layer and bacterial products in the gut.

Overall, mice lacking the core 1 glycosylation solely in their IECs (IEC *C1galt1 -/-)* displayed a similar susceptibility phenotype to that seen in mice completely deficient in the mucin *Muc2* (*Muc2 -/-* mice), indicating that the key function of Muc2 in our model appears to reflect its glycosylation. Notably, *C1galt1 -/-* mice still produce and secrete Muc2, although in lower concentrations and with a different structure than in WT mice. Thus, proper Muc2 glycosylation rather than the mere presence of the Muc2 mucin appears to control *C. rodentium* burdens in the intestinal mucosa and its translocation to systemic sites. These results confirm previous data that

Muc2 production and secretion are critical host defense mechanisms that regulate the interactions of *C. rodentium* with the intestinal mucosal surface (130).

Curiously, loss of core 3 derived O-glycans did not overtly impact susceptibility to *C. rodentium* infection in our studies, in contrast to the major effect seen with *C1galt1 -/-* mice. *C3GnT -/-* mice showed a similar phenotype as WT mice, which was characterized by modest weight loss and mild histological damage such as intestinal crypt elongation, immune cell infiltration, and goblet cell depletion (126,129). This finding is consistent with previous studies describing core 3 O-glycosylation of Muc2 playing an important role in humans, but a less important role in promoting murine host defense against enteric pathogens such as *C. rodentium* and *S.* Typhimurium (217,268,269).

Previous data from our research group has shown a dysbiotic intestinal microbiota in *Muc2 -/-* mice with reduced numbers of *Firmicutes* bacteria, involving specific deficits in *Lactobacillus* spp. along with a trend towards more γ-*Proteobacteria* (130,177). In keeping with the loss of such microbes, it is already well known that metabolites such SCFA are key products to confer protection against colitis. Considering the importance of dysbiosis in triggering inflammatory responses, we assessed the three major SCFAs – acetate, propionate and butyrate – in *Muc2 -/-* and *C1galt1 -/-* mice before infection with *C. rodentium*, to better define whether these SCFAs might play a role in infection susceptibility. Interestingly, *Muc2 -/-* mice displayed lower levels of propionate and butyrate under baseline conditions, suggesting an imbalanced microbiota with a reduction in obligate anaerobes such as butyrate producers and an increase in facultative anaerobes such as *E. coli* and most pathogenic/pathobiont bacteria. Although not significantly different from

their controls, *C1galt1 -/-* mice also showed a trend towards lower levels of butyrate that may be a reflection of their thinner mucus layer, rather than the complete absence of Muc2. Nevertheless, further studies investigating their microbiota composition are necessary to confirm the hypothesis that specific microbes and their products regulate/limit *C. rodentium* infection.

Since SCFAs – especially butyrate - have been successful applied in murine models of intestinal inflammation as well as in IBD patients (270–273), we administered the triglyceride tributyrin for three consecutive days after *C. rodentium* infection and compared the disease severity in treated mice with mice given only the tributyrin carrier matrix glycerol. We noted that mice supplemented with tributyrin proved less susceptible to *C. rodentium* infection, as indicated by them suffering only modest body weight loss, carrying relatively small pathogen burdens as well as suffering only mild histological damage in their colonic tissues. We also noted that *Muc2-/-* and *C1galt1 -/-* mice again showed a similar phenotype, ie. both mouse strains benefitted from the tributyrin. These findings are supported by other studies showing that oral supplementation of tributyrin acts as more than just an energy source for colonocytes but also controls immune responses and interferes in immune cell migration and mucosal healing (260). Another study found that *S.* Typhimurium was able to expand in the gut following antibiotic treatment due to elevated epithelial oxygenation as a consequence of the loss of butyrate-producing Clostridial bacteria (187). As reviewed by Allaire and colleagues (34), butyrate directly impacts gut homeostasis by influencing intestinal pro- and anti-inflammatory mechanisms, colonocyte metabolism, and consequently the intestinal microbiota composition.

The present study provides additional evidence of the critical protective role of the mucus layer in providing host defense against *C. rodentium* infection. It also demonstrates that the protective role played by Muc2 in this model reflects its glycosylation, rather than the protein itself. Furthermore, our data also provides evidence that tributyrin supplementation could benefit the host during enteric infections – putatively by shifting oxygen and butyrate gradients in the gut, and potentially through changes in the gut microbiota. These findings underscore the need for further exploration of the mechanisms by which the mucus layer protects the intestinal tract from bacterial pathogens and other noxious stimuli.

Chapter 4: Conclusions

4.1 The big picture: potential application of the research findings

Animal models have been widely used to study several GI human diseases, such as IBD and enteric infections. Although animals are not completely identical to humans in terms of anatomy and physiology, they share most human genes and are biologically similar. Therefore, murine models have served as important tools to examine underlying mechanisms involved in both the initiation and the progression of IBD. Moreover, these models have contributed towards novel therapeutic strategies for intestinal diseases before these strategies are tested in humans. Thus, the animal models used in this research project, along with the results we obtained, provide interesting insights into the development of novel probiotics to prevent and treat intestinal conditions. Moreover, the use of mice lacking specific genes, such as the *Muc2*$^{-/-}$ mice and the *C1galt1*$^{-/-}$ mice, allow us to isolate particular targets from the complex intestinal environment that might be playing a more important role in host defense.

Over the last years, several approaches have been studied to positively modulate the gut microbiome and therefore prevent gastrointestinal and metabolic diseases related to a dysbiotic state. One of the most common strategies currently used is the administration of beneficial microbes known as probiotics. Probiotics can be incorporated into the daily diet through the consumption of fermented food or as dietary supplement. Although the use of probiotics has proven beneficial in different conditions such as cow's milk allergy (274), antibiotic associated diarrhea (275), infant colic and constipation (276), respiratory tract infections (277,278), among others, their efficacy in IBD is still controversial. The commercial probiotic VSL#3 seems to be

effective in inducing remission in active UC patients as well as in preventing disease relapse in these patients. However, more evidence is required for the use of probiotics in CD patients as its efficacy remains uncertain in several studies (279).

The work described in this thesis, proposes a novel strategy – i.e. a personalized probiotic approach that we believe is highly promising for treating different dysbiotic related diseases. Considering the particular characteristics of an individual's microbiome, as well as the environmental factors that influence this microbial community during life, we believe that therapies should focus on the host's endogenous commensal microbes. By isolating personalized probiotics, rather than trying to incorporate new microorganisms from exogenous sources, we propose this strategy will prove more effective as compared to the "one size fits all" approach that has been used for several decades and yet has not proven sufficiently reproducible to affect clinical practice in IBD. Our personalized approach did protect mice against the intestinal inflammation caused by DSS, and to a greater extent than that seen with the commercial probiotic LGG.

Although further studies are necessary to define the exact mechanism of this protection, our hypothesis is that this beneficial effect reflects an increase in the numbers of these endogenous commensal bacteria in the gut after their *ex vivo* propagation and delivery. Previous studies have shown IBD patients, as well as people suffering from enteric infections undergo dramatic shifts in the makeup of their microbiomes. Thus, by orally providing/restoring large numbers of these microbes to intestinal diseases patients, we hypothesize this will reduce the dysbiosis, as well as provides beneficial factors to reduce inflammation, such as short chain fatty acids.

Another important factor in shaping the composition of the intestinal microbiome is the colonic mucus layer. Besides acting as a primary defense barrier against enteric pathogens, the colonic mucus layer also serves as a nutrient source for commensal bacteria, therefore shaping the gut microbial community. Recent findings have described that in the absence of Muc2, the major gel-like forming mucin in the colonic mucus layer, mice are significantly more susceptible to enteric infections (130,217). Moreover, patients with IBD often have a thinner and more penetrable mucus layer, which could be either cause or consequence for a dysbiotic state within the gut. These findings intrigued us to explore in more detail the enzymes that control the glycosylation patterns of the mucin Muc2, and how their absence would affect mice challenged with *C. rodentium*.

We found that mice deficient in Core 1 derived O-glycans (*C1galt1* $^{-/-}$) showed very similar susceptibility to *C. rodentium* infection as that seen with the complete absence of the mucin Muc2 (*Muc2* $^{-/-}$ mice). Conversely, mice lacking the Core 3 derived O-glycans had only minor effects on pathogen burdens and inflammation, showing a roughly similar phenotype to that displayed by WT mice. The different data obtained from the two major glycosylations of Muc2 may reflect the specific role of each O-glycan in the models studied, since Core 1 is the most prominent glycosylation in mice while Core 3 is most prominent in humans. Our results thus indicate that Core 1 glycosylation may play the key role in protecting mice against *C. rodentium* rather the protein core of the mucin Muc2. We also demonstrated that the administration of tributyrin was effective in decreasing pathogen burdens during infection in both *Muc2* $^{-/-}$ and *C1galt1* $^{-/-}$ mice. Future studies are needed to verify the mechanisms of this protective role, however, we believe the supplementation of tributyrin shifts the intestinal environment to a more anaerobic state, thus creating a disadvantageous habitat for the expansion of facultative anaerobes such as *C. rodentium*.

Overall, these findings are important to establish a central role for Muc2 and its glycosylation in intestinal homeostasis. Besides confirming previous discoveries of the importance of Muc2 in host health and disease, my studies provide novel insights regarding the complex interactions between mucus, enteric bacteria and host defense.

4.2 Future directions

Since dysbiosis has been associated with the pathogenesis of numerous intestinal diseases, future studies focusing on how to positively modulate the intestinal ecosystem may provide new therapies for patients with IBD as well as those suffering from enteric infections. Furthermore, additional discoveries regarding the mechanistic role of the microbiota in these disorders may assist in developing novel therapies for intestinal conditions that directly benefit the overall health and general well-being of individuals.

Regarding the personalized probiotics strategy, we understand that, like all novel ideas, additional experiments are necessary to validate and confirm the positive effects described in this thesis. Even so, we believe the manuscript published with our findings will initiate a productive discussion regarding optimal strategies for personalized therapies in the microbiome field. We believe our study is the first one demonstrating a promising result when shifting the strategy of microbiota manipulation from a general "one size fits all" approach to a more personalized approach for each patient. To prove this, we suggest further comparisons of personalized probiotics against commercially available probiotics containing different formulations and applications.

Additionally, we recognize that the selection process will be more challenging in humans as compared to murine models due to the complexity of the human microbiome. A few strategies have been discussed by our research group seeking an approach that will be effective in clinical practice. First, the selection of personalized commensals at an early age in children genetically predisposed to develop IBD (for example - and before any disease has developed), could represent a promising idea. These bacteria could be stratified based on their potential probiotic capacity (*in vitro* tests and characterization) and then kept in a microbiota biobank as proposed in Chapter 2. Since several diseases have been liked to a dysbiotic state in the gut we see the microbiota bank as an opportunity to treat both intestinal and extra-intestinal conditions. Second, we suggest the selection of beneficial commensal bacteria during the remission phase of inflammatory conditions such as IBD or any other relapsing condition. This period is known to have a more diverse microbiota as compared to that found during acute disease, thus allowing the selection of different genera/species/strains. Lastly, we propose for future studies the isolation and selection of commensal bacteria during the acute phase of IBD or any other inflammatory/dysbiotic disease, in combination (or not) with the ones isolated when the patient is in remission/healthy. Although microbial diversity is affected during the disease/inflammatory phase, the potentially beneficial strains that survive within this noxious inflammatory environment could be the most promising ones to act as probiotic bacteria due to their capacity to survive through adverse conditions.

With reference to the mucus layer and its importance in intestinal homeostasis, several additional experiments are necessary to further elucidate the mechanistic roles of the mucin Muc2 and its glycosylations in host defense. It is fundamental to better understand the complex dynamics of the major Muc2 glycosylations before and during infection as some specific steps of these

glycosylations pathways may impact in IBD susceptibility. Furthermore, we should examine the microbiota composition of the different mouse strains studied in Chapter 3, before and over the course of infection. Such analysis may bring new insights regarding specific groups of microbes affected by the mucus layer and by *C. rodentium*. Analysis using high-throughput techniques, such as 16S sequencing, may help us to understand in more detail how different microbes behave in the absence of mucus or its glycosylations as well as how they affect host health. Additionally, the investigation of bacterial metabolites such as SCFA also contributes towards our understanding of changes in the microbial gut composition and the impact of this complex community on health and disease. Moreover, future studies in our lab will focus on the supplementation of tributyrin through the diet, as this represents a better model of how SCFA might be modified in humans.

Lastly, we believe that focusing on oxygen levels in the gut is extremely important for future research projects as the availability of oxygen, along with other electron acceptors, may impact host physiology and control the luminal expansion of Enterobacteriaceae (280). As previously mentioned in Chapter 3, the oxygen-butyrate gradient within the colon provides important information about the microbial community in the gut, as well as influencing the host resident and transient microorganisms and their impact on health and disease.

4.3 Final remarks

Overall, the work described in this thesis has provided numerous contributions towards the advancement of strategies to positively modulate the intestinal microbiota, either by developing a new and personalized strategy for using probiotics or by improving our understanding of the

interactions between the mucus layer and enteric bacteria as well as their metabolites. Besides the novelty and the positive effects found when using endogenous commensal microbes to prevent intestinal inflammation, the personalized probiotic strategy opens an interesting discussion in the field regarding the focus on the particularities of each person's unique microbiota. Furthermore, the role of *Muc2* and *Core 1* glycosylation in controlling susceptibility to bacterial infections provides new research directions in the lab to continue investigating how the intestinal microbial community is affected by the mucus components and bacterial metabolites, and how these interactions are reflected in the health or disease of the host.

References

1. Clemente JC, Ursell LK, Parfrey LW, Knight R. The impact of the gut microbiota on human health: an integrative view. Cell [Internet]. NIH Public Access; 2012 Mar 16 [cited 2018 Jul 27];148(6):1258–70. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22424233
2. Sender R, Fuchs S, Milo R. Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans. Cell [Internet]. Elsevier; 2016 Jan 28 [cited 2018 Aug 14];164(3):337–40. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0092867416000532
3. Wang B, Yao M, Lv L, Ling Z, Li L. The Human Microbiota in Health and Disease. Engineering [Internet]. Elsevier; 2017 Feb 1 [cited 2018 Jul 25];3(1):71–82. Available from: https://www.sciencedirect.com/science/article/pii/S2095809917301492#bib7
4. Consortium THMP, Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, et al. Structure, function and diversity of the healthy human microbiome. Nature [Internet]. Nature Publishing Group; 2012 Jun 14 [cited 2018 Jul 25];486(7402):207–14. Available from: http://www.nature.com/articles/nature11234
5. Young VB. The role of the microbiome in human health and disease: an introduction for clinicians. BMJ [Internet]. British Medical Journal Publishing Group; 2017 Mar 15 [cited 2018 Jul 25];356:j831. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28298355
6. Arnold JW, Roach J, Azcarate-Peril MA. Emerging Technologies for Gut Microbiome Research. Trends Microbiol [Internet]. NIH Public Access; 2016 [cited 2018 Aug 1];24(11):887–901. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27426971
7. Laupland KB, Valiquette L. The changing culture of the microbiology laboratory. Can J Infect Dis Med Microbiol = J Can des Mal Infect la Microbiol medicale [Internet]. Hindawi Limited; 2013 [cited 2018 Jul 27];24(3):125–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24421822
8. NIH HMP Working Group TNHW, Peterson J, Garges S, Giovanni M, McInnes P, Wang L, et al. The NIH Human Microbiome Project. Genome Res [Internet]. Cold Spring Harbor Laboratory Press; 2009 Dec [cited 2018 Jul 25];19(12):2317–23. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/19819907

9. Human Microbiome Project Consortium BA, Nelson KE, Pop M, Creasy HH, Giglio MG, Huttenhower C, et al. A framework for human microbiome research. Nature [Internet]. NIH Public Access; 2012 Jun 13 [cited 2018 Jul 27];486(7402):215–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22699610
10. Gilbert JA, Dupont CL. Microbial Metagenomics: Beyond the Genome. Ann Rev Mar Sci [Internet]. 2011 Jan 15 [cited 2018 Jul 27];3(1):347–71. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21329209
11. Oulas A, Pavloudi C, Polymenakou P, Pavlopoulos GA, Papanikolaou N, Kotoulas G, et al. Metagenomics: tools and insights for analyzing next-generation sequencing data derived from biodiversity studies. Bioinform Biol Insights [Internet]. SAGE Publications; 2015 [cited 2018 Aug 1];9:75–88. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25983555
12. Jovel J, Patterson J, Wang W, Hotte N, O'Keefe S, Mitchel T, et al. Characterization of the Gut Microbiome Using 16S or Shotgun Metagenomics. Front Microbiol [Internet]. Frontiers Media SA; 2016 [cited 2018 Aug 1];7:459. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27148170
13. Ursell LK, Metcalf JL, Parfrey LW, Knight R. Defining the human microbiome. Nutr Rev [Internet]. NIH Public Access; 2012 Aug [cited 2018 Jul 25];70 Suppl 1(Suppl 1):S38-44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22861806
14. Lloyd-Price J, Abu-Ali G, Huttenhower C. The healthy human microbiome. Genome Med [Internet]. BioMed Central; 2016 Dec 27 [cited 2018 Aug 1];8(1):51. Available from: http://genomemedicine.biomedcentral.com/articles/10.1186/s13073-016-0307-y
15. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature [Internet]. NIH Public Access; 2012 Sep 13 [cited 2018 Apr 20];489(7415):220–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22972295
16. Consortium THMP, Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, et al. Structure, function and diversity of the healthy human microbiome. Nature [Internet]. Nature Publishing Group; 2012 Jun 14 [cited 2018 Jul 27];486(7402):207–14. Available

from: http://www.nature.com/articles/nature11234

17. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature [Internet]. 2010 Mar 4 [cited 2018 Aug 1];464(7285):59–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20203603
18. Belda-Ferre P, Alcaraz LD, Cabrera-Rubio R, Romero H, Simón-Soro A, Pignatelli M, et al. The oral metagenome in health and disease. ISME J [Internet]. Nature Publishing Group; 2012 Jan [cited 2018 Aug 7];6(1):46–56. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21716308
19. Duran-Pinedo AE, Chen T, Teles R, Starr JR, Wang X, Krishnan K, et al. Community-wide transcriptome of the oral microbiome in subjects with and without periodontitis. ISME J [Internet]. Nature Publishing Group; 2014 Aug [cited 2018 Aug 7];8(8):1659–72. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24599074
20. Kianoush N, Adler CJ, Nguyen K-AT, Browne G V, Simonian M, Hunter N. Bacterial profile of dentine caries and the impact of pH on bacterial population diversity. PLoS One [Internet]. Public Library of Science; 2014 [cited 2018 Aug 7];9(3):e92940. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24675997
21. Grice EA, Kong HH, Conlan S, Deming CB, Davis J, Young AC, et al. Topographical and temporal diversity of the human skin microbiome. Science [Internet]. American Association for the Advancement of Science; 2009 May 29 [cited 2018 Aug 7];324(5931):1190–2. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19478181
22. Chen YE, Fischbach MA, Belkaid Y. Skin microbiota-host interactions. Nature [Internet]. NIH Public Access; 2018 [cited 2018 Aug 7];553(7689):427–36. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29364286
23. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SSK, McCulle SL, et al. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci U S A [Internet]. National Academy of Sciences; 2011 Mar 15 [cited 2018 Aug 7];108 Suppl 1(Supplement 1):4680–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20534435
24. Fettweis JM, Brooks JP, Serrano MG, Sheth NU, Girerd PH, Edwards DJ, et al. Differences in vaginal microbiome in African American women versus women of

European ancestry. Microbiology [Internet]. Microbiology Society; 2014 Oct [cited 2018 Aug 7];160(Pt 10):2272–82. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25073854

25. Celiberto LS, Graef FA, Healey GR, Bosman ES, Jacobson K, Sly LM, et al. Inflammatory Bowel Disease and Immunonutrition: Novel Therapeutic Approaches Through Modulation of Diet and the Gut Microbiome. Immunology [Internet]. 2018 Apr 25 [cited 2018 Apr 30]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/29693729
26. Saffrey MJ. Aging of the mammalian gastrointestinal tract: a complex organ system. Age (Omaha) [Internet]. Springer Netherlands; 2014 Jun 20 [cited 2018 May 15];36(3):9603. Available from: http://link.springer.com/10.1007/s11357-013-9603-2
27. Bellmann S, Carlander D, Fasano A, Momcilovic D, Scimeca JA, Waldman WJ, et al. Mammalian gastrointestinal tract parameters modulating the integrity, surface properties, and absorption of food-relevant nanomaterials. Wiley Interdiscip Rev Nanomedicine Nanobiotechnology [Internet]. 2015 Sep [cited 2018 May 4];7(5):609–22. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25641962
28. Thomson A, Shaffer E. First Principles of Gastroenterology [Internet]. [cited 2018 May 17]. Available from: https://www.cag-acg.org/publications/first-principles-of-gastroenterology
29. Fawley J, Gourlay DM. Intestinal alkaline phosphatase: a summary of its role in clinical disease. J Surg Res [Internet]. NIH Public Access; 2016 May 1 [cited 2018 May 17];202(1):225–34. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27083970
30. Mowat AM, Agace WW. Regional specialization within the intestinal immune system. Nat Rev Immunol [Internet]. Nature Publishing Group; 2014 Oct 19 [cited 2018 May 18];14(10):667–85. Available from: http://www.nature.com/articles/nri3738
31. Laurin M, Everett M Lou, Parker W. The Cecal Appendix: One More Immune Component With a Function Disturbed By Post-Industrial Culture. Anat Rec Adv Integr Anat Evol Biol [Internet]. 2011 Apr [cited 2018 May 18];294(4):567–79. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21370495
32. Young B (Pathologist), O'Dowd G, Woodford P. Wheater's functional histology : a text and colour atlas. 433 p.

33. Jejunum's Function in the Small Intestine and Digestive System: [Internet]. [cited 2018 Oct 16]. Available from: https://www.healthpages.org/anatomy-function/anatomy-function-digestive-system/
34. F F M Allaire DI, Crowley SM, Law HT, Chang S-Y, Ko H-J, Vallance BA. The Intestinal Epithelium: Central Coordinator of Mucosal Immunity. Trends Immunol [Internet]. 2018 [cited 2018 May 4]; Available from: https://www.cell.com/trends/immunology/pdf/S1471-4906(18)30068-1.pdf
35. Crosnier C, Stamataki D, Lewis J. Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. Nat Rev Genet [Internet]. Nature Publishing Group; 2006 May 1 [cited 2018 Aug 7];7(5):349–59. Available from: http://www.nature.com/articles/nrg1840
36. Hao W-L, Lee Y-K. Microflora of the Gastrointestinal Tract: A Review. In: Public Health Microbiology [Internet]. New Jersey: Humana Press; 2004 [cited 2018 May 15]. p. 491–502. Available from: http://link.springer.com/10.1385/1-59259-766-1:491
37. Cénit MC, Matzaraki V, Tigchelaar EF, Zhernakova A. Rapidly expanding knowledge on the role of the gut microbiome in health and disease. Biochim Biophys Acta - Mol Basis Dis [Internet]. Elsevier B.V.; 2014 Oct;1842(10):1981–92. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0925443914001513
38. Johansson ME V., Hansson GC. Immunological aspects of intestinal mucus and mucins. Nat Rev Immunol [Internet]. Nature Publishing Group; 2016 Oct 8 [cited 2018 May 17];16(10):639–49. Available from: http://www.nature.com/articles/nri.2016.88
39. Allaire JM, Morampudi V, Crowley SM, Stahl M, Yu H, Bhullar K, et al. Frontline defenders: goblet cell mediators dictate host-microbe interactions in the intestinal tract during health and disease. Am J Physiol Liver Physiol [Internet]. 2018 Mar 1 [cited 2018 Aug 8];314(3):G360–77. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29122749
40. Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K, et al. Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature [Internet]. 2011 Jan 27 [cited 2018 Jul 18];469(7331):543–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21270894
41. Candela M, Perna F, Carnevali P, Vitali B, Ciati R, Gionchetti P, et al. Interaction of

probiotic Lactobacillus and Bifidobacterium strains with human intestinal epithelial cells: Adhesion properties, competition against enteropathogens and modulation of IL-8 production. Int J Food Microbiol [Internet]. 2008 Jul 31 [cited 2018 Jul 18];125(3):286–92. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18524406

42. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature [Internet]. 2012 Jun 9 [cited 2018 Jul 19];486(7402):222–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22699611
43. LeBlanc JG, Milani C, de Giori GS, Sesma F, van Sinderen D, Ventura M. Bacteria as vitamin suppliers to their host: a gut microbiota perspective. Curr Opin Biotechnol [Internet]. 2013 Apr [cited 2018 Jul 19];24(2):160–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22940212
44. Furusawa Y, Obata Y, Fukuda S, Endo T a, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 2013;504(7480):446–50.
45. Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, et al. Microbial Exposure During Early Life Has Persistent Effects on Natural Killer T Cell Function. Science (80-) [Internet]. 2012 Apr 27 [cited 2018 Jul 19];336(6080):489–93. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22442383
46. Dominguez-Bello MG, Blaser MJ. The Human Microbiota as a Marker for Migrations of Individuals and Populations. Annu Rev Anthropol [Internet]. Annual Reviews ; 2011 Oct 21 [cited 2018 Aug 7];40(1):451–74. Available from: http://www.annualreviews.org/doi/10.1146/annurev-anthro-081309-145711
47. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: Human gut microbes associated with obesity. Nature [Internet]. 2006 Dec 21 [cited 2018 Apr 20];444(7122):1022–3. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17183309
48. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the Human Intestinal Microbial Flora. Science (80-) [Internet]. 2005 Jun 10 [cited 2018 Aug 22];308(5728):1635–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15831718

49. Martin R, Nauta AJ, Amor K Ben, Knippels LMJ, Knol J, Garssen J. Early life : gut microbiota and immune development in infancy. 2010;1(December):367–82.
50. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: Human gut microbes associated with obesity. Nature. 2006;444:1022–3.
51. Kabeerdoss J, Jayakanthan P, Pugazhendhi S, Ramakrishna BS. Alterations of mucosal microbiota in the colon of patients with inflammatory bowel disease revealed by real time polymerase chain reaction amplification of 16S ribosomal ribonucleic acid. Indian J Med Res [Internet]. Wolters Kluwer -- Medknow Publications; 2015 Jul [cited 2018 Aug 22];142(1):23–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26261163
52. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, et al. Enterotypes of the human gut microbiome. Nature [Internet]. Nature Publishing Group; 2011 May 12 [cited 2018 Aug 16];473(7346):174–80. Available from: http://www.nature.com/doifinder/10.1038/nature09944
53. Mariat D, Firmesse O, Levenez F, Guimarães V, Sokol H, Doré J, et al. The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. BMC Microbiol [Internet]. BioMed Central; 2009 Jun 9 [cited 2018 Aug 22];9(1):123. Available from: http://bmcmicrobiol.biomedcentral.com/articles/10.1186/1471-2180-9-123
54. Woodmansey EJ. Intestinal bacteria and ageing. J Appl Microbiol [Internet]. 2007 May [cited 2018 Aug 22];102(5):1178–86. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17448153
55. Hébuterne X. Gut changes attributed to ageing: effects on intestinal microflora. Curr Opin Clin Nutr Metab Care [Internet]. 2003 Jan [cited 2018 Aug 22];6(1):49–54. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12496680
56. Kirjavainen* P V., Kalliomäki M, Salminen SJ, Isolauri E. Postnatal effects of obstetrical epidural anesthesia on allergic sensitization. Allergy [Internet]. Wiley/Blackwell (10.1111); 2007 Jan 1 [cited 2018 Aug 22];62(1):88–9. Available from: http://doi.wiley.com/10.1111/j.1398-9995.2006.01259.x
57. Heikkilä MP, Saris PEJ. Inhibition of Staphylococcus aureus by the commensal bacteria of human milk. J Appl Microbiol [Internet]. 2003 [cited 2018 Aug 22];95(3):471–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12911694

58. Dominguez-bello MG, Costello EK, Contreras M, Magris M, Hidalgo G. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. 2010;107(26):11971–5.
59. Martín R, Heilig GHJ, Zoetendal EG, Smidt H, Rodríguez JM. Diversity of the Lactobacillus group in breast milk and vagina of healthy women and potential role in the colonization of the infant gut. J Appl Microbiol [Internet]. Wiley/Blackwell (10.1111); 2007 Jul 20 [cited 2018 Aug 22];103(6):2638–44. Available from: http://doi.wiley.com/10.1111/j.1365-2672.2007.03497.x
60. Liepke C, Adermann K, Raida M, Mägert H-J, Forssmann W-G, Zucht H-D. Human milk provides peptides highly stimulating the growth of bifidobacteria. Eur J Biochem [Internet]. Wiley/Blackwell (10.1111); 2002 Feb 1 [cited 2018 Aug 23];269(2):712–8. Available from: http://doi.wiley.com/10.1046/j.0014-2956.2001.02712.x
61. Dethlefsen L, Huse S, Sogin ML, Relman DA. The Pervasive Effects of an Antibiotic on the Human Gut Microbiota, as Revealed by Deep 16S rRNA Sequencing. Eisen JA, editor. PLoS Biol [Internet]. 2008 Nov 18 [cited 2018 Aug 22];6(11):e280. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19018661
62. Jernberg C, Löfmark S, Edlund C, Jansson JK. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. ISME J [Internet]. 2007 May 1 [cited 2018 Aug 23];1(1):56–66. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18043614
63. Löfmark S, Jernberg C, Billström H, Andersson DI, Edlund C. Restored fitness leads to long-term persistence of resistant Bacteroides strains in the human intestine. Anaerobe [Internet]. 2008 Jun [cited 2018 Aug 23];14(3):157–60. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18434218
64. Sjölund M, Wreiber K, Andersson DI, Blaser MJ, Engstrand L. Long-term persistence of resistant Enterococcus species after antibiotics to eradicate Helicobacter pylori. Ann Intern Med [Internet]. 2003 Sep 16 [cited 2018 Aug 23];139(6):483–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/13679325
65. Bernalier-Donadille A. Fermentative metabolism by the human gut microbiota. Gastroentérologie Clin Biol [Internet]. Elsevier Masson; 2010 Sep 1 [cited 2018 Oct

10];34:S16–22. Available from: https://www.sciencedirect.com/science/article/pii/S0399832010700166

66. Briefel RR, Johnson CL. Secular trends in dietary intake in the United States. Annu Rev Nutr. 2004;24(1):401–31.

67. Thevaranjan N, Puchta A, Schulz C, Naidoo A, Szamosi JC, Verschoor CP, et al. Age-Associated Microbial Dysbiosis Promotes Intestinal Permeability, Systemic Inflammation, and Macrophage Dysfunction. Cell Host Microbe [Internet]. 2017 Apr 12 [cited 2018 Jan 6];21(4):455–466.e4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28407483

68. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci [Internet]. 2005 Aug 2 [cited 2018 Oct 10];102(31):11070–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16033867

69. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature [Internet]. 2006 Dec 21 [cited 2018 May 1];444(7122):1027–131. Available from: http://www.nature.com/doifinder/10.1038/nature05414

70. Turnbaugh PJ, Bäckhed F, Fulton L, Gordon JI. Diet-Induced Obesity Is Linked to Marked but Reversible Alterations in the Mouse Distal Gut Microbiome. Cell Host Microbe [Internet]. 2008 Apr 17 [cited 2018 Apr 20];3(4):213–23. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18407065

71. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med [Internet]. NIH Public Access; 2009 Nov 11 [cited 2018 Oct 10];1(6):6ra14. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20368178

72. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic Endotoxemia Initiates Obesity and Insulin Resistance. Diabetes [Internet]. 2007 Jul 1 [cited 2018 Oct 10];56(7):1761–72. Available from: http://diabetes.diabetesjournals.org/cgi/doi/10.2337/db06-1491

73. Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, et al. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia [Internet]. 2007

Oct 1 [cited 2018 Oct 10];50(11):2374–83. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17823788

74. Neyrinck AM, Possemiers S, Verstraete W, De Backer F, Cani PD, Delzenne NM. Dietary modulation of clostridial cluster XIVa gut bacteria (Roseburia spp.) by chitin–glucan fiber improves host metabolic alterations induced by high-fat diet in mice. J Nutr Biochem [Internet]. 2012 Jan [cited 2018 Oct 10];23(1):51–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21411304

75. Nava GM, Carbonero F, Ou J, Benefiel AC, O'Keefe SJ, Gaskins HR. Hydrogenotrophic microbiota distinguish native Africans from African and European Americans. Environ Microbiol Rep [Internet]. 2012 Jun [cited 2018 Oct 10];4(3):307–15. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23760794

76. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y, Keilbaugh SA, et al. Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes. Science (80-) [Internet]. 2011 Oct 7 [cited 2018 Oct 10];334(6052):105–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21885731

77. Conlon MA, Bird AR. The impact of diet and lifestyle on gut microbiota and human health. Nutrients [Internet]. Multidisciplinary Digital Publishing Institute (MDPI); 2014 Dec 24 [cited 2018 Oct 10];7(1):17–44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25545101

78. Louis P, Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiol Lett [Internet]. 2009 May [cited 2018 Oct 10];294(1):1–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19222573

79. Segain JP, Raingeard de la Blétière D, Bourreille A, Leray V, Gervois N, Rosales C, et al. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. Gut [Internet]. 2000 Sep [cited 2018 Oct 10];47(3):397–403. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10940278

80. Fava F, Danese S. Intestinal microbiota in inflammatory bowel disease: Friend of foe? World J Gastroenterol [Internet]. 2011 Feb 7 [cited 2018 Oct 10];17(5):557. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21350704

81. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nat Rev Gastroenterol Hepatol [Internet]. Nature Publishing Group; 2017 Jun 14 [cited 2018 Jul 11];14(8):491. Available from: http://www.nature.com/doifinder/10.1038/nrgastro.2017.75
82. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol [Internet]. Nature Publishing Group; 2014 Aug 10 [cited 2018 Jul 11];11(8):506–14. Available from: http://www.nature.com/articles/nrgastro.2014.66
83. Ontario L. Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food Guidelines for the Evaluation of Probiotics in Food Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food. [cited 2018 Apr 21]; Available from: http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf
84. Suez J, Korem T, Zeevi D, Zilberman-Schapira G, Thaiss CA, Maza O, et al. Artificial sweeteners induce glucose intolerance by altering the gut microbiota. Nature. Nature Research; 2014 Sep;514(7521):181.
85. Krishnan S, Alden N, Lee K. Pathways and functions of gut microbiota metabolism impacting host physiology. Curr Opin Biotechnol [Internet]. NIH Public Access; 2015 Dec [cited 2018 Jul 10];36:137–45. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26340103
86. Koboziev I, Reinoso Webb C, Furr KL, Grisham MB. Role of the enteric microbiota in intestinal homeostasis and inflammation. Free Radic Biol Med [Internet]. 2014 Mar [cited 2018 Oct 10];68:122–33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24275541
87. Chung H, Pamp SJ, Hill JA, Surana NK, Edelman SM, Troy EB, et al. Gut Immune Maturation Depends on Colonization with a Host-Specific Microbiota. Cell [Internet]. 2012 Jun 22 [cited 2018 Oct 10];149(7):1578–93. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/22726443

88. Ivanov II, Littman DR. Modulation of immune homeostasis by commensal bacteria. Curr Opin Microbiol [Internet]. 2011 Feb [cited 2018 Oct 10];14(1):106–14. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21215684

89. Alam M, Midtvedt T, Uribe A. Differential cell kinetics in the ileum and colon of germfree rats. Scand J Gastroenterol [Internet]. 1994 May [cited 2018 Oct 10];29(5):445–51. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8036460

90. Gustafsson BE, Midtvedt T, Strandberg K. Effects of microbial contamination on the cecum enlargement of germfree rats. Scand J Gastroenterol [Internet]. 1970 [cited 2018 Oct 10];5(4):309–14. Available from: http://www.ncbi.nlm.nih.gov/pubmed/5429896

91. Walters WA, Xu Z, Knight R. Meta-analyses of human gut microbes associated with obesity and IBD. FEBS Lett. Federation of European Biochemical Societies; 2014 Oct;588(22):4223–33.

92. Dicksved J, Halfvarson J, Rosenquist M, Järnerot G, Tysk C, Apajalahti J, et al. Molecular analysis of the gut microbiota of identical twins with Crohn's disease. ISME J [Internet]. 2008 Jul 10 [cited 2018 Apr 20];2(7):716–27. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18401439

93. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci U S A [Internet]. National Academy of Sciences; 2007 Aug 21 [cited 2018 Apr 20];104(34):13780–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17699621

94. Rodiño-Janeiro BK, Vicario M, Alonso-Cotoner C, Pascua-García R, Santos J. A Review of Microbiota and Irritable Bowel Syndrome: Future in Therapies. Adv Ther [Internet]. 2018 Mar 1 [cited 2018 Apr 20];35(3):289–310. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29498019

95. Wu X, Ma C, Han L, Nawaz M, Gao F, Zhang X, et al. Molecular Characterisation of the Faecal Microbiota in Patients with Type II Diabetes. Curr Microbiol [Internet]. 2010 Jul 20 [cited 2018 Apr 20];61(1):69–78. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20087741

96. Arrieta M-C, Arévalo A, Stiemsma L, Dimitriu P, Chico ME, Loor S, et al. Associations between infant fungal and bacterial dysbiosis and childhood atopic wheeze in a nonindustrialized setting. J Allergy Clin Immunol [Internet]. 2017 Dec 30 [cited 2018 Apr 20]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/29241587
97. Ohigashi S, Sudo K, Kobayashi D, Takahashi O, Takahashi T, Asahara T, et al. Changes of the intestinal microbiota, short chain fatty acids, and fecal pH in patients with colorectal cancer. Dig Dis Sci. 2013;58(6):1717–26.
98. Lupton JR. Microbial Degradation Products Influence Colon Cancer Risk: the Butyrate Controversy. J Nutr [Internet]. 2004 Feb 1 [cited 2018 Apr 20];134(2):479–82. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14747692
99. Lau LHS, Wong SH. Microbiota, Obesity and NAFLD. In: Advances in experimental medicine and biology [Internet]. 2018 [cited 2018 Jul 10]. p. 111–25. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29956210
100. Jia W, Rajani C. The Influence of Gut Microbial Metabolism on the Development and Progression of Non-alcoholic Fatty Liver Disease. In: Advances in experimental medicine and biology [Internet]. 2018 [cited 2018 Jul 10]. p. 95–110. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29956209
101. Gonzalez A, Stombaugh J, Lozupone C, Turnbaugh PJ, Gordon JI, Knight R. The mind-body-microbial continuum. Dialogues Clin Neurosci [Internet]. 2011 [cited 2018 Apr 20];13(1):55–62. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21485746
102. Pulikkan J, Maji A, Dhakan DB, Saxena R, Mohan B, Anto MM, et al. Gut Microbial Dysbiosis in Indian Children with Autism Spectrum Disorders. Microb Ecol [Internet]. 2018 Mar 21 [cited 2018 Apr 20]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/29564487
103. Andoh A, Kuzuoka H, Tsujikawa T, Nakamura S, Hirai F, Suzuki Y, et al. Multicenter analysis of fecal microbiota profiles in Japanese patients with Crohn's disease. J Gastroenterol. 2012;47(12):1298–307.
104. Ott SJ, Musfeldt M, Wenderoth DF, Hampe J, Brant O, Fölsch UR, et al. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. Gut. 2004;53(5):685–93.

105. Joossens M, Huys G, Cnockaert M, De Preter V, Verbeke K, Rutgeerts P, et al. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. Gut. 2011;60(5):631–7.
106. Sokol H, Seksik P, Furet JP, Firmesse O, Nion-Larmurier I, Beaugerie L, et al. Low counts of Faecalibacterium prausnitzii in colitis microbiota. Inflamm Bowel Dis. 2009;15(8):1183–9.
107. Sokol H, Lepage P, Seksik P, Doré J, Marteau P. Temperature gradient gel electrophoresis of fecal 16S rRNA reveals active Escherichia coli in the microbiota of patients with ulcerative colitis. J Clin Microbiol. 2006;44(9):3172–7.
108. Darfeuille-Michaud A, Boudeau J, Bulois P, Neut C, Glasser AL, Barnich N, et al. High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. Gastroenterology. 2004;127(2):412–21.
109. Rodemann JF, Dubberke ER, Reske KA, Seo DH, Stone CD. Incidence of Clostridium difficile infection in inflammatory bowel disease. Clin Gastroenterol Hepatol. 2007;5(3):339–44.
110. Huda-Faujan N, Abdulamir AS, Fatimah AB, Anas OM, Shuhaimi M, Yazid AM, et al. The impact of the level of the intestinal short chain fatty acids in inflammatory bowel disease patients versus healthy subjects. Open Biochem J. 2010;4(Ic):53–8.
111. Taurog JD, Richardson JA, Croft JT, Simmons WA, Zhou M, Fernández-Sueiro JL, et al. The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. J Exp Med [Internet]. 1994 Dec 1 [cited 2018 Jan 6];180(6):2359–64. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7964509
112. Dianda L, Hanby AM, Wright NA, Sebesteny A, Hayday AC, Owen MJ. T cell receptor-alpha beta-deficient mice fail to develop colitis in the absence of a microbial environment. Am J Pathol [Internet]. American Society for Investigative Pathology; 1997 Jan [cited 2018 Jan 6];150(1):91–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9006326
113. Hudcovic T, Stěpánková R, Cebra J, Tlaskalová-Hogenová H. The role of microflora in the development of intestinal inflammation: acute and chronic colitis induced by dextran sulfate in germ-free and conventionally reared immunocompetent and immunodeficient mice. Folia Microbiol (Praha) [Internet]. 2001 [cited 2018 Jan 6];46(6):565–72. Available

from: http://www.ncbi.nlm.nih.gov/pubmed/11898350

114. Gophna U, Sommerfeld K, Gophna S, Doolittle WF, Veldhuyzen van Zanten SJO. Differences between Tissue-Associated Intestinal Microfloras of Patients with Crohn's Disease and Ulcerative Colitis. J Clin Microbiol [Internet]. 2006 Nov 1 [cited 2018 Jan 6];44(11):4136–41. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16988016
115. Frank DN, St. Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci [Internet]. 2007 Aug 21 [cited 2018 Jan 6];104(34):13780–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17699621
116. Perše M, Cerar A. Dextran sodium sulphate colitis mouse model: traps and tricks. J Biomed Biotechnol [Internet]. 2012 [cited 2018 May 7];2012:718617. Available from: http://www.hindawi.com/journals/bmri/2012/718617/
117. Martín R, Chain F, Miquel S, Motta J-P, Vergnolle N, Sokol H, et al. Using murine colitis models to analyze probiotics–host interactions. FEMS Microbiol Rev [Internet]. 2017 Aug 1 [cited 2018 Apr 21];41(Supp_1):S49–70. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28830096
118. Wirtz S, Neufert C, Weigmann B, Neurath MF. Chemically induced mouse models of intestinal inflammation. Nat Protoc [Internet]. Nature Publishing Group; 2007 Mar 1 [cited 2018 Oct 2];2(3):541–6. Available from: http://www.nature.com/articles/nprot.2007.41
119. Kawada M, Arihiro A, Mizoguchi E. Insights from advances in research of chemically induced experimental models of human inflammatory bowel disease. World J Gastroenterol [Internet]. 2007 Nov 14 [cited 2018 Oct 2];13(42):5581–93. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17948932
120. Dieleman LA, Ridwan BU, Tennyson GS, Beagley KW, Bucy RP, Elson CO. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. Gastroenterology [Internet]. W.B. Saunders; 1994 Dec 1 [cited 2018 Oct 2];107(6):1643–52. Available from: https://www.sciencedirect.com/science/article/pii/0016508594908036
121. Yoda K, Miyazawa K, Hosoda M, Hiramatsu M, Yan F, He F. Lactobacillus GG-fermented milk prevents DSS-induced colitis and regulates intestinal epithelial

homeostasis through activation of epidermal growth factor receptor. Eur J Nutr [Internet]. NIH Public Access; 2014 Feb [cited 2018 Oct 16];53(1):105–15. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23468308

122. Sham HP, Yu EYS, Gulen MF, Bhinder G, Stahl M, Chan JM, et al. SIGIRR, a Negative Regulator of TLR/IL-1R Signalling Promotes Microbiota Dependent Resistance to Colonization by Enteric Bacterial Pathogens. Philpott DJ, editor. PLoS Pathog [Internet]. Public Library of Science; 2013 Aug 8 [cited 2018 Oct 2];9(8):e1003539. Available from: http://dx.plos.org/10.1371/journal.ppat.1003539
123. Loh G, Blaut M. Role of commensal gut bacteria in inflammatory bowel diseases. Gut Microbes [Internet]. 2012 Nov 16 [cited 2018 Oct 2];3(6):544–55. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23060017
124. ECKMANN L. Animal Models of Inflammatory Bowel Disease: Lessons from Enteric Infections. Ann N Y Acad Sci [Internet]. 2006 Aug 1 [cited 2018 Oct 2];1072(1):28–38. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17057188
125. Mundy R, MacDonald TT, Dougan G, Frankel G, Wiles S. Citrobacter rodentium of mice and man. Cell Microbiol [Internet]. 2005 Dec [cited 2018 Oct 2];7(12):1697–706. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16309456
126. Bhinder G, Sham HP, Chan JM, Morampudi V, Jacobson K, Vallance BA. The Citrobacter rodentium mouse model: studying pathogen and host contributions to infectious colitis. J Vis Exp [Internet]. MyJoVE Corporation; 2013 Feb 19 [cited 2018 Aug 13];(72):e50222. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23462619
127. Petty NK, Bulgin R, Crepin VF, Cerdeno-Tarraga AM, Schroeder GN, Quail MA, et al. The Citrobacter rodentium Genome Sequence Reveals Convergent Evolution with Human Pathogenic Escherichia coli. J Bacteriol [Internet]. 2010 Jan 15 [cited 2018 Oct 2];192(2):525–38. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19897651
128. Vallance BA, Deng W, Jacobson K, Finlay BB. Host susceptibility to the attaching and effacing bacterial pathogen Citrobacter rodentium. Infect Immun [Internet]. 2003 Jun [cited 2018 Oct 2];71(6):3443–53. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12761129
129. Bosman ES, Chan JM, Bhullar K, Vallance BA. Investigation of Host and Pathogen

Contributions to Infectious Colitis Using the Citrobacter rodentium Mouse Model of Infection. In: Methods in molecular biology (Clifton, NJ) [Internet]. 2016 [cited 2018 Oct 2]. p. 225–41. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27246037

130. Bergstrom KSB, Kissoon-Singh V, Gibson DL, Ma C, Montero M, Sham HP, et al. Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa. PLoS Pathog [Internet]. Public Library of Science; 2010 May 13 [cited 2018 Aug 13];6(5):e1000902. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20485566
131. Linden SK, Sutton P, Karlsson NG, Korolik V, McGuckin MA. Mucins in the mucosal barrier to infection. Mucosal Immunol [Internet]. 2008 May 5 [cited 2018 Oct 2];1(3):183–97. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19079178
132. Dharmani P, Srivastava V, Kissoon-Singh V, Chadee K. Role of Intestinal Mucins in Innate Host Defense Mechanisms against Pathogens. J Innate Immun [Internet]. 2009 [cited 2018 Oct 2];1(2):123–35. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20375571
133. Lebeer S, Vanderleyden J, De Keersmaecker SCJ. Genes and Molecules of Lactobacilli Supporting Probiotic Action. Microbiol Mol Biol Rev [Internet]. 2008 Dec 1 [cited 2018 Jan 6];72(4):728–64. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19052326
134. Borchers AT, Selmi C, Meyers FJ, Keen CL, Gershwin ME. Probiotics and immunity. J Gastroenterol [Internet]. 2009 Jan 22 [cited 2018 Jan 6];44(1):26–46. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19159071
135. Wells JM. Immunomodulatory mechanisms of lactobacilli. Microb Cell Fact [Internet]. 2011 Aug 30 [cited 2018 Jan 7];10(Suppl 1):S17. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21995674
136. Mann ER, You J, Horneffer-van der Sluis V, Bernardo D, Omar Al-Hassi H, Landy J, et al. Dysregulated Circulating Dendritic Cell Function in Ulcerative Colitis Is Partially Restored by Probiotic Strain *Lactobacillus casei* Shirota. Mediators Inflamm [Internet]. 2013 [cited 2018 Jan 7];2013:1–12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23970814
137. Mann ER, Bernardo D, Ng SC, Rigby RJ, Al-Hassi HO, Landy J, et al. Human Gut

Dendritic Cells Drive Aberrant Gut-specific T-cell Responses in Ulcerative Colitis, Characterized by Increased IL-4 Production and Loss of IL-22 and IFNγ. Inflamm Bowel Dis [Internet]. 2014 Dec [cited 2018 Jan 7];20(12):2299–307. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25397892

138. Wu Y, Zhu C, Chen Z, Chen Z, Zhang W, Ma X, et al. Protective effects of Lactobacillus plantarum on epithelial barrier disruption caused by enterotoxigenic Escherichia coli in intestinal porcine epithelial cells. Vet Immunol Immunopathol [Internet]. 2016 Apr [cited 2018 Jan 7];172:55–63. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27032504
139. Roselli M, Finamore A, Nuccitelli S, Carnevali P, Brigidi P, Vitali B, et al. Prevention of TNBS-induced colitis by different Lactobacillus and Bifidobacterium strains is associated with an expansion of γδT and regulatory T cells of intestinal intraepithelial lymphocytes. Inflamm Bowel Dis [Internet]. 2009 Oct [cited 2018 Jan 7];15(10):1526–36. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19504616
140. Hacini-Rachinel F, Nancey S, Boschetti G, Sardi F, Doucet-Ladeveze R, Durand P-Y, et al. CD4+ T Cells and Lactobacillus casei Control Relapsing Colitis Mediated by CD8+ T Cells. J Immunol [Internet]. 2009 Nov 1 [cited 2018 Jan 7];183(9):5477–86. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19843933
141. Dai C, Zheng C-Q, Meng F, Zhou Z, Sang L, Jiang M. VSL#3 probiotics exerts the anti-inflammatory activity via PI3k/Akt and NF-κB pathway in rat model of DSS-induced colitis. Mol Cell Biochem [Internet]. 2013 Feb 23 [cited 2018 Jan 7];374(1–2):1–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23271629
142. Salim SY, Young PY, Lukowski CM, Madsen KL, Sis B, Churchill TA, et al. VSL#3 probiotics provide protection against acute intestinal ischaemia/reperfusion injury. Benef Microbes [Internet]. 2013 Dec 1 [cited 2018 Jan 7];4(4):357–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24240573
143. Talero E, Bolivar S, Ávila-Román J, Alcaide A, Fiorucci S, Motilva V. Inhibition of Chronic Ulcerative Colitis-associated Adenocarcinoma Development in Mice by VSL#3. Inflamm Bowel Dis [Internet]. 2015 May [cited 2018 Jan 7];21(5):1027–37. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25793324
144. Schultz M, Veltkamp C, Dieleman LA, Grenther WB, Wyrick PB, Tonkonogy SL, et al.

Lactobacillus plantarum 299V in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice. Inflamm Bowel Dis [Internet]. 2002 Mar [cited 2018 Jan 7];8(2):71–80. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11854603

145. DuPont A, Richards DM, Jelinek KA, Krill J, Rahimi E, Ghouri Y. Systematic review of randomized controlled trials of probiotics, prebiotics, and synbiotics in inflammatory bowel disease. Clin Exp Gastroenterol [Internet]. 2014 Dec [cited 2018 Jan 7];7:473. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25525379
146. Whelan K, Quigley EMM. Probiotics in the management of irritable bowel syndrome and inflammatory bowel disease. Curr Opin Gastroenterol [Internet]. 2013 Mar [cited 2018 Jan 7];29(2):184–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23286925
147. Veerappan GR, Betteridge J, Young PE. Probiotics for the Treatment of Inflammatory Bowel Disease. Curr Gastroenterol Rep [Internet]. 2012 Aug 13 [cited 2018 Jan 7];14(4):324–33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22581276
148. Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, et al. Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. Science [Internet]. 2000 Aug 25 [cited 2018 Jan 7];289(5483):1352–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10958782
149. Braat H, Rottiers P, Hommes DW, Huyghebaert N, Remaut E, Remon J, et al. A Phase I Trial With Transgenic Bacteria Expressing Interleukin-10 in Crohn's Disease. Clin Gastroenterol Hepatol [Internet]. 2006 Jun [cited 2018 Jan 7];4(6):754–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16716759
150. Vandenbroucke K, Hans W, Van Huysse J, Neirynck S, Demetter P, Remaut E, et al. Active delivery of trefoil factors by genetically modified Lactococcus lactis prevents and heals acute colitis in mice. Gastroenterology [Internet]. 2004 Aug [cited 2018 Jan 7];127(2):502–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15300583
151. Maldonado-Gómez MX, Martínez I, Bottacini F, O'Callaghan A, Ventura M, van Sinderen D, et al. Stable Engraftment of Bifidobacterium longum AH1206 in the Human Gut Depends on Individualized Features of the Resident Microbiome. Cell Host Microbe [Internet]. 2016 Oct 12 [cited 2018 Jan 7];20(4):515–26. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27693307

152. Zmora N, Zeevi D, Korem T, Segal E, Elinav E. Taking it Personally: Personalized Utilization of the Human Microbiome in Health and Disease. Cell Host Microbe [Internet]. Elsevier; 2016 Jan 13 [cited 2018 Jan 10];19(1):12–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26764593
153. Jameson JL, Longo DL. Precision Medicine — Personalized, Problematic, and Promising. N Engl J Med [Internet]. 2015 Jun 4 [cited 2018 Apr 21];372(23):2229–34. Available from: http://www.nejm.org/doi/10.1056/NEJMsb1503104
154. Rebbeck TR, Friebel T, Lynch HT, Neuhausen SL, van 't Veer L, Garber JE, et al. Bilateral prophylactic mastectomy reduces breast cancer risk in BRCA1 and BRCA2 mutation carriers: the PROSE Study Group. J Clin Oncol [Internet]. American Society of Clinical Oncology; 2004 Mar 15 [cited 2018 May 1];22(6):1055–62. Available from: http://ascopubs.org/doi/10.1200/JCO.2004.04.188
155. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature [Internet]. Nature Publishing Group; 2009 Jan 22 [cited 2018 May 1];457(7228):480–4. Available from: http://www.nature.com/doifinder/10.1038/nature07540
156. Arrieta M-C, Stiemsma LT, Dimitriu PA, Thorson L, Russell S, Yurist-Doutsch S, et al. Early infancy microbial and metabolic alterations affect risk of childhood asthma. Sci Transl Med [Internet]. American Association for the Advancement of Science; 2015 Sep 30 [cited 2018 May 1];7(307):307ra152. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26424567
157. Taneja V. Arthritis susceptibility and the gut microbiome. FEBS Lett [Internet]. Wiley-Blackwell; 2014 Nov 17 [cited 2018 May 1];588(22):4244–9. Available from: http://doi.wiley.com/10.1016/j.febslet.2014.05.034
158. Cornick S, Tawiah A, Chadee K. Roles and regulation of the mucus barrier in the gut. Tissue barriers [Internet]. Taylor & Francis; 2015 [cited 2018 Oct 6];3(1–2):e982426. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25838985
159. Johansson ME V, Sjövall H, Hansson GC. The gastrointestinal mucus system in health and disease. Nat Rev Gastroenterol Hepatol [Internet]. NIH Public Access; 2013 Jun [cited 2018 Oct 7];10(6):352–61. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/23478383

160. Johansson ME V., Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc Natl Acad Sci [Internet]. 2008 Sep 30 [cited 2018 Oct 7];105(39):15064–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18806221

161. van Klinken BJ, Einerhand AW, Duits LA, Makkink MK, Tytgat KM, Renes IB, et al. Gastrointestinal expression and partial cDNA cloning of murine Muc2. Am J Physiol [Internet]. 1999 Jan [cited 2018 Oct 16];276(1 Pt 1):G115-24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9886986

162. Tytgat KM, Büller HA, Opdam FJ, Kim YS, Einerhand AW, Dekker J. Biosynthesis of human colonic mucin: Muc2 is the prominent secretory mucin. Gastroenterology [Internet]. 1994 Nov [cited 2018 Oct 16];107(5):1352–63. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7926500

163. Hattrup CL, Gendler SJ. Structure and Function of the Cell Surface (Tethered) Mucins. Annu Rev Physiol [Internet]. 2008 Mar [cited 2018 Oct 16];70(1):431–57. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17850209

164. Jonckheere N, Skrypek N, Van Seuningen I. Mucins and pancreatic cancer. Cancers (Basel) [Internet]. Multidisciplinary Digital Publishing Institute (MDPI); 2010 Oct 25 [cited 2018 Oct 21];2(4):1794–812. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24281201

165. Perez-Vilar J, Hill RL. The structure and assembly of secreted mucins. J Biol Chem [Internet]. 1999 Nov 5 [cited 2018 Oct 16];274(45):31751–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10542193

166. McGuckin MA, Lindén SK, Sutton P, Florin TH. Mucin dynamics and enteric pathogens. Nat Rev Microbiol [Internet]. 2011 Apr 1 [cited 2018 Oct 16];9(4):265–78. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21407243

167. Johansson ME V., Larsson JMH, Hansson GC. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. Proc Natl Acad Sci [Internet]. 2011 Mar 15 [cited 2018 Oct 16];108(Supplement_1):4659–65. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/20615996

168. Specian RD, Oliver MG. Functional biology of intestinal goblet cells. Am J Physiol Physiol [Internet]. 1991 Feb [cited 2018 Oct 16];260(2):C183–93. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1996606

169. Arike L, Hansson GC. The Densely O-Glycosylated MUC2 Mucin Protects the Intestine and Provides Food for the Commensal Bacteria. J Mol Biol [Internet]. NIH Public Access; 2016 [cited 2018 May 3];428(16):3221–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26880333

170. Bennett EP, Mandel U, Clausen H, Gerken TA, Fritz TA, Tabak LA. Control of mucin-type O-glycosylation: A classification of the polypeptide GalNAc-transferase gene family. Glycobiology [Internet]. 2012 Jun 1 [cited 2018 Oct 16];22(6):736–56. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22183981

171. Bergstrom KSB, Xia L. Mucin-type O-glycans and their roles in intestinal homeostasis. Glycobiology [Internet]. Oxford University Press; 2013 Sep [cited 2018 Aug 13];23(9):1026–37. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23752712

172. Thomsson KA, Holmén-Larsson JM, Ångström J, Johansson ME, Xia L, Hansson GC. Detailed O-glycomics of the Muc2 mucin from colon of wild-type, core 1- and core 3-transferase-deficient mice highlights differences compared with human MUC2. Glycobiology [Internet]. 2012 Aug [cited 2018 Oct 16];22(8):1128–39. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22581805

173. Holmén Larsson JM, Karlsson H, Sjövall H, Hansson GC. A complex, but uniform O-glycosylation of the human MUC2 mucin from colonic biopsies analyzed by nanoLC/MSn. Glycobiology [Internet]. 2009 Jul [cited 2018 Oct 16];19(7):756–66. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19321523

174. Rokhsefat S, Lin A, Comelli EM. Mucin–Microbiota Interaction During Postnatal Maturation of the Intestinal Ecosystem: Clinical Implications. Dig Dis Sci [Internet]. Springer US; 2016 Jun 20 [cited 2018 Oct 20];61(6):1473–86. Available from: http://link.springer.com/10.1007/s10620-016-4032-6

175. Johansson MEV, Jakobsson HE, Holmén-Larsson J, Schütte A, Ermund A, Rodríguez-Piñeiro AM, et al. Normalization of Host Intestinal Mucus Layers Requires Long-Term

Microbial Colonization. Cell Host Microbe [Internet]. Elsevier; 2015 Nov 11 [cited 2018 Oct 20];18(5):582–92. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1931312815004175

176. Arike L, Holmén-Larsson J, Hansson GC. Intestinal Muc2 mucin O-glycosylation is affected by microbiota and regulated by differential expression of glycosyltranferases. Glycobiology [Internet]. Oxford University Press; 2017 Apr 1 [cited 2018 May 3];27(4):318–28. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28122822
177. Morampudi V, Dalwadi U, Bhinder G, Sham HP, Gill SK, Chan J, et al. The goblet cell-derived mediator RELM-β drives spontaneous colitis in Muc2-deficient mice by promoting commensal microbial dysbiosis. Mucosal Immunol [Internet]. 2016 Sep 27 [cited 2018 Jul 25];9(5):1218–33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26813339
178. Tailford LE, Crost EH, Kavanaugh D, Juge N. Mucin glycan foraging in the human gut microbiome. Front Genet [Internet]. 2015 Mar 19 [cited 2018 Oct 20];6:81. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25852737
179. Martens EC, Chiang HC, Gordon JI. Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. Cell Host Microbe [Internet]. NIH Public Access; 2008 Nov 13 [cited 2018 Oct 21];4(5):447–57. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18996345
180. Xu J, Bjursell MK, Himrod J, Deng S, Carmichael LK, Chiang HC, et al. A Genomic View of the Human-Bacteroides thetaiotaomicron Symbiosis. Science (80-) [Internet]. 2003 Mar 28 [cited 2018 Oct 21];299(5615):2074–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12663928
181. Collado MC, Derrien M, Isolauri E, de Vos WM, Salminen S. Intestinal integrity and Akkermansia muciniphila, a mucin-degrading member of the intestinal microbiota present in infants, adults, and the elderly. Appl Environ Microbiol [Internet]. American Society for Microbiology (ASM); 2007 Dec [cited 2018 Oct 21];73(23):7767–70. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17933936
182. Derrien M, Collado MC, Ben-Amor K, Salminen S, de Vos WM. The Mucin degrader Akkermansia muciniphila is an abundant resident of the human intestinal tract. Appl

Environ Microbiol [Internet]. American Society for Microbiology (ASM); 2008 Mar [cited 2018 Oct 21];74(5):1646–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18083887

183. Derrien M, Belzer C, de Vos WM. Akkermansia muciniphila and its role in regulating host functions. Microb Pathog [Internet]. 2017 May [cited 2018 Jan 10];106:171–81. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26875998

184. Fujita K, Oura F, Nagamine N, Katayama T, Hiratake J, Sakata K, et al. Identification and Molecular Cloning of a Novel Glycoside Hydrolase Family of Core 1 Type *O* -Glycan-specific Endo-α- *N* -acetylgalactosaminidase from *Bifidobacterium longum*. J Biol Chem [Internet]. 2005 Nov 11 [cited 2018 Oct 22];280(45):37415–22. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16141207

185. Turroni F, Bottacini F, Foroni E, Mulder I, Kim J-H, Zomer A, et al. Genome analysis of Bifidobacterium bifidum PRL2010 reveals metabolic pathways for host-derived glycan foraging. Proc Natl Acad Sci [Internet]. 2010 Nov 9 [cited 2018 Oct 22];107(45):19514–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20974960

186. Sun M, Wu W, Liu Z, Cong Y. Microbiota metabolite short chain fatty acids, GPCR, and inflammatory bowel diseases. J Gastroenterol [Internet]. Springer Japan; 2017 Jan 23 [cited 2018 Oct 22];52(1):1–8. Available from: http://link.springer.com/10.1007/s00535-016-1242-9

187. Rivera-Chávez F, Zhang LF, Faber F, Lopez CA, Byndloss MX, Olsan EE, et al. Depletion of Butyrate-Producing Clostridia from the Gut Microbiota Drives an Aerobic Luminal Expansion of Salmonella. Cell Host Microbe [Internet]. Elsevier; 2016 Apr 13 [cited 2018 Jan 15];19(4):443–54. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1931312816300968

188. Kelly CJ, Zheng L, Campbell EL, Saeedi B, Scholz CC, Bayless AJ, et al. Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function. Cell Host Microbe [Internet]. Cell Press; 2015 May 13 [cited 2018 Oct 22];17(5):662–71. Available from: https://www.sciencedirect.com/science/article/pii/S1931312815001225

189. Levy M, Kolodziejczyk AA, Thaiss CA, Elinav E. Dysbiosis and the immune system. Nat

Rev Immunol [Internet]. 2017 Apr 6 [cited 2018 Jul 10];17(4):219–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28260787

190. Petersen C, Round JL. Defining dysbiosis and its influence on host immunity and disease. Cell Microbiol [Internet]. Wiley-Blackwell; 2014 Jul [cited 2018 Jul 10];16(7):1024–33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24798552
191. Hooks KB, O'Malley MA. Dysbiosis and Its Discontents. MBio [Internet]. American Society for Microbiology (ASM); 2017 [cited 2018 Jul 10];8(5). Available from: http://www.ncbi.nlm.nih.gov/pubmed/29018121
192. Vangay P, Ward T, Gerber JS, Knights D. Antibiotics, pediatric dysbiosis, and disease. Cell Host Microbe [Internet]. NIH Public Access; 2015 May 13 [cited 2018 Jul 10];17(5):553–64. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25974298
193. Borchers AT, Selmi C, Meyers FJ, Keen CL, Gershwin ME. Probiotics and immunity. J Gastroenterol [Internet]. 2009 Jan 22 [cited 2018 Apr 21];44(1):26–46. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19159071
194. Khalesi S, Bellissimo ● Nick, Vandelanotte ● Corneel, Williams S, Stanley D, Irwin C. A review of probiotic supplementation in healthy adults: helpful or hype? Eur J Clin Nutr [Internet]. [cited 2018 Apr 11]; Available from: https://doi.org/10.1038/s41430-018-0135-9
195. Fedorak RN, Madsen KL. Probiotics and the management of inflammatory bowel disease. Inflamm Bowel Dis. 2004;10(3):286–99.
196. Boirivant M, Strober W. The mechanism of action of probiotics. Curr Opin Gastroenterol [Internet]. 2007 Nov [cited 2018 Aug 12];23(6):679–92. Available from: https://insights.ovid.com/crossref?an=00001574-200711000-00016
197. Marco ML, Pavan S, Kleerebezem M. Towards understanding molecular modes of probiotic action. Curr Opin Biotechnol [Internet]. Elsevier Current Trends; 2006 Apr 1 [cited 2018 Aug 12];17(2):204–10. Available from: https://www.sciencedirect.com/science/article/pii/S0958166906000309?_rdoc=1&_fmt=high&_origin=gateway&_docanchor=&md5=b8429449ccfc9c30159a5f9aeaa92ffb
198. Celiberto LS, Bedani R, Rossi EA, Cavallini DCU. Probiotics: The Scientific Evidence in the Context of Inflammatory Bowel Disease. Crit Rev Food Sci Nutr [Internet]. 2015;0.

Available from: http://www.ncbi.nlm.nih.gov/pubmed/25996176

199. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol [Internet]. 2014 Aug 10 [cited 2018 Apr 20];11(8):506–14. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24912386

200. Reid G. Probiotics: definition, scope and mechanisms of action. Best Pract Res Clin Gastroenterol [Internet]. Baillière Tindall; 2016 Feb 1 [cited 2018 Jul 11];30(1):17–25. Available from: https://www.sciencedirect.com/science/article/pii/S1521691815001651

201. Parker EA, Roy T, D'Adamo CR, Wieland LS. Probiotics and gastrointestinal conditions: An overview of evidence from the Cochrane Collaboration. Nutrition [Internet]. Elsevier; 2018 Jan 1 [cited 2018 Jul 11];45:125–134.e11. Available from: https://www.sciencedirect.com/science/article/pii/S0899900717301351

202. Sommer F, Anderson JM, Bharti R, Raes J, Rosenstiel P. The resilience of the intestinal microbiota influences health and disease. Nat Rev Microbiol [Internet]. 2017 Jun 19 [cited 2018 Apr 20];15(10):630–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28626231

203. McNaughton SJ. Diversity and Stability of Ecological Communities: A Comment on the Role of Empiricism in Ecology [Internet]. Vol. 111, The American Naturalist. The University of Chicago PressThe American Society of Naturalists; [cited 2018 Apr 21]. p. 515–25. Available from: https://www.jstor.org/stable/2460237

204. Naeem S, Li S. Biodiversity enhances ecosystem reliability. Nature [Internet]. Nature Publishing Group; 1997 Dec 4 [cited 2018 Apr 21];390(6659):507–9. Available from: http://www.nature.com/articles/37348

205. Kort R. Personalized therapy with probiotics from the host by TripleA. Trends Biotechnol [Internet]. Elsevier Ltd; 2014;32(6):291–3. Available from: http://dx.doi.org/10.1016/j.tibtech.2014.04.002

206. Pelczar MJ. Microbiology [Internet]. Tata McGraw-Hill; 2003 [cited 2018 Apr 20]. 918 p. Available from: https://books.google.ca/books/about/Microbiology.html?id=BKq5iXPHMlcC

207. Heilig HGHJ, Zoetendal EG, Vaughan EE, Marteau P, Akkermans ADL, de Vos WM. Molecular Diversity of Lactobacillus spp . and Other Lactic Acid Bacteria in the Human Intestine as Determined by Specific Amplification of 16S Ribosomal DNA Molecular Diversity of Lactobacillus spp . and Other Lactic Acid Bacteria in the Human Intestine. Appl Environ Microbiol. 2002;68(1):114–23.
208. Vincent D, Roy D, Mondou F, Déry C. Characterization of bifidobacteria by random DNA amplification. Int J Food Microbiol [Internet]. Elsevier; 1998 Sep 8 [cited 2018 Apr 20];43(3):185–93. Available from: https://www.sciencedirect.com/science/article/pii/S0168160598001093?via%3Dihub
209. Liserre AM, Ré MI, Franco BDGM. Microencapsulation of *Bifidobacterium animalis* subsp. *lactis* in Modified Alginate-chitosan Beads and Evaluation of Survival in Simulated Gastrointestinal Conditions. Food Biotechnol [Internet]. Taylor & Francis Group ; 2007 Mar 6 [cited 2018 Apr 20];21(1):1–16. Available from: http://www.tandfonline.com/doi/abs/10.1080/08905430701191064
210. Buriti FCA, Castro IA, Saad SMI. Viability of Lactobacillus acidophilus in synbiotic guava mousses and its survival under in vitro simulated gastrointestinal conditions. Int J Food Microbiol [Internet]. 2010 Feb 28 [cited 2018 Apr 20];137(2–3):121–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20056289
211. Clinical and Laboratory Standards Institute. [cited 2018 Apr 20]; Available from: http://demo.nextlab.ir/getattachment/27407437-3d73-4048-8239-81857d68cf3d/CLSI-M2-A9.aspx
212. Performance Standards for Antimicrobial Susceptibility Testing An informational supplement for global application developed through the Clinical and Laboratory Standards Institute. [cited 2018 Apr 20]; Available from: http://ljzx.cqrmhospital.com/upfiles/201601/20160112155335884.pdf
213. Segers ME, Lebeer S. Towards a better understanding of Lactobacillus rhamnosus GG - host interactions. Microb Cell Fact [Internet]. 2014 Aug 29 [cited 2018 May 1];13(Suppl 1):S7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25186587
214. Szajewska H, Kołodziej M. Systematic review with meta-analysis: *Lactobacillus rhamnosus* GG in the prevention of antibiotic-associated diarrhoea in children and adults.

Aliment Pharmacol Ther [Internet]. 2015 Nov [cited 2018 May 1];42(10):1149–57. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26365389

215. Cooper HS, Murthy SN, Shah RS, Sedergran DJ. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. Lab Invest [Internet]. 1993 Aug [cited 2018 Jul 2];69(2):238–49. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8350599
216. Ryz NR, Lochner A, Bhullar K, Ma C, Huang T, Bhinder G, et al. Dietary vitamin D3 deficiency alters intestinal mucosal defense and increases susceptibility to Citrobacter rodentium-induced colitis. Am J Physiol Gastrointest Liver Physiol [Internet]. American Physiological Society; 2015 Nov 1 [cited 2018 Jul 2];309(9):G730-42. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26336925
217. Zarepour M, Bhullar K, Montero M, Ma C, Huang T, Velcich A, et al. The mucin Muc2 limits pathogen burdens and epithelial barrier dysfunction during Salmonella enterica serovar Typhimurium colitis. Infect Immun [Internet]. American Society for Microbiology (ASM); 2013 Oct [cited 2018 Jul 2];81(10):3672–83. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23876803
218. Eissa N, Hussein H, Wang H, Rabbi MF, Bernstein CN, Ghia J-E. Stability of Reference Genes for Messenger RNA Quantification by Real-Time PCR in Mouse Dextran Sodium Sulfate Experimental Colitis. PLoS One [Internet]. Public Library of Science; 2016 [cited 2018 Jul 2];11(5):e0156289. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27244258
219. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc [Internet]. 2008 [cited 2018 Jul 2];3(6):1101–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18546601
220. Goldstein EJC, Tyrrell KL, Citron DM. Lactobacillus Species: Taxonomic Complexity and Controversial Susceptibilities. Clin Infect Dis [Internet]. Oxford University Press; 2015 May 15 [cited 2018 May 6];60(suppl_2):S98–107. Available from: http://academic.oup.com/cid/article/60/suppl_2/S98/379146/Lactobacillus-Species-Taxonomic-Complexity-and
221. Muñoa FJ, Pares R. Selective medium for isolation and enumeration of Bifidobacterium spp. Appl Environ Microbiol [Internet]. American Society for Microbiology (ASM); 1988

Jul [cited 2018 May 6];54(7):1715–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3415235

222. Xiao L, Feng Q, Liang S, Sonne SB, Xia Z, Qiu X, et al. A catalog of the mouse gut metagenome. Nat Biotechnol [Internet]. 2015 Oct 28 [cited 2018 May 6];33(10):1103–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26414350
223. Chen S, Chen L, Chen L, Ren X, Ge H, Li B, et al. Potential probiotic characterization of Lactobacillus reuteri from traditional Chinese highland barley wine and application for room-temperature-storage drinkable yogurt. J Dairy Sci [Internet]. 2018 Apr 25 [cited 2018 May 7]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/29705426
224. Zmora N, Zilberman-Schapira G, Suez J, Mor U, Dori-Bachash M, Bashiardes S, et al. Personalized Gut Mucosal Colonization Resistance to Empiric Probiotics Is Associated with Unique Host and Microbiome Features. Cell [Internet]. Elsevier; 2018 Sep 6 [cited 2018 Sep 8];174(6):1388–1405.e21. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0092867418311024
225. De Vuyst L, Neysens P. The sourdough microflora: biodiversity and metabolic interactions. Trends Food Sci Technol [Internet]. Elsevier; 2005 Jan 1 [cited 2018 Jul 2];16(1–3):43–56. Available from: https://www.sciencedirect.com/science/article/abs/pii/S092422440400192X
226. Ehrmann MA, Müller MRA, Vogel RF. Molecular analysis of sourdough reveals Lactobacillus mindensis sp. nov. Int J Syst Evol Microbiol [Internet]. 2003 Jan 1 [cited 2018 Jul 2];53(1):7–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12656145
227. Hayford AE, Petersen A, Vogensen FK, Jakobsen M. Use of conserved randomly amplified polymorphic DNA (RAPD) fragments and RAPD pattern for characterization of Lactobacillus fermentum in Ghanaian fermented maize dough. Appl Environ Microbiol [Internet]. 1999 Jul [cited 2018 Jul 2];65(7):3213–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10388723
228. Venturi M, Guerrini S, Granchi L, Vincenzini M. Typing of Lactobacillus sanfranciscensis isolates from traditional sourdoughs by combining conventional and multiplex RAPD–PCR profiles. Int J Food Microbiol [Internet]. Elsevier; 2012 May 15 [cited 2018 Apr 23];156(2):122–6. Available from:

https://www.sciencedirect.com/science/article/pii/S0168160512001304#bb0050

229. Gueimonde M, Sánchez B, G. de los Reyes-Gavilán C, Margolles A. Antibiotic resistance in probiotic bacteria. Front Microbiol [Internet]. 2013 [cited 2018 Jul 2];4:202. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23882264

230. Zhou JS, Pillidge CJ, Gopal PK, Gill HS. Antibiotic susceptibility profiles of new probiotic Lactobacillus and Bifidobacterium strains. Int J Food Microbiol [Internet]. 2005 Feb 1 [cited 2018 Jul 3];98(2):211–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15681048

231. Mayrhofer S, Mair C, Kneifel W, Domig KJ. Susceptibility of Bifidobacteria of Animal Origin to Selected Antimicrobial Agents. Chemother Res Pract [Internet]. 2011 [cited 2018 Jul 3];2011:1–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22312561

232. Perše M, Cerar A. Dextran sodium sulphate colitis mouse model: traps and tricks. J Biomed Biotechnol [Internet]. Hindawi Limited; 2012 [cited 2018 Jul 23];2012:718617. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22665990

233. Eichele DD, Kharbanda KK. Dextran sodium sulfate colitis murine model: An indispensable tool for advancing our understanding of inflammatory bowel diseases pathogenesis. World J Gastroenterol [Internet]. Baishideng Publishing Group Inc; 2017 Sep 7 [cited 2018 Jul 23];23(33):6016–29. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28970718

234. König J, Wells J, Cani PD, García-Ródenas CL, MacDonald T, Mercenier A, et al. Human Intestinal Barrier Function in Health and Disease. Clin Transl Gastroenterol [Internet]. Nature Publishing Group; 2016 Oct 20 [cited 2018 Jul 23];7(10):e196–e196. Available from: http://www.nature.com/articles/ctg201654

235. Michielan A, D'Incà R. Intestinal Permeability in Inflammatory Bowel Disease: Pathogenesis, Clinical Evaluation, and Therapy of Leaky Gut. Mediators Inflamm [Internet]. Hindawi Limited; 2015 [cited 2018 Jul 23];2015:628157. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26582965

236. Chang J, Leong RW, Wasinger VC, Ip M, Yang M, Phan TG. Impaired Intestinal Permeability Contributes to Ongoing Bowel Symptoms in Patients With Inflammatory Bowel Disease and Mucosal Healing. Gastroenterology [Internet]. 2017 Sep [cited 2018

Jul 23];153(3):723–731.e1. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28601482

237. Fukui H. Increased Intestinal Permeability and Decreased Barrier Function: Does It Really Influence the Risk of Inflammation? Inflamm Intest Dis [Internet]. Karger Publishers; 2016 [cited 2018 Jul 23];1(3):135–45. Available from: https://www.karger.com/Article/FullText/447252

238. Mijan M Al, Lim BO. Diets, functional foods, and nutraceuticals as alternative therapies for inflammatory bowel disease: Present status and future trends. World J Gastroenterol [Internet]. Baishideng Publishing Group Inc; 2018 Jul 7 [cited 2018 Jul 23];24(25):2673–85. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29991873

239. Celiberto LS, Bedani R, Dejani NN, Ivo de Medeiros A, Sampaio Zuanon JA, Spolidorio LC, et al. Effect of a probiotic beverage consumption (Enterococcus faecium CRL 183 and Bifidobacterium longum ATCC 15707) in rats with chemically induced colitis. Smidt H, editor. PLoS One [Internet]. Public Library of Science; 2017 Apr 24 [cited 2018 May 4];12(4):e0175935. Available from: http://dx.plos.org/10.1371/journal.pone.0175935

240. Seo S, Shin JS, Lee WS, Rhee YK, Cho CW, Hong H Do, et al. Anti-colitis effect of Lactobacillus sakei K040706 via suppression of inflammatory responses in the dextran sulfate sodium-induced colitis mice model. J Funct Foods [Internet]. Elsevier Ltd; 2017;29(December):256–68. Available from: http://dx.doi.org/10.1016/j.jff.2016.12.045

241. Shigemori S, Watanabe T, Kudoh K, Ihara M, Nigar S, Yamamoto Y, et al. Oral delivery of Lactococcus lactis that secretes bioactive heme oxygenase-1 alleviates development of acute colitis in mice. Microb Cell Fact [Internet]. 2015 Dec 25 [cited 2018 Jul 23];14(1):189. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26608030

242. Park J-S, Joe I, Rhee PD, Jeong C-S, Jeong G. A lactic acid bacterium isolated from kimchi ameliorates intestinal inflammation in DSS-induced colitis. J Microbiol [Internet]. 2017 Apr 26 [cited 2018 Jul 23];55(4):304–10. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28124779

243. Camuesco D, Peran L, Comalada M, Nieto A, Di Stasi LC, Rodriguez-Cabezas ME, et al. Preventative effects of lactulose in the trinitrobenzenesulphonic acid model of rat colitis. Inflamm Bowel Dis [Internet]. 2005 Mar [cited 2018 May 29];11(3):265–71. Available

from: http://www.ncbi.nlm.nih.gov/pubmed/15735433

244. Osman N, Adawi D, Molin G, Ahrne S, Berggren A, Jeppsson B. Bifidobacterium infantis strains with and without a combination of oligofructose and inulin attenuate inflammation in DSS-induced colitis in rats. BMC Gastroenterol. 2006;6:31.

245. Krieglstein CF, Anthoni C, Cerwinka WH, Stokes KY, Russell J, Grisham MB, et al. Role of Blood- and Tissue-Associated Inducible Nitric-Oxide Synthase in Colonic Inflammation. Am J Pathol [Internet]. 2007 Feb [cited 2018 Jul 21];170(2):490–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17255317

246. Alex P, Zachos NC, Nguyen T, Gonzales L, Chen T-E, Conklin LS, et al. Distinct Cytokine Patterns Identified from Multiplex Profiles of Murine DSS and TNBS-Induced Colitis. Inflamm Bowel Dis [Internet]. 2009 Mar [cited 2018 Jul 21];15(3):341–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18942757

247. Ramasamy S, Nguyen DD, Eston MA, Alam SN, Moss AK, Ebrahimi F, et al. Intestinal alkaline phosphatase has beneficial effects in mouse models of chronic colitis. Inflamm Bowel Dis [Internet]. NIH Public Access; 2011 Feb [cited 2018 Jul 21];17(2):532–42. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20645323

248. Vallance BA, Chan C, Robertson ML, Finlay BB. Enteropathogenic and enterohemorrhagic Escherichia coli infections: emerging themes in pathogenesis and prevention. Can J Gastroenterol [Internet]. 2002 Nov [cited 2018 Aug 13];16(11):771–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12464970

249. Das S, Jayaratne R, Barrett KE. The Role of Ion Transporters in the Pathophysiology of Infectious Diarrhea. Cell Mol Gastroenterol Hepatol [Internet]. 2018 [cited 2018 Aug 13];6(1):33–45. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29928670

250. Fyderek K, Strus M, Kowalska-Duplaga K, Gosiewski T, Wedrychowicz A, Jedynak-Wasowicz U, et al. Mucosal bacterial microflora and mucus layer thickness in adolescents with inflammatory bowel disease. World J Gastroenterol [Internet]. 2009 Nov 14 [cited 2018 Aug 13];15(42):5287–94. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19908336

251. Schwerbrock NMJ, Makkink MK, van der Sluis M, Büller HA, Einerhand AWC, Sartor RB, et al. Interleukin 10-deficient mice exhibit defective colonic Muc2 synthesis before

and after induction of colitis by commensal bacteria. Inflamm Bowel Dis [Internet]. 2004 Nov [cited 2018 Aug 13];10(6):811–23. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15626900

252. Johansson ME V, Gustafsson JK, Holmén-Larsson J, Jabbar KS, Xia L, Xu H, et al. Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. Gut [Internet]. 2014 Feb [cited 2018 Aug 13];63(2):281–91. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23426893
253. Fu J, Wei B, Wen T, Johansson MEV, Liu X, Bradford E, et al. Loss of intestinal core 1–derived O-glycans causes spontaneous colitis in mice. J Clin Invest [Internet]. 2011 Apr 1 [cited 2018 Aug 13];121(4):1657–66. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21383503
254. An G, Wei B, Xia B, McDaniel JM, Ju T, Cummings RD, et al. Increased susceptibility to colitis and colorectal tumors in mice lacking core 3–derived O-glycans. J Exp Med [Internet]. 2007 Jun 11 [cited 2018 Aug 20];204(6):1417–29. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17517967
255. Li H, Limenitakis JP, Fuhrer T, Geuking MB, Lawson MA, Wyss M, et al. The outer mucus layer hosts a distinct intestinal microbial niche. Nat Commun [Internet]. Nature Publishing Group; 2015 Sep 22 [cited 2018 Jan 10];6:8292. Available from: http://www.nature.com/doifinder/10.1038/ncomms9292
256. Huang EY, Inoue T, Leone VA, Dalal S, Touw K, Wang Y, et al. Using Corticosteroids to Reshape the Gut Microbiome. Inflamm Bowel Dis [Internet]. 2015 May [cited 2018 Aug 20];21(5):963–72. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25738379
257. Finnie IA, Dwarakanath AD, Taylor BA, Rhodes JM. Colonic mucin synthesis is increased by sodium butyrate. Gut [Internet]. 1995 Jan [cited 2018 Aug 20];36(1):93–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7890244
258. Shimotoyodome A, Meguro S, Hase T, Tokimitsu I, Sakata T. Short chain fatty acids but not lactate or succinate stimulate mucus release in the rat colon. Comp Biochem Physiol A Mol Integr Physiol [Internet]. 2000 Apr [cited 2018 Aug 20];125(4):525–31. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10840229
259. Gaudier E, Jarry A, Blottière HM, De Coppet P, Buisine MP, Aubert JP, et al. Butyrate

specifically modulates MUC gene expression in intestinal epithelial goblet cells deprived of glucose. [cited 2018 May 3]; Available from: https://www.physiology.org/doi/pdf/10.1152/ajpgi.00219.2004

260. Leonel AJ, Teixeira LG, Oliveira RP, Santiago AF, Batista N V., Ferreira TR, et al. Antioxidative and immunomodulatory effects of tributyrin supplementation on experimental colitis. Br J Nutr [Internet]. 2013 Apr 20 [cited 2018 Aug 20];109(08):1396–407. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22906779

261. Zhao G, Nyman M, Åke Jönsson J. Rapid determination of short-chain fatty acids in colonic contents and faeces of humans and rats by acidified water-extraction and direct-injection gas chromatography. Biomed Chromatogr [Internet]. 2006 Aug [cited 2018 Nov 6];20(8):674–82. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16206138

262. Alipour M, Zaidi D, Valcheva R, Jovel J, Martínez I, Sergi C, et al. Mucosal Barrier Depletion and Loss of Bacterial Diversity are Primary Abnormalities in Paediatric Ulcerative Colitis. J Crohn's Colitis [Internet]. 2016 Apr [cited 2018 Oct 23];10(4):462–71. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26660940

263. Kasprzak A, Siodła E, Andrzejewska M, Szmeja J, Seraszek-Jaros A, Cofta S, et al. Differential expression of mucin 1 and mucin 2 in colorectal cancer. World J Gastroenterol [Internet]. 2018 Sep 28 [cited 2018 Oct 23];24(36):4164–77. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30271081

264. Johansson ME V, Larsson JMH, Hansson GC. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. Proc Natl Acad Sci U S A [Internet]. National Academy of Sciences; 2011 Mar 15 [cited 2018 Oct 16];108 Suppl 1(Suppl 1):4659–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20615996

265. Desai MS, Seekatz AM, Koropatkin NM, Kamada N, Hickey CA, Wolter M, et al. A Dietary Fiber-Deprived Gut Microbiota Degrades the Colonic Mucus Barrier and Enhances Pathogen Susceptibility. Cell [Internet]. NIH Public Access; 2016 [cited 2018 May 3];167(5):1339–1353.e21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27863247

266. Maaser C, Housley MP, Iimura M, Smith JR, Vallance BA, Finlay BB, et al. Clearance of

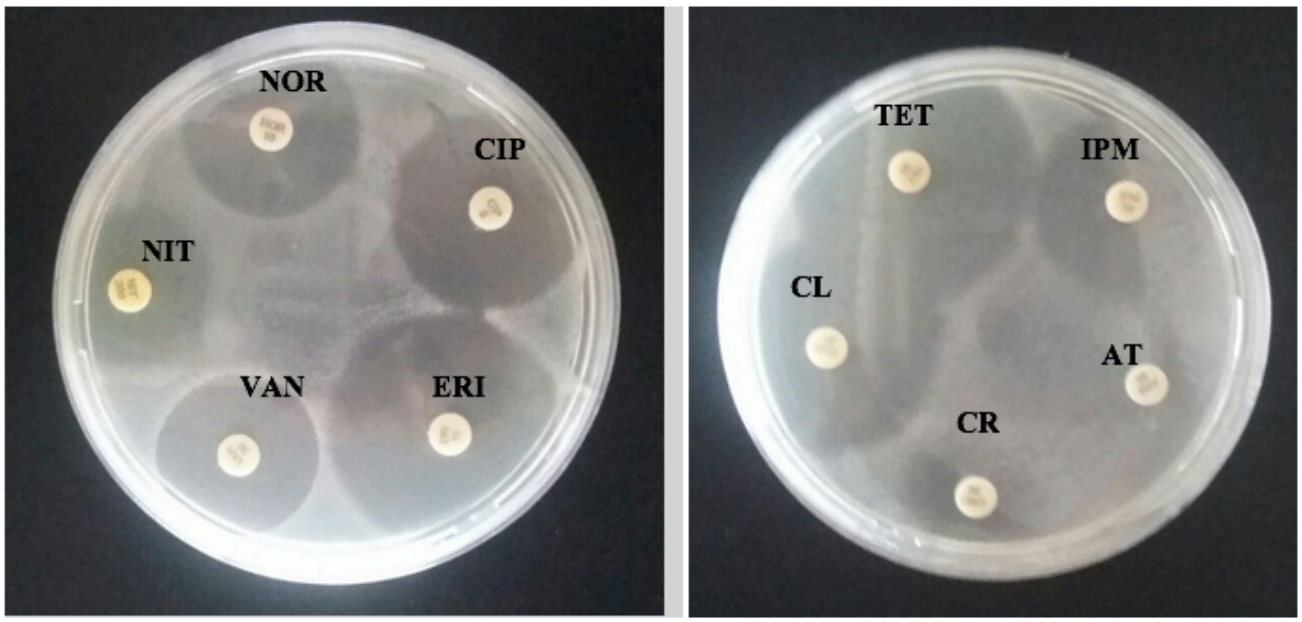
Citrobacter rodentium Requires B Cells but Not Secretory Immunoglobulin A (IgA) or IgM Antibodies. Infect Immun [Internet]. 2004 Jun 1 [cited 2018 Oct 24];72(6):3315–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15155635

267. Bry L, Brigl M, Brenner MB. CD4+-T-Cell Effector Functions and Costimulatory Requirements Essential for Surviving Mucosal Infection with Citrobacter rodentium. Infect Immun [Internet]. 2006 Jan 1 [cited 2018 Oct 24];74(1):673–81. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16369024
268. An G, Wei B, Xia B, McDaniel JM, Ju T, Cummings RD, et al. Increased susceptibility to colitis and colorectal tumors in mice lacking core 3–derived O-glycans. J Exp Med [Internet]. 2007 Jun 11 [cited 2018 Oct 24];204(6):1417–29. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17517967
269. Robbe C, Capon C, Coddeville B, Michalski J-C. Structural diversity and specific distribution of O-glycans in normal human mucins along the intestinal tract. Biochem J [Internet]. Portland Press Ltd; 2004 Dec 1 [cited 2018 Oct 24];384(Pt 2):307–16. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15361072
270. Vieira ELM, Leonel AJ, Sad AP, Beltrão NRM, Costa TF, Ferreira TMR, et al. Oral administration of sodium butyrate attenuates inflammation and mucosal lesion in experimental acute ulcerative colitis. J Nutr Biochem. Elsevier Inc.; 2012;23(5):430–6.
271. Scheppach W, Sommer H, Kirchner T, Paganelli GM, Bartram P, Christl S, et al. Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. Gastroenterology [Internet]. 1992 Jul [cited 2018 Oct 24];103(1):51–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1612357
272. Scheppach W. Treatment of distal ulcerative colitis with short-chain fatty acid enemas. A placebo-controlled trial. German-Austrian SCFA Study Group. Dig Dis Sci [Internet]. 1996 Nov [cited 2018 Oct 24];41(11):2254–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8943981
273. Patz J, Jacobsohn WZ, Gottschalk-Sabag S, Zeides S, Braverman DZ. Treatment of refractory distal ulcerative colitis with short chain fatty acid enemas. Am J Gastroenterol [Internet]. 1996 Apr [cited 2018 Oct 24];91(4):731–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8677939

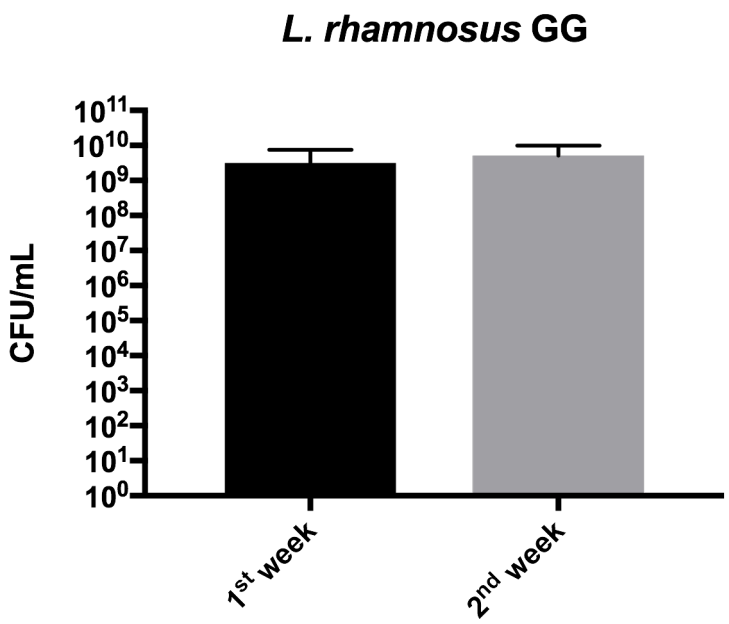
274. Guest JF, Kobayashi RH, Mehta V, Neidich G. Cost-effectiveness of using an extensively hydrolyzed casein formula containing *Lactobacillus rhamnosus* GG in managing infants with cow's milk allergy in the US. Curr Med Res Opin [Internet]. Taylor & Francis; 2018 Sep 2 [cited 2018 Dec 4];34(9):1539–48. Available from: https://www.tandfonline.com/doi/full/10.1080/03007995.2017.1400962
275. Lenoir-Wijnkoop I, Nuijten MJC, Craig J, Butler CC. Nutrition economic evaluation of a probiotic in the prevention of antibiotic-associated diarrhea. Front Pharmacol [Internet]. Frontiers; 2014 Feb 17 [cited 2018 Dec 4];5:13. Available from: http://journal.frontiersin.org/article/10.3389/fphar.2014.00013/abstract
276. Indrio F, Di Mauro A, Riezzo G, Civardi E, Intini C, Corvaglia L, et al. Prophylactic Use of a Probiotic in the Prevention of Colic, Regurgitation, and Functional Constipation. JAMA Pediatr [Internet]. American Medical Association; 2014 Mar 1 [cited 2018 Dec 4];168(3):228. Available from: http://archpedi.jamanetwork.com/article.aspx?doi=10.1001/jamapediatrics.2013.4367
277. Lenoir-Wijnkoop I, Gerlier L, Roy D, Reid G. The Clinical and Economic Impact of Probiotics Consumption on Respiratory Tract Infections: Projections for Canada. Arez AP, editor. PLoS One [Internet]. Public Library of Science; 2016 Nov 10 [cited 2018 Dec 4];11(11):e0166232. Available from: http://dx.plos.org/10.1371/journal.pone.0166232
278. Lenoir-Wijnkoop I, Gerlier L, Bresson J-L, Le Pen C, Berdeaux G. Public Health and Budget Impact of Probiotics on Common Respiratory Tract Infections: A Modelling Study. Foligne B, editor. PLoS One [Internet]. 2015 Apr 10 [cited 2018 Dec 4];10(4):e0122765. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25859849
279. Derwa Y, Gracie DJ, Hamlin PJ, Ford AC. Systematic review with meta-analysis: the efficacy of probiotics in inflammatory bowel disease. Aliment Pharmacol Ther [Internet]. 2017 Aug [cited 2018 Dec 4];46(4):389–400. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28653751
280. Rivera-Chávez F, Lopez CA, Bäumler AJ. Oxygen as a driver of gut dysbiosis. Free Radic Biol Med [Internet]. 2017 Apr [cited 2018 Dec 4];105:93–101. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27677568

Appendix A

Addendum to Chapter 2.



Supplemental Figure A.1. Representative image of one selected strain challenged with different antibiotics.
CRO=ceftriaxone 30 µg, IPM=imipenem 10 µg, ATM=aztreonam 30 µg, ERI=erythromycin 15 µg, VAN=vancomycin 30 µg, CLO=chloramphenicol 30 µg, TET=tetracycline 30 µg, NIT=nitrofurantoin 300 µg, NOR=norfloxacin 10 µg e CIP=ciprofloxacin 5 µg.



Supplemental Figure A.2. Weekly average CFU counts of *Lactobacillus rhamnosus* GG.
1st week: before the DSS administration; 2nd week: During DSS administration. Values are represented as the average of four CFU counts along the week. No significant difference between the groups using t-student test.

Supplemental Table A.1. Weekly viability of the strains isolated from the DSS+PP group in log_{10}CFU.

Animal	Strain	1st week	2nd week
	Bifidobacterium spp.	9.23±0.02	9.97±0.02
1	*Bifidobacterium* spp.	9.21±0.02	9.57±0.01
	Lactobacillus spp.	9.64±0.06	9.85±0.03
	Bifidobacterium spp.	9.34±0.02	9.62±0.02
2	*Bifidobacterium* spp.	9.00±0.03	9.35±0.02
	Lactobacillus spp.	9.22±0.03	9.19±0.02
	Bifidobacterium spp.	9.52±0.03	9.80±0.01
3	*Bifidobacterium* spp.	9.32±0.02	9.62±0.06
	Lactobacillus spp.	9.30±0.04	9.61±0.09
	Bifidobacterium spp.	9.40±0.06	9.17±0.09
4	*Bifidobacterium* spp.	9.94±0.02	9.17±0.06
	Lactobacillus spp.	9.66±0.01	9.62±0.02
	Bifidobacterium spp.	9.28±0.02	9.91±0.02
5	*Bifidobacterium* spp.	9.52±0.02	9.97±0.01
	Lactobacillus spp.	9.28±0.01	9.72±0.02
	Bifidobacterium spp.	9.12±0.04	9.17±0.03
6	*Bifidobacterium* spp.	9.18±0.01	9.76±0.01
	Lactobacillus spp.	9.83±0.03	9.90±0.06
	Bifidobacterium spp.	9.62±0.08	9.70±0.03
7	*Bifidobacterium* spp.	9.38±0.03	9.80±0.04
	Lactobacillus spp.	9.20±0.04	9.80±0.04
	Bifidobacterium spp.	9.24±0.02	9.80±0.04
8	*Bifidobacterium* spp.	9.22±0.06	9.77±0.02
	Lactobacillus spp.	9.90±0.04	9.96±0.02
	Bifidobacterium spp.	9.25±0.02	9.50±0.02
9	*Bifidobacterium* spp.	9.64±0.02	9.99±0.01
	Lactobacillus spp.	9.32±0.01	9.57±0.06
	Bifidobacterium spp.	9.40±0.02	9.66±0.05
10	*Bifidobacterium* spp.	9.62±0.04	9.62±0.04
	Lactobacillus spp.	9.50±0.08	9.90±0.02

Results correspond to the weekly average CFU counts ± standard deviation (SD) of each isolate from group DSS+PP.