The Impact of *Bifidobacterium bifidum* and its Surface Protein BopA on the Murine Intestinal Barrier and Endogenous Microbiota Composition

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Nutritional Sciences
University of Toronto

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Abstract

*Bifidobacterium bifidum* MIMBb75 is a novel strain whose behavior within the intestinal tract is not known. This work attempts to determine the survival and molecular determinant responsible for its colonization through two studies involving administration to mice of 1) *B. bifidum* MIMBb75 or 2) engineered *B. longum* NCC2705 expressing the *B. bifidum*-specific surface protein BopA. MIMBb75 was able to transiently colonize the murine intestinal tract. As a result, endogenous bifidobacteria increased significantly in the proximal colon whereas *Clostridia* clusters were differentially affected in a region-dependent fashion. Genetically engineered *B. longum* NCC2705, expressing the BopA gene, did not impact endogenous bacterial groups in the proximal colon or feces, albeit, it down-regulated the gene expression of KC and mucin 4 in the proximal colon. These outcomes indicate that MIMBb75 encompasses characteristics of a probiotic, but further studies are required to assess the role of BopA as the molecular determinant of its action.
Acknowledgements

Two years ago I walked into the office of a woman I had met only a handful of times for very brief moments, asking her to take a chance on a recent graduate with no lab experience, and whom she had known very little about. After the conversation concluded I was officially a volunteer of the lab, who was given the opportunity to prove my work ethic and willingness to learn, and the rest is history. This anecdote was meant to illustrate the kind, open-mindedness, compassionate characteristics Dr. Elena Comelli encompasses. For this I would like to thank you. You not only provided me with this great opportunity to better myself, and gain experience that is truly priceless, you have been a great mentor and someone I could always depend on for help, whether it be writing proposals, reports, making posters or presentations, or answering any questions, of which we know there were plenty. During the writing of this dissertation, you put me first by providing great feedback quickly ensuring there was enough time left for me to finish even though you had your own deadlines to meet. I can only hope that I did not disappoint you and made your decision to take me on as a Masters student worthwhile. I could never thank you enough with words, but I’ll say it anyways: thank you so much I do not think you can really understand how much I appreciate everything you have done for me.

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This acknowledgement section would not be complete without thanking my parents. This is not just acknowledging their help for the last two years, but for my whole life. They have always provided unconditional love and support. I can honestly say that without them I would be nothing. Anything worth anything that I have achieved in my life so far, I owe to them. I cannot thank you enough, I love you both very much.
I would also like to take this opportunity to thank my lab mates. I use the term ‘lab mates’ loosely as a means to acknowledge people I had the pleasure of working with for the past two years. In my heart I consider each and every one of you a close friend. From the very first day coming into your lab you treated me as a friend. I have not forgotten all those hours spent teaching me how to perform what must have been in your eyes as mindless tasks; I would not have the patience to put up with what you did. A special thanks goes to Natasha Singh and Raha Jahani for being great friends, always there willing to help. I know we will remain lifelong friends.

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# Table of Contents

Acknowledgements ........................................................................................................................ iii

Table of Contents ............................................................................................................................. v

List of Tables ..................................................................................................................................... ix

List of Figures .................................................................................................................................... x

List of Abbreviations ....................................................................................................................... xiii

Chapter 1 Introduction .....................................................................................................................1

1 Introduction ...................................................................................................................................1

Chapter 2 Literature Review ............................................................................................................4

2 Literature Review ........................................................................................................................4

2.1 The Intestinal Barrier ..............................................................................................................4

2.1.1 Physical Barrier: Cellular and Stromal Components and their Function ..........................4

2.1.1.1 The Apical Junction Complex ....................................................................................5

2.1.1.2 Intestinal Epithelial Cell Types and Function .............................................................7

2.1.1.3 The Mucus Layer ........................................................................................................7

2.1.1.4 The Gut Endogenous Microbiota ..............................................................................11

2.1.2 Chemical Barrier: Digestive Secretions and Antimicrobial Peptides .............................11

2.1.3 Immunological Barrier: Architecture of the Intestinal Immune System (IIS) ...............12

2.1.3.1 Cellular Composition and Function of the IIS ..........................................................12

2.1.3.1.1 Pattern Recognition Receptors (PRRs) ...............................................................15

2.1.3.2 Innate Immunity versus Acquired Immunity ...........................................................16

2.1.3.2.1 Th1 versus Th2 Immune Response ....................................................................17

2.2 Microbiota within the Gastrointestinal Tract ........................................................................19

2.2.1 Developmental Acquisition of the Enteric Microbiota and Changes Throughout Life as a Result of Environmental and Genetic Factors .........................................................20

2.2.1.1 Tissier’s Second Phase: Mode of Delivery ...............................................................20

2.2.1.2 Tissier’s Third Phase: Infant Diet .............................................................................20

2.2.1.3 Tissier’s Fourth Phase: Weaning and Culture-based Diets ......................................21
2.4.4.2 Impact on Immunity ................................................................. 39
2.4.4.3 Synergistic Interactions with Commensal Bacteria .................. 39
2.4.5 Bifidobacteria bifidum .............................................................. 40
  2.4.5.1 Health Benefits Associated with B. bifidum ......................... 41
  2.4.5.2 Colonization Methods ......................................................... 42
2.5 Mouse Model ........................................................................... 45

Chapter 3 Rationale, Hypothesis, Objectives ........................................ 46
  3 Rationale, Hypothesis, Objective .................................................. 46
    3.1 Rationale .............................................................................. 46
    3.2 Hypothesis and Objectives ..................................................... 46

Chapter 4 B. bifidum MIMBb75 transiently colonizes the large intestine and impacts on endogenous microbiota composition in a region-dependent manner ................. 48
  4.1 Abstract .................................................................................. 49
  4.2 Introduction ............................................................................. 50
  4.3 Materials and Methods ............................................................. 52
  4.4 Results .................................................................................... 56
  4.5 Discussion .............................................................................. 72

Chapter 5 Genetically engineered Bifidobacterium longum NCC2705 expressing the Bifidobacterium bifidum-specific cell surface adhesion protein BopA affects gut microbiota composition and impacts colonic intestinal barrier gene expression in mouse ................................................... 78
  5.1 Abstract .................................................................................. 79
  5.2 Introduction ............................................................................. 80
  5.3 Materials and Methods ............................................................. 82
  5.4 Results .................................................................................... 87
  5.5 Discussion .............................................................................. 102

Chapter 6 Concluding Remarks .......................................................... 106
  6 Concluding Remarks .................................................................. 106
6.1 Major Overall Findings from the *B. bifidum* and BopA studies ..............................................106
6.2 General Discussion ..................................................................................................................107
6.3 Limitations and Future Directions ......................................................................................108
6.4 Implications .........................................................................................................................110
Chapter 7 Conclusions ..............................................................................................................112
7 Conclusions ..........................................................................................................................112
References ..................................................................................................................................113
List of Tables

Chapter 2

Table 2.1: Intestinal mucins.................................................................9
Table 2.2: Effects of pathogenic and probiotic bacteria on intestinal mucins .................10
Table 2.3: Cytokines of interest in this study..................................................18
Table 2.4: Excerpt from the Guide to Food Labelling and Advertising, Canadian Food Inspection Agency..................................................................................33
Table 2.5: Clinically Tested Probiotics in Canada.............................................34
Table 2.6: Current Bifidobacteria species isolated from human intestine and feces........35
Table 2.7: Isolated B. bifidum strains and their source......................................41
Table 2.8: B. bifidum associated effects on the host........................................43

Chapter 4

Table 4.4.1: Analysis of the microbial composition between control and treatment groups in the feces of mice on day 0.................................................................57

Chapter 5

Table 5.4.1: Microbial composition analysis between BopA- and BopA+ groups on day -7.....88
List of Figures

Chapter 2

Figure 2.1: Summary of the Intestinal Barrier Functions.............................................6

Chapter 4

Figure 4.3.1: Study Design.........................................................................................55

Figure 4.4.1: Clearance time of MIMBb75 on day 14 between control and treatment groups....59

Figure 4.4.2: Comparing absolute cell counts of MIMBb75 per gram wet feces between
treatment and control groups at 24 hours on day 0, 7, 14, and 21.................................61

Figure 4.4.3: Regional enumeration of *B. bifidum* species within the large intestine and feces of
mice on day 14..............................................................................................................63

Figure 4.4.4: Microbial composition analysis in the feces on day 14...............................65

Figure 4.4.5: Microbial composition analysis as a percentage of total bacteria in the feces on day
14....................................................................................................................................66

Figure 4.4.6: Microbial composition analysis in the proximal colon contents on day 14........68

Figure 4.4.7: Microbial composition analysis as a percentage of total bacteria in the proximal
colon contents on day 14.............................................................................................69

Figure 4.4.8: *B. bifidum* MIMBb75 effect on proximal colon cytokine mRNA gene expression on
day 14............................................................................................................................71

Chapter 5

Figure 5.3.1: Study Design.........................................................................................86

Figure 5.4.1: Enumeration of bacterial colonies grown on selective medium containing
chloramphenicol in the feces and caecal contents on day 13 and day 14, respectively........90

Figure 5.4.2: Microbial composition analysis in the feces on day 11...............................92

Figure 5.4.3: Microbial composition analysis in the feces as a percentage of total bacteria on day
11.....................................................................................................................................93

Figure 5.4.4: Detection of Bifidobacteria in the feces of mice on day -7 versus day 11 for both
BopA- and BopA+ groups.............................................................................................94

Figure 5.4.5: Microbial composition analysis in the proximal colon contents on day 14........95
Figure 5.4.6: Microbial composition analysis as a percentage of total bacteria in the proximal colon contents on day 14.

Figure 5.4.7: Comparison of the regional enumeration of *Bifidobacteria* within the feces and large intestine of mice on day 11 and 14, respectively, between BopA- and BopA+ groups.

Figure 5.4.8: Detection of *B. longum* species in the feces at day -7 versus day 11.

Figure 5.4.9: Cytokines gene expression in the proximal colon of mice receiving *B. longum* BopA+ (black bars) or *B. longum* BopA- (white bars).

Figure 5.4.10: Mucin gene expression in the proximal colon of mice receiving *B. longum* BopA+ (black bars) or *B. longum* BopA- (white bars).
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Term</th>
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<tbody>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AJ</td>
<td>Adherens Junction</td>
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<td>AJC</td>
<td>Apical Junction Complex</td>
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<tr>
<td>APCs</td>
<td>Antigen Presenting Cells</td>
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<tr>
<td>BopA</td>
<td>Bifidobacteria Outer Protein A</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CFB</td>
<td>Cytophaga-Flavobacterium-Bacteroides</td>
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<tr>
<td>CpG</td>
<td>Cytosine Phosphoryl Guanine</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic Cells</td>
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<tr>
<td>FAE</td>
<td>Follicle Associated Epithelium</td>
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<tr>
<td>GALT</td>
<td>Gut Associated Lymphoid Tissue</td>
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<tr>
<td>GIT</td>
<td>Gastrointestinal Tract</td>
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<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
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<tr>
<td>IEC</td>
<td>Intestinal Epithelial Cell</td>
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<td>IEL</td>
<td>Intra-epithelial lymphocytes</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>IgA</td>
<td>Immunoglobulin A</td>
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<td>IIS</td>
<td>Intestinal Immune System</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>ITF</td>
<td>Intestinal Trefoil Factor</td>
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<td>LP</td>
<td>Lamina Propria</td>
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<td>KC</td>
<td>Keratinocyte Chemoattractant</td>
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<td>Abbreviation</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>Microbial Associated Molecular Patterns</td>
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<td>Microfold-cells</td>
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<td>MLNs</td>
<td>Mesenteric Lymph Nodes</td>
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<td>Major Histocompatibility Complex Class II</td>
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<td>Nuclear Factor κβ</td>
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<td>NLRs</td>
<td>Nod-like Receptors</td>
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<tr>
<td>NOD</td>
<td>Nucleotide-binding Oligomerization Domain</td>
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<td>Phosphate Buffer Solution</td>
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<td>PGN</td>
<td>Peptidoglycan</td>
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<td>Peyer’s patches</td>
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<td>Potentially Pathogenic Organisms</td>
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<td>PRR</td>
<td>Pattern Recognition Receptor</td>
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<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
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<tr>
<td>SCFAs</td>
<td>Short Chain Fatty Acids</td>
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<td>Subepithelial Dome</td>
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<td>sIgA</td>
<td>Secretory Immunoglobulin A</td>
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<td>TAs</td>
<td>Teichoic Acids</td>
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<td>Tight Junction</td>
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<td>T-helper</td>
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<td>TLRs</td>
<td>Toll-like Receptors</td>
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<td>TNF-α</td>
<td>Tumor Necrosis Factor Alpha</td>
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<td>T-regulatory Cells</td>
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Chapter 1
Introduction

1 Introduction

Both chronic and acute disorders affiliated with gastrointestinal diseases have enormous impact on the Canadian populace. The Canadian Communicable Disease Report estimated that 1.3 cases of acute gastrointestinal illness per person-year are associated with an annual cost of approximately 3.5 billion dollars [1]. Moreover, inflammatory bowel disease (IBD) impinges on some 170,000 people in Canada with an estimated 9,000 new cases diagnosed annually [2]. These staggering statistics necessitate a deeper appreciation of the gastrointestinal ecological unit, including the environmental niche and the microbial community that resides there, which may be responsible for the development of such illnesses if homeostasis is not maintained.

The human intestinal tract is comprised of approximately $10^{14}$ bacterial cells, which translates to roughly ten to twenty times more than the host eukaryotic cells [3]. Several physical, chemical and immunological mechanisms have evolved to ensure the vast quantity of bacteria residing in the intestinal lumen does not translocate into the systemic circulation. For instance, the viscoelastic mucus layer lining the underlying epithelial cells, found to be thickest in the colon where the majority of bacteria reside, acts as a protective barrier by serving as the preliminary blockade between environmental microorganisms and the host [4]. However, there remains a continuous interaction between the mucosal surface and a heterogeneous population of microorganisms, which include the endogenous microbiota, food and other environmental microbes. Consequentially, the primary concern of the host is to distinguish between pathogenic and commensal microorganisms. To alleviate potential infection, a primary line of defense is provided by non-immune cells offering physical, chemical and dynamic processes including mucin secretion (mainly by goblet cells and enterocytes to establish the mucus layer), and release of antimicrobial peptides (mainly $\alpha$-defensins by Paneth cells). Additionally, the presence of commensal microbiota prevent the establishment of pathogenic bacteria through competition for binding sites and energy sources [4]. In addition, innate and adaptive immune defenses are stimulated in tandem by mucosal sentinel cells representative of epithelial cells, macrophages, and intraepithelial dendritic cells (DCs) in circumstances in which the non-immune defense fails to protect the host efficiently [4].
Bifidobacteria are one of several indigenous bacterial inhabitants of the human colon and comprise some of the most important probiotics that are currently commercially available. Specifically, Bifidobacterium bifidum strains have been linked with several beneficial health effects including, but not limited to, immunomodulation [5, 6], antibacterial activity [7, 8] and reduction of inflammation [9]. It has been postulated that B. bifidum is able to accomplish this in two ways. First, B. bifidum directly adheres to attachment sites on mucins, which may be commonly utilized by many different bacterial species found within the lumen, thereby establishing a healthy microbiota composition by averting the peristaltic elimination of beneficial Bifidobacterial species, while at the same time preventing pathogens from attaching to the gut mucosa [10]. Secondly, B. bifidum modulates barrier host-derived components including mediator molecule secretion such as cytokines and chemokines, via epithelial cell gene expression [11, 12].

A novel B. bifidum strain called MIMBb75 was isolated from healthy adult feces by Guglielmetti and colleagues whom reported that MIMBb75 is a highly adherent bacterium to both Caco-2 and HT-29 cells in vitro [13, 14], which led to our hypothesis that the MIMBb75 strain would behave similarly in vivo. In a first study, the novel MIMBb75 strain was intragastrically gavaged daily to C57Bl/6J male mice for two weeks followed by a one-week washout period. We tested colonization of MIMBb75 within the gut, the effects on the endogenous microbial composition, and the effects on cytokine gene expression using both absolute and relative qPCR. The results of this study can be found in the results and discussion portion of Chapter 4.

In addition, the recent discovery of a novel cell surface lipoprotein called BopA has been identified and characterized as being the specific molecular determinant involved in B. bifidum adhesion to intestinal epithelial cells in vitro [13]. Furthermore, although proinflammatory cytokines including IL-6, TNF-α, or IL-8 (murine homologue KC) are characteristically secreted in an inflammatory milieu, it appears that purified BopA supports the production of IL-8 in vitro [13], which may have a potentially protective role in inflammation [15]. However, it is unknown if this occurs in vivo and through which mechanisms. Therefore, the intention of this project was also to elucidate the role of BopA in vivo, in terms of modulating the epithelial barrier through a potential shift in the gut microecology and modulation of a subset of epithelial barrier genes including cytokines and mucins. The expectation is that by completing this project, a greater
understanding of the probiotic effect of *B. bifidum*, at the molecular level, will be obtained supporting its inclusion in functional food products.
Chapter 2
Literature Review

2 Literature Review

2.1 The Intestinal Barrier

The cross-sectional stratum of the intestinal epithelium, from the basolateral to apical region, consists of the serosa, muscularis externa, sub-mucosa and mucosa. The intestinal mucosa is characteristic of a relatively large surface area of approximately 400\(m^2\) in an unfolded, flat disposition, which represents a major site of potential infection due to the large interface with the external environment. The mucosa is comprised of both cellular and stromal components, which operate to both physically and chemically separate the internal milieu of the host from foreign substances and microorganisms within the intestinal lumen, thereby maintaining homeostasis. Three main protective barriers exist within the gastrointestinal (GI) tract, which include the physical barrier, chemical barrier and receptor mediated immune barrier (Figure 2.1). Although innate and adaptive immunity plays a critical role in defending the host, non-immune structural and dynamic processes provide a primary line of defense.

2.1.1 Physical Barrier: Cellular and Stromal Components and their Function

The mucosa is a tightly knit barrier comprised of tight, adherens, and desmosomal junctions in conjunction with a general class of cells called epithelial cells that derive from stem cells. Stem cells dwell at the junction of the villus and crypt [16]. Stem cells migrate towards the luminal surface or the base of the crypt while undergoing proliferation, differentiation and maturation into one of several differentially classified epithelial cells (i.e. colonocytes, goblet cells) before undergoing programmed cell death and shedding into the intestinal lumen [17]. The process of epithelial apoptosis and exfoliation is collectively known as cell turnover. Epithelial cells, specifically enterocytes, have the distinctive ability to rapidly and constantly turnover (approximately every 2-3 days) via the proliferation of stem cells [18]. The rate by which villi and crypt cells undergo apoptosis is balanced by the rate that stem cells are replicated to ensure that repopulation is sustained. However, the rate of stem cell replication may also be affected by pathogenic invasion wherein epithelial cell turnover rate increases in an attempt to expel
parasitic microbes such as is observed in the presence of *Trichuris* in the colon [19]. With the collaborative occurrence of both luminal loss of epithelial cells due to an increased rate of cell turnover and the exudation of fluid from the crypt, pathogens adhering to the mucosa are literally washed away, which provides protection of the epithelial surface from bacterial invasion [4]. The capacity of the stem cells to protect the host is provided by their ability to differentiate into several genres of cell types, each with a unique function and expression pattern throughout the gastrointestinal tract. The principal cells making up the small intestine are represented by the enterocytes, goblet cells, microfold cells (or M-cells) and Paneth cells, whereas the large intestine is primarily composed of colonocytes and goblet cells.

2.1.1.1 The Apical Junction Complex

The intestinal epithelium serves as a barrier, which effectively segregates constituents within the lumen of the intestine from the underlying systemic circulation. This is accomplished via the apical junction complex (AJC), which is comprised of two zones; the proximal zone known as the tight junctions and distal to that, the adherence junctions. The AJC is comprised of several complex structural proteins, most notably occludin and claudins, that interact with complementary molecules on adjacent cells via tetraspanning membrane proteins. The binding characteristic of the AJC serves to polarize the epithelium by creating a molecular ‘fence’ and ensuring the intestine maintains their apical and basolateral regions, or in other words, its cell polarity. The AJC helps to prevent the paracellular passage of pathogens and extremely small molecules (>2kDa) between the epithelial cells [20]. This is supported by the observation that the expression of occludin was down-regulated in patients affected by inflammatory bowel disease (IBD), which was associated with enhanced paracellular permeability and neutrophil transmigration [21].
Figure 2.1: Summary of the Intestinal Barrier Functions. The figure displays the physical, chemical, and immune-receptor mediated barriers of the intestine. The physical barrier comprises of the inner (sterile) and outer mucus layers, which entrap potentially pathogenic organisms (PPOs), and act as receptors for resident bacteria to attach and use as a carbohydrate source. It also consists of the commensal bacteria that can prevent overgrowth of PPOs through competition for binding sites and nutrients, while at the same time releasing their own antimicrobial peptides. The chemical barrier includes the release of host-derived antimicrobial peptides, which prevent the overgrowth of opportunistic and pathogenic bacteria. Lastly, the immune-receptor mediated barrier is quite complex and is shown in steps 1 through 7. In step (1), bacteria within the inner mucus layer are detected by pattern recogonition receptors (PRRs), which causes a downstream signaling cascade that converges on a transcription factor [NF-kB] (2), which is responsible for the synthesis and secretion of cytokines/chemokines and/or antimicrobial peptides (3, 5). The cytokines/chemokines can act as chemoattractants for immune cells such as neutrophiles that then eradicate any bacteria that may have translocated into the gut associated lymphoid tissue (GALT). Finally, dendritic cells may scan the lumen and outer mucus layer for over-growth of bacteria via their finger-like projections (6), and then ‘ingest’ a sample of these live bacteria to initiate synthesis and secretion of cytokines (7) again leading to the recruitment of immune cells (8).
2.1.1.2 Intestinal Epithelial Cell Types and Function

The intestinal epithelium is composed of four cell lineages that originate from a pluripotent stem cell progenitor located at the base of intestinal crypts [16]. These include enteroendocrine cells, enterocytes, goblet cells, and Paneth cells. Other cells found within the GI tract include intraepithelial lymphocytes, and microfold-cells (M-cells), but these will be discussed in detail in section 2.1.3. Briefly, the enteroendocrine cells are not generally categorized as part of the physical barrier of the intestinal epithelium, but rather as a hormonal-producing cell type. Enteroendocrine cells are characterized by their ability to produce hormones including serotonin, motilin, cholecystokinin, vasoactive intestinal peptide and enteroglucagon, among others, depending on their type and location. The enterocytes, goblet cells, and Paneth cells are considered part of the epithelial physical barrier and will be described in more detail.

The enterocytes (or colonocytes as they are referred to within the colon) are the predominant cell type of the epithelium with a lifespan of 2-3 days [4]. Enterocytes and colonocytes are both typified primarily as absorptive cells; however, they are distinguishable from each other by their physical structure. Enterocytes contain approximately 3000 microvilli at their luminal surface within the small intestine, making them exceptional transporters of diet-derived substances [4], while the colonocytes lack the microvilli structure and are generally classified as simple columnar cells. Both enterocytes and colonocytes are capable of producing and secreting endogenous antimicrobial peptides into the lumen that have been proven to be an important mechanism in innate immune defense [4].

Unlike the modest discrimination between the aforementioned absorptive cells, goblet cells have similar physical and functional features in both regions of the intestine. They contribute to the formation of the mucosal barrier by releasing a combination of trefoil peptides, mucin glycoproteins and surfactant lipids [22-24].

2.1.1.3 The Mucus Layer

Together, the mucins, surfactant lipids, trefoil peptides, ions and water are essential for the establishment of the gel-like mucus layer. The mucus layer is a continuous gel that covers the epithelial surface and acts as a protective barrier by physically separating the intestinal cell surfaces from the luminal environment, thereby preventing microbial translocation. The mucus
layer varies in thickness depending on the gastrointestinal segment of interest. The mucus layer can reach up to 450 µm in the stomach while the small intestine is covered with a much thinner mucus layer characteristic of discontinuous sections above the Peyer’s patches [4]. However, the hydrophobicity and thickness of the mucus layer gradually increases from the ascending colon to the rectum where it reaches 285µm [25, 26].

The mucus layer is composed of two distinct layers within the murine [27] and human [22] colon, identified by histochemistry. The inner, firmly attached, mucus layer is found at the luminal interface and is commonly referred to as the “protected zone.” Under healthy circumstances, this layer is devoid of luminal bacteria because bacteria are unable to actively reach and colonize the inner layer due to the production and secretion of antibodies and antimicrobial peptides by the underlying intestinal epithelial cells. The outer mucus layer is less firmly adherent and has been shown to contain high numbers of bacteria [22, 27]. The distinct mucus layers are similar in terms of their composition, being mainly composed of the secretory mucin 2 proteins. The outer mucus layer is twice as thick as the inner mucus layer, providing ample space for microbial functions and interactions with the host and each other [28].

Mucins are characterized by O-linked glycosylated regions, which makes up 60-90% of the polymer’s molecular weight. The mucus layer has both a heterogeneous and dynamic nature that is dependent on the regulation of mucin genes and the mucin subtypes that vary spatially throughout the entire gastrointestinal tract [4]. Twelve epithelial mucin genes have been identified to date within the intestine of both humans and mice (Table 2.1) [29]. The physical structure of the mucus layer also depends on the composition of the mucin glycan chains, which in turn depends on the panel of expressed glycosyltransferase enzymes responsible for their synthesis. Mucin glycans contribute to the visco-elastic properties of the mucus layer and are also a carbohydrate source for endogenous microorganisms. Moreover, mucins’ propensity to entrap microbes with their oligosaccharide chains, contributes to their defensive influence in the gut by ensuring microbes do not reach the mucosal surface. In addition, commensal bacteria are able to colonize the gut via attachment to specific mucins [4].

The regulatory expression of mucin genes, proteins and secretions is largely determined by both commensal bacterial and intestinal pathogens [30]. For example, Lactobacillus plantarum 299v increases mucin expression and secretion of MUC2 and MUC3 in vitro, and as a result, impedes
colonization of entero-hermorrhagic *Escherichia coli* O157:H7 via steric hindrance, or through competitive inhibition for attachment sites on mucins [31]. In addition, *Clostridium difficile* toxin A initiates a dose-dependent decline of mucin exocytosis resulting in hampered mucus integrity [32]. A summary of the effects of both pathogenic and probiotic type bacteria on mucin synthesis and secretion can be found in Table 2.2.

**Table 2.1: Intestinal mucins.**

<table>
<thead>
<tr>
<th>Mucin Gene</th>
<th>Species</th>
<th>Secreted</th>
<th>Membrane-bound</th>
<th>Intestinal Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC1</td>
<td>H, M</td>
<td>NO</td>
<td>YES</td>
<td>SI, LI</td>
</tr>
<tr>
<td>MUC2</td>
<td>H, M</td>
<td>YES</td>
<td>NO</td>
<td>SI, LI</td>
</tr>
<tr>
<td>MUC3A</td>
<td>H, M</td>
<td>YES</td>
<td>YES</td>
<td>SI, LI</td>
</tr>
<tr>
<td>MUC3B</td>
<td>H, M</td>
<td>YES</td>
<td>YES</td>
<td>SI, LI</td>
</tr>
<tr>
<td>MUC4</td>
<td>H, M</td>
<td>NO</td>
<td>YES</td>
<td>SI, LI</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>H, M</td>
<td>YES</td>
<td>NO</td>
<td>LI</td>
</tr>
<tr>
<td>MUC6</td>
<td>H, M</td>
<td>YES</td>
<td>NO</td>
<td>LI</td>
</tr>
<tr>
<td>MUC12</td>
<td>H, M</td>
<td>NO</td>
<td>YES</td>
<td>SI, LI</td>
</tr>
<tr>
<td>MUC13</td>
<td>H, M</td>
<td>YES</td>
<td>YES</td>
<td>SI, LI</td>
</tr>
<tr>
<td>MUC15</td>
<td>H, M</td>
<td>NO</td>
<td>YES</td>
<td>SI, LI</td>
</tr>
<tr>
<td>MUC16</td>
<td>H, M</td>
<td>NO</td>
<td>YES</td>
<td>SI, LI</td>
</tr>
<tr>
<td>MUC17</td>
<td>H, M</td>
<td>NO</td>
<td>YES</td>
<td>SI, LI</td>
</tr>
</tbody>
</table>

H=human; M=mouse; SI=small intestine; LI= large intestine
Modified from Dhamani et al. (2009) and McGuckin et al. (2011) [29, 33]
Table 2.2 Effects of pathogenic and probiotic bacteria on intestinal mucins.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Type</th>
<th>Effect on Mucins</th>
<th>Mechanism</th>
<th>Study</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vibrio cholera</em></td>
<td>Pathogenic</td>
<td>triggers mucin secretion</td>
<td>cholera toxin affects cAMP-dependent pathway</td>
<td><em>in vitro</em></td>
<td>[34, 35]</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>Pathogenic</td>
<td>induces mucin exocytosis</td>
<td>toxin listeriolysin O binds to brush border-associated receptor</td>
<td><em>in vitro</em></td>
<td>[36]</td>
</tr>
<tr>
<td><em>Entamoeba histolytica</em></td>
<td>Pathogenic</td>
<td>depletes goblet cell mucin stores</td>
<td>unknown</td>
<td><em>in vivo</em></td>
<td>[37]</td>
</tr>
<tr>
<td><em>Entamoeba histolytica</em></td>
<td>Pathogenic</td>
<td>directly trigger mucin release</td>
<td>protein kinase C pathway</td>
<td><em>in vitro</em></td>
<td>[38]</td>
</tr>
<tr>
<td><em>Clostridium difficile</em></td>
<td>Pathogenic</td>
<td>inhibits mucin exocytosis</td>
<td>unknown</td>
<td><em>in vitro</em></td>
<td>[32]</td>
</tr>
<tr>
<td><em>Helicobacter pylori</em></td>
<td>Pathogenic</td>
<td>prolonged exposure decreases mucin secretion; decreases expression of MUC1 and MUC5AC</td>
<td>through lipopolysaccharide exposure</td>
<td><em>in vitro</em></td>
<td>[39, 40]</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em></td>
<td>Probiotic</td>
<td>Increased expression of MUC2 and MUC3 genes</td>
<td>unknown</td>
<td><em>in vitro</em></td>
<td>[31]</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus GG</em></td>
<td>Probiotic</td>
<td>Increased expression of MUC2 and MUC3 genes</td>
<td>unknown</td>
<td><em>in vitro</em></td>
<td>[31]</td>
</tr>
<tr>
<td>VSL#3</td>
<td>Probiotic</td>
<td>Increased expression of MUC2 gene</td>
<td>unknown</td>
<td><em>in vitro, in vivo</em></td>
<td>[41]</td>
</tr>
</tbody>
</table>
2.1.1.4 The Gut Endogenous Microbiota

The gut endogenous microbiota is yet another integral part of the physical barrier of the intestine. Some studies have shown that animals bred in a germ-free (absence of bacteria) environment have increased susceptibility to infection in comparison to conventionally raised animals [42]. For example, endogenous \textit{C. difficile}, associated with colitis and antibiotic-associated diarrhea, is able to achieve a population of over $10^8$ CFU per gram of caecum luminal contents in gnotobiotic mice when colonized alone, but conventionally raised mice suppress its growth to undetectable levels [43]. Moreover, antibiotic-induced disruption of the indigenous microbes, for example via clindamycin antibiotic treatment, is often associated with colitis and diarrhea due to overgrowth of opportunistic bacteria such as \textit{Clostridium difficile} as a result of enteric microbe dysbiosis [44]. These studies shed light on the importance of the presence of autochthonous microbes and further contribute to the idea that they are, in fact, active participants in the epithelial barrier function. The enteric microbiota may be active participants in providing the host with protection through their ability to compete with potential luminal pathogens for binding sites and nutritional sources, inducing unfavourable environmental conditions by lowering pH levels and secreting antimicrobial substances, and by modulating the immune response, which will be discussed in detail in section 2.2.

2.1.2 Chemical Barrier: Digestive Secretions and Antimicrobial Peptides

Digestive secretions and antimicrobial peptides are generally regarded as the intestinal chemical barrier. In addition to facilitating digestion of foods and enhancing the absorption of nutrients (iron and calcium), the digestive secretions including gastric acids and pancreatic secretions, have been found to exhibit bactericidal properties due to low pH (<3.0) [4]. Particularly, the multiplication rates of \textit{Shigella flexneri} increased three-fold after neutralization of gastric juices via the addition of sodium bicarbonate [25]. Paneth cells are also capable of producing and secreting bactericidal molecules called antimicrobial peptides, such as the alpha-defensins [45, 46]. The antimicrobial peptides exhibit electrostatic interactions with both Gram-positive and Gram-negative bacteria either leading to a fatal depolarization, production of physical holes, or disrupting the bacterial cell membrane via the disturbance of the distribution of lipids [4].
2.1.3 Immunological Barrier: Architecture of the Intestinal Immune System (IIS)

The IIS is distinct from that of the peripheral immune system in that it is in constant contact with enormous quantities of antigens (Ags), innocuous food molecules and intestinal bacteria, yet maintains homeostasis under healthy circumstances. Ergo, the IIS has a dual responsibility: to avoid an unnecessary inflammatory response while simultaneously protecting the host from potentially pathogenic bacteria and their toxins. The small intestine and colon are principal locales of the IIS, although better characterized within the small intestine. Within the small intestine, the gut associated lymphoid tissue (GALT) is compartmentalized into two distinct regions: (i) inducing sites, which comprises of organized aggregated lymphoid tissue, Peyer’s patches (PPs), and mesenteric lymph nodes (MLN); and (ii) effector sites or lamina propria (LP), consisting of numerous mature B and T cells (60% CD4+T cells), plasma cells (antibody producers), and intra-epithelial lymphocytes (IELs) (cytotoxic T-cells or otherwise known as CD8+ T-cells) [47-50].

2.1.3.1 Cellular Composition and Function of the IIS

Located within the submucosa of the distal ileum are the PPs, which are macroscopically visible lymphoid aggregates with a characteristic dome shape, comprised of B-cell follicles and T-cells, responsible for the collection of antigens from epithelial surfaces. This sub-epithelial dome (SED) is rich in dendritic cells, while the area situated beneath is the lymphoid tissue dense in B- and T-cells [51]. The SED is separated from the intestinal lumen by a monolayer composed of columnar epithelial cells characterized by a less distinct brush border, fewer digestive enzymes, and high numbers of immune cells [51] and is generally referred to as follicle associated epithelium (FAE). A distinctive feature of the FAE is the presence of M-cells. M-cells lack microvilli, mucus layer and cellular lysozymes commonly found on the apical surface of the intestinal epithelium [48]. These cells superimpose the SED and are primarily responsible for the transport of antigens and microorganisms within the lumen to specialized antigen presenting cells (APCs), via vesicular transport [52]. The antigens are then presented to the underlying B- and T-cells found in the lymphoid tissue by DCs from the SED [51]. Thus, M-cells are suggested to be active participants in the initiation of mucosal immunity responses.
The MLNs lie between the layers of the mesentery, which is the double-layered section of the peritoneum lining, and providing lubrication to, the small intestine. Lymphocyte migration to the MLN is mediated by cell surface receptors, which bind to ligands on high endothelial venules thereby allowing passage from the blood into the MLN [53]. Most lymphocytes circulating within the blood share the same receptors, therefore immune interactions between the periphery and GALT may share responsibility in directing responses between the gut and entire host [53].

Immune cells, particularly DCs and T-cells, are key contributors to initiation of either an inflammatory response or tolerance. This decision is based on two factors: for one, the inherent properties of the antigen and secondly, by professional antigen presenting cells (APCs) such as DCs. Several DCs are located both within inductive and effector sites within the GALT, and are believed to play a role in antigen uptake. Various subsets of T-cell such as IELs and some DCs are strategically localized between IECs in order to sample luminal contents [54-56]. Within the intestinal villi, at the subepithelial level in the lamina propria, activated DCs can penetrate the epithelium, and send dendrites to the epithelial surface to sample luminal Ags and present them to IEL and other lymphocytes (Figure 2.1) [48, 50]. DCs are the major players in directing innate and adaptive immune responses within the GIT [57]. The difference in subsets of DCs is based on their differential expression of specific cell surface markers and localization [58]. For instance, PPs DCs produce IL-10, promoting an anti-inflammatory response, but given the same activation signals within the spleen, DCs produce pro-inflammatory IL-12 [59], illustrating the difference in immune responses within the GIT versus systemic system. DCs have also been shown to directly activate, expand and induce T-cells within the GALT via migration into interfollicular areas of the PP (T-areas) or by means of the lymphatic to stimulate T-cells in the MLN [51]. Antigens that are taken up by DCs or other APCs, are processed into immunogenic peptides, which are then displayed to T-cell receptors by Major Histocompatibility Complex Class II (MHCII) molecules at the cell surface of APCs [4].

Several subsets of T-cells exist, but those involved in tolerogenic responses are recognized as T-regulatory (T_R), and have the capacity to suppress other subsets of T-cells including Th1 and Th2 cells (also known as CD4+ cells). Like DCs, several subsets of T_R have been identified according to cellular markers and mode of suppression [51]. Despite their location, stimulation of T-cells
depends on a given Ag, but they are constantly in circulation along the lamina propria and epithelium where they can execute their purpose.

B-cells mature as a consequence of being triggered by a matching antigen. Once triggered, a B-cell engulfs the antigen, degrades it, and exposes the fragments at its cell surface bound to MHCII molecules. The combination of antigen and MHCII molecules recruits T-cells with matching antigen receptors. T-cell secretion of cytokines aids in the maturation and differentiation of B-cells into antibody secreting plasma cells. Up to 80% of all plasma B-cells are located within the GIT, and secrete antibodies including the major immunoglobulin isotype sIgA [60]. The sIgA plays a crucial role in the anti-inflammatory response by playing an important protective role against opportunistic bacteria such as *Salmonella* and *Shigella*. This is accomplished by preventing microbial adhesion, viral multiplication in enterocytes, neutralization of toxins, impeding the translocation ability of both pathogenic and commensal microorganisms [61], and binding innocuous antigens thus preventing their ability to activate a compliment with corresponding CD4+ or CD8+ T-cells [51]. sIgA can also act on its own within the mucus to screen the luminal area of the intestine for PPOs [62]. It is evident that IgA is critical in the homeostasis of the host. Humans deficient in IgA were shown to be prone to gastro-intestinal infections, ulcerative colitis (UC) and IBD [63, 64]. Deficiency in IgA has also been associated with presence of commensal micobiota; for example, IgA deficiency in humans is associated with an increase in the quantity of *E. coli* strains with potentially pro-inflammatory properties [65].

Granted that the DCs, T-cells and B-cells within the GALT and MLNs are the primary tissues involved in the IIS; however, epithelial cells also play a part in regulating the immune response. It has been postulated that the intestinal epithelial cells may function as antigen-presenting cells given that they have been shown to express MHC class II and non-classical MHC class I molecules (presentation of peptides derived from the cytosol as opposed to extracellular proteins presented by MHC class II molecules) [21]. Moreover, they also have the capacity to secrete immune signaling molecules such as cytokines via pattern recognition receptors such as TLRs and NLRs.
2.1.3.1.1 Pattern Recognition Receptors (PRRs)

The protective role of the mucosal barrier stems from its ability to distinguish between the endogenous microbiota and pathogenic microorganisms. This is accomplished mainly through the expression of PRRs. PPRs have been identified in DCs, macrophages, monocytes and T-cells, but have also been found to be expressed in non-professional immune cells (those not expressing the MHC class-II receptors) such as endothelial cells, and epithelial cells, among others [66, 67]. Two specific PRR families have a pivotal role in detecting microbial associated molecular patterns (MAMPs), which include toll-like receptors (TLRs) and nucleotide oligomerization domains (NODs) [68]. Through a complex signaling cascade in response to invading bacteria, stimulation of PRRs by MAMPs can ultimately result in the secretion of cytokines, through the convergence on the transcription factor NF-κB [69]. Conversely, stimulation of PRRs via commensals can initiate epithelial cell proliferation [70], promote the secretion of sIgA into the lumen of the intestine [71], ensure maintenance of tight junctions [72, 73], increase expression of trefoil factors such as trefoil factor 3 [74], inhibit epithelial cell apoptosis in a colitis model [74], and increase expression and secretion of antimicrobial peptides and lectins via Paneth cells [71, 75, 76].

MAMPs tend to be considered highly conserved molecular structures required for microbial survival [77] and include bacterial lipoproteins (ligand for TLR2), Gram-negative LPS (ligand for TLR4), Gram-positive TAs (ligand for TLR2), Gram-negative/positive PGNs (ligand for TLR2; NOD1; NOD2), flagellin (ligand for TLR5) and unmethylated CpG motifs of DNA found only in bacterial DNA (ligand for TLR9) [78]. The general concept that the host is tolerant toward the MAMPs housed by the commensal microbiota, stems from the hypothesis that the microbiota are able to stimulate specific molecular programming of the innate immune system via their MAMPs [79, 80].

The TLRs are transmembrane proteins characteristic of an extracellular domain made of leucine-rich repeats and an intracytoplasmic domain containing a highly conserved Toll/IL-1 receptor (TIR) domain [81]. The disparity in the leucine-rich repeats provides the TLRs with the capacity to distinguish between many of the MAMPs that exist [82]. Thirteen mammalian TLRs have been identified to date and have been labeled 1-13 [81]. The expression patterns of the TLRs are polarized within the GIT and expression patterns are specific to cell type and thus either located
on the apical cell surface (TLR2, -4, -5, -9), basolateral side (TLR2, -3, -4, -5, -9) or within the intracellular compartments (TLR1, -2, -4, -5, -9). Only TLRs 1, -2, -3, -4, -5, and -9 are located within the GIT in healthy humans and rodents. In the presence of pathogenic microbes or pro-inflammatory cytokines, the expression of TLRs commonly increases [68]. However, in the presence of an anti-inflammatory cytokine, the expression of TLRs are inhibited [68].

NODs are a family of cytosolic proteins that contain three distinct functional domains including an amino-terminal effector-binding domain, a centrally located NOD, and a carboxyl-terminal ligand-recognition domain [81]. Only NOD1 (also named CARD4) and NOD2 (also named CARD15) are involved in a signaling cascade that leads to an inflammatory response by acting as PRRs [81]. NODs are structurally similar to TLRs in that they have leucine-rich repeats in their carboxyl terminal [81]. NODs recognize intra-cytoplasmic MAMPs and can only recognize NOD ligands once bacteria reach the cytosol [83]. Several studies have shown that TLRs and NODs act synergistically, and in circumstances resulting in PRRs dysfunction, chronic diseases likely develop. For example, NOD2 polymorphism results in defective IL-10 secretion leading to an uncontrolled pro-inflammatory response, which is associated with Crohn’s disease [84]. In addition, NOD2 signaling has been shown to inhibit the activation of NF-κB via TLR2 [85]. In situations where NOD2 is mutated or there is a deficiency in NOD2, there was also an associated increase in NF-κB triggered by TLR2, resulting in an excessive T\textsubscript{H}1 reaction attributable to Crohn’s disease [85].

2.1.3.2 Innate Immunity versus Acquired Immunity

Innate immunity is the first line of defense in the event that the intestinal physical and chemical barriers fail to protect the host efficiently. It is characterized as being a quick, non-specific response, which lacks “memory.” Sentinel cells, including monocytes, macrophages, DCs, natural killer cells, and neutrophils, detect danger signals in the form of MAMPs via PPRs [86], or cytokines, and signal to other cells via the synthesis of molecules. Epithelial cells have also been found to express TLR2 and TLR4 and therefore may also have the capacity to produce and secrete cytokines thus playing a role in the innate immune response [87]. Through the release of cytokines, sentinel cells have the capacity to modulate other cells to produce and secrete their own molecules including antimicrobial peptides, cytokines, or they can recruit them to the site of action, as is the case with neutrophils in the event IL-8 is secreted. If the inflammatory response
is heightened past a threshold, B and T cells are activated, which in turn sets up an acquired response.

Innate and acquired immunity are intimately associated. Sentinel cells of the innate immune system activate the acquired immune response through the presentation of an epitope (small part of an Ag) to T-cells and via the production of cytokines, which participate in the orientation of the immune response. Acquired responses involve both humoral (antibody production) and cell-mediated (T-cell co-stimulation) responses. Three lineages of cells are involved in the acquired immune response: APCs (mainly macrophages and DCs), T-cells and B-cells. Cellular responses of acquired immunity involve APCs and CD8+ T-cells, while CD4+ T-cells, and B-cells are required for the humoral responses.

2.1.3.2.1 Th1 versus Th2 Immune Response

The classification of different subsets of CD4+ T-cells is determined based on cytokine profiles produced after activation [88]. Three main subsets of CD4+ T-cells have been identified, with a fourth recently discovered. These include the Th-0 (or T

R

-cells), Th-1, Th-2, and Th-17 cells. Under normal circumstances, when cells are functioning without any interference, cytokine secretion is low or non-existent but can rapidly increase during a pathogenic invasion [89]. Th-1 type cytokines, are associated with a pro-inflammatory response [90]. These reactions are a necessity for the suppression of PPOs and the abolishment of infection [90], while other MAMPs induce anti-inflammatory cytokines, also known as Th-2 type cytokines, leading to a tolerogenic response. The Th-1 class cytokines Include TNF-α, IL-1, IL-6, IL-8, and IL-12, whereas Th-2 type cytokines include IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, that lead to tolerance of the invading organism/molecule [91].

The roles and functions of cytokines are perplexing and still being sorted out. Cytokines are signalling molecules that function as a network for cellular communication. Specifically, DCs can elicit an inflammatory or tolerogenic response depending on the Ags present [4]. Cytokines may be classified as interferons (IFNs) (IFN-alpha, -beta, and -gamma), interleukins (ILs) (i.e. IL-6, IL-10), chemokines (i.e. IL-8), or inflammatory cytokines (i.e. TNF-alpha and –beta). They range between 6 to 70 kilodaltons, with some sharing amino acid sequence homology from anywhere between 20-70% [92]. Depending on their structures, they can be sub-divided further
into two main sub-groups: α or C-X-C and β or C-C groups [92]. The presence of an additional amino acid (amino acid “X”) separating the first two cysteine residues is what is used to classify whether they are an α (presence of amino acid) or β (absence of amino acid) [89, 93-95]. For the purposes of this study, primary focus will be directed on IL-6, IL-10, TNF-α and IL-8 (Table 2.3).

**Table 2.3: Cytokines of interest in this study.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin-8 (IL-8)</td>
<td>monocytes, lymphocytes, enterocytes</td>
<td>Chemotaxis (neutrophil recruitment)</td>
<td>[96, 97]</td>
</tr>
<tr>
<td>Tumor Necrosis Factor</td>
<td>monocytess, lymphocytes, mast cells, NK cells, DCs</td>
<td>Activate macrophages, granulocytes, cytotoxic cells, release of acute phase proteins, MHC-I &amp; II, induction of IL-6, up-regulate transcription of IL-8 [human] KC [murine] through activation of NF-κB</td>
<td>[98-101]</td>
</tr>
<tr>
<td>Interleukin-6 (IL-6)</td>
<td>T-cells, B-cells, endothelial cells, monocytes, macrophages, DCs</td>
<td>B-cell differentiation, release of acute-phase proteins IL-6 can promote the release and activate chemokines (i.e. IL-8) by modulating lymphocyte differentiation and adhesion molecule expression Can act as a anti-inflammatory cytokine by inhibiting the expression of TNF-α, while at the same time activating IL-10.</td>
<td>[102]</td>
</tr>
<tr>
<td>Interleukin-10 (IL-10)</td>
<td>T and B-cells, macrophages, DCs, mast cells</td>
<td>Inhibition of cytokine production (IL-6, TNF-α), favour a Th-2 response, Ag presentation ↓ by blocking translocation of Ag-MHC-II complex, attenuates the stimulatory capacity of DCs</td>
<td>[103]</td>
</tr>
</tbody>
</table>
2.2 Microbiota within the Gastrointestinal Tract

The adult human gastrointestinal tract is home to a diverse community of microorganisms that represent all three domains of life including bacteria, archaea and eukarya [104]. The microbial population is estimated to total approximately $10^{12}$ to $10^{14}$ bacterial cells/gram of luminal contents, translating to roughly 10 times greater the total number of host somatic and germ cells [104, 105]. Together, the microbiota contain 100 times as many genes as our own genome [106]. Members residing within the GI tract are classified into two groups: the autochthonous organisms, or resident members, occupy a given ecological niche, while allochthonous organisms are transient members and pass through the gut. Cultivation methods and small subunit ribosomal RNA gene-based approaches (culture-independent) have shown the enormous complexity of the gut associated microbiota. Greater than 99% of the enteric microbes are composed of species within four bacterial phylum divisions. These include the two major bacterial divisions, the Cytophaga-Flavobacterium-Bacteroides (CFB) (i.e. Bacteroides-Prevoteolla group representing 28-93% of total Bacteroidetes) representing 10-30% of total microbes in human feces, and the Firmicutes, representing 46-58% of total bacteria in human feces [107]. The two main groups of Firmicutes include the Clostridium coccoides-Eubacterium rectal group (or Clostridium rRNA subcluster XIVa representing 39.1-48.3% of total Firmicutes), and Clostridium leptum group (or Clostridium rRNA subcluster IV representing 36.4-43.4% of total Firmicutes). In addition, although the phylum Actinobacteria (representing 8-17% of total bacteria detected in human feces), which contain Bifidobacteria (representing 4.4-4.8% of total bacteria in human feces), may not constitute a significant portion of the bacteria that inhabit the gut in terms of quantity when compared to Bacteroidetes and Firmicutes, they too are considered a dominant population and their health-promoting effects are essential. Lastly, Proteobacteria is considered the fourth phylum detected in healthy humans (i.e. 0.1-0.2% Enterobacteriaceae represented as a percentage of total bacteria detected). Eight additional phyla have been detected in the GIT [108], although rare, together with previously known phyla, bring the total number represented in the mammalian intestine to 12 out of the known 55.
2.2.1 Developmental Acquisition of the Enteric Microbiota and Changes Throughout Life as a Result of Environmental and Genetic Factors

Tissier described four phases of microbial acquisition from infancy until about 2 years of age [109]. The first, refers to the initial hours after birth when no fecal bacteria are detectable. The second phase corresponds to the tenth and twentieth hour of life, wherein the fecal bacteria now have a heterogeneous composition (mode of delivery). The third phase refers to a Bifidobacterial-predominanted fecal composition in breast-fed infants that he had named Bacillus bifidus (infant diet) [110, 111]. The fourth phase, corresponds to the modulation of enteric microbial composition of breast-fed infants toward an adult-type subsequent to introduction of solid food, which is characteristic of a complex conglomerate of microorganisms [112, 113]. Though ahead of his time, Tissier did not illustrate the importance of host genetic make-up as a mode through which the micobiota establish themselves as permanent residents within the GI tract, something that current sequencing techniques and knock-out models can now provide insight on.

2.2.1.1 Tissier’s Second Phase: Mode of Delivery

Although the adult microbiota is relatively stable for months at a time [114], the infant gut microbiota is compositionally variable and much less stable until about two years of age. In conventional birth, the inoculum is largely delivered from the mother’s vaginal and fecal flora [115-117]. However, in the event that a child is born via Caesarean section, colonization is delayed and mainly originate from the environment [116]. In fact, delivery from Caesarean section versus conventional birth has been shown to lead to a dissimilar composition of enteric microbes. For example, children born of a Caesarean section displayed greater numbers of Clostridia in comparison to children delivered vaginally, and this trend persisted even after 7 years of age [118].

2.2.1.2 Tissier’s Third Phase: Infant Diet

Initially, facultative bacterial strains, such as Escherichia coli, and Streptococci, are transferred from mother to infant creating a reduced environment, which in turn becomes suitable for the development of obligate anaerobic species that later dominate the gut including Clostridia, Bifidobacteria and Bacteroides. As the infant grows, the compositional development of the
infant gut microbiota is largely dependent on the feeding regimen of the child. Tissier was the first to highlight the significant differences in microbial composition between breast-fed and formula-fed babies [109]. The prevalence of *Bifidobacterium* that easily outcompete other genera in the gut of breast-fed infants is a consequence of prebiotic factors such as certain glycoproteins and oligosaccharides found in the mother’s breast milk [119, 120]. Tissier was limited by cultivation techniques, which may have led to some bias. However, the present use of molecular techniques provides more comprehensive results. It is now generally accepted that breast-fed infants harbor a predominant bifidobacterial fecal microbiota, whereas formulae-fed infants harbor a greater bacterial load with greater bacterial heterogeneity (i.e. higher levels of *Bacteroides, Clostridia,* and *Enterobacteria*) probably in part due to a lack of antimicrobial substances found in the mother’s breast milk. However, infants fed a formulae-based meal similar in composition to that of breast-milk, that is with the inclusion of certain oligosaccharides and iron, also have a similar microbial composition to that of breast-fed infants [117, 121]. Through molecular techniques, *bifidobacteria* was found to represent between 60-91% of the total bacteria in breast-fed infants, whereas formulae-fed infants comprised of 28-75% *bifidobacteria* after day six [122]. There is a similar succession of bacterial groups colonizing the GIT in neonatal mice. Mice 21 to 40 days old, corresponding to suckling and maturity periods, respectively, showed distinct increases in bacterial diversity during each of these critical developmental stages [123]. In addition, *Enterobacteria, Lactobacilli,* and *Bifidobacteria* changed with age in the large intestine of mice between the ages of 12 to 45 days old [124].

2.2.1.3 Tissier’s Fourth Phase: Weaning and Culture-based Diets

Upon being introduced to solid food carbohydrates such as rice cereal, infant bacterial populations switch from facultative anaerobes to strict anaerobes where Bacteroidetes and Firmicutes numbers dramatically rise [125]. The mean ratio of strict anaerobes to facultative anaerobes increase from 1:10 at one week to 60:1 at one year until strict anaerobes outnumber facultative anaerobes by a factor of 100 to 1000 in adult humans [126]. The microbial community of the infant resembles that of the adult after 2 years of age, meaning that the microbial community is more diverse and dominated by strict anaerobes of which Firmicutes and Bacteroides constitute the largest proportion, and they remain stable unless chronic drug use or
drastic change in diet occurs [127]. As a result, the microbial community established during early life may have profound effects in adult years [128].

Culture-based diets also have marked differences in fecal microbiota composition, which can be observed between Japanese vegetarians and those on an omnivorous diet, where a mere 6% of *Bacteroides* were detected in those consuming a vegetarian diet, whereas *F. prausnitzii* was not detected by either cultivation or molecular techniques using 16S rRNA in individuals consuming a vegetarian diet [129]. These findings were confirmed by Finegold et al. [130], where *F. prausnitzii* was not detected in the feces of individuals consuming a vegetarian diet. A comparative study analyzing the fecal matter of European individuals found that the Swedish group, consuming a diet rich in fish and meat, had the greatest quantity of *F. prausnitzii* [131]. In addition, the microbiome has also been shown to shift as a result of high dietary fat intake. Feeding mice a ‘Westernized’ diet induced obesity and resulted in an increased proportion of Erysipelotrichaceae member (one family of the Firmicutes), which is related to the human-associated *Eubacterium dolichum*, although the relative abundance of this member was reduced once the mouse diet was changed to usual mouse chow [132]. Moreover, mice conventionalized with human fecal microbiota, were found to have increased numbers of erysipelotrichi when consuming a high-fat diet [133]. Others have found similar findings with respect to erysipelotrichi response to dietary fat. For example, four clades of this family illustrated dissimilar behaviour in the presence of either high or low-fat diets in mice [134]. Furthermore, changes in dietary carbohydrates has also been shown to modulate the microbiome in humans, for example lowering carbohydrate intake in the diet resulted in a reduction in population levels of butyrate-producing *Roseburia* spp. [135]. Interestingly, the ability of *Bacteroides* spp. to use specific substrates such as inulin can be used to predict the outcomes of competitive interactions between the species [136] and other microbes such as *Bifidobacteria*, which have been shown to increase in numbers after prebiotic stimulation with inulin [137].

Although the microbiome remains relatively stable throughout adulthood, as individuals become elderly the microbial composition shifts once more. Mariat and colleagues showed that species age-related changes were detected in humans via qPCR, where infants were generally characterized with low total bacteria, and highly represented *C. coccoides* and *C. leptum* clusters, whereas elderly subjects exhibited high levels of *E. coli* and *Bacteroidetes* [138]. In addition, the
Firmicutes to Bacteroidetes ratio evolves throughout different life stages in healthy humans, where infants and elderly subjects have a similarly lower ratio (0.4 and 0.6, respectively) compared to middle-aged counterparts (10.9) [138]. In a study analyzing the bacterial composition of an aged European population versus younger adults within the same geographical location, high proportions of enterobacteria and lower levels of \textit{Bacteroides} were observed in all elderly recruits [131]. In Asian populations, higher numbers of enterobacteria with corresponding decreases in anaerobic bacteria were observed in the elderly [60]. From these studies it appears that as one ages, the microbial composition reverts back to that often observed in early infancy.

2.2.1.4 Host Genotype

Apart from environmental factors, bacterial diversity within the GIT appears to also be influenced by the host genotype. A study conducted by Wang et al. [139], found that the fecal microbial compositional profile of 11 different individuals were significantly dissimilar in all cases. Other studies have shown similar results [114, 140, 141]. Furthermore, a study by Zoetendal et al. [142], found that identical twins had greater similarity in their microbial profiles in comparison to unrelated individuals. In addition, marital partners also showed low similarity in their denaturing gradient gel electrophoresis profiles targeting fecal bacteria 16S rRNA sequences, although their environmental habitat was similar [142]. These results were corroborated in a study by Steward et al. [143], where microbial profiles of identical twins were significantly more similar versus fraternal twins, and fraternal twins were found to be more similar than unrelated individuals.

Furthermore, several host genes have been shown to have an impact on the microbial composition. For example, mutations in NOD2 have been implicated in increased risk for ileal Crohn’s disease risk in humans [144] and have been significantly associated with shifts in microbial composition [145], specifically via increased abundance of \textit{Dorea} spp. and \textit{Subdoligranulum} spp [146]. In addition, NOD2-deficient mice harbour a greater bacterial load belonging to Firmicutes and Bacteroidetes than wild-type mice [146]. These types of correlative observations often raise the question as to whether or not the microbiota have a causative role in disease, or if dysbiosis is a consequence of the disease. Although some studies show that microbiota contributes to the disease via transplantation experiments of a microbial composition
originating from a diseased animal to those that are considered healthy [132], further work is necessary to draw an accurate conclusion.

2.2.2 Regional Colonization of the Gastrointestinal Tract

The capacity by which the microbial community is able to establish itself within a specific niche of the GI tract depends on certain factors such as gastric, pancreatic, and biliary secretions, redox potential, pH, transit time, mucin secretion, and nutrient availability characteristic of that environment [147]. The quantity of microbes becomes larger from the proximal to distal portions of the GI tract. For example, the stomach houses the fewest organisms ($10^3$-$10^4$ bacterial per ml) due to the secretion of gastric juices thereby sustaining a low pH, swift peristalsis, and production of alpha-defensins by Paneth cells [105], while the colon permits the highest quantities of resident microbes ($10^{11}$-$10^{12}$ bacteria per ml) due to neutral pH, strictly anaerobic environment, readily available nutrients, and relatively slow transit time [148].

2.2.3 Microbial Colonization Methods

The enteric microbiota presents the host with several metabolic and protective advantages, which would not be possible without their inherent colonization properties that serve to ensure evasion of PPOs. Establishment at an early phase during infancy forges the symbiotic relationship between host and the microbial community; however, this raises questions about how the enteric microbial consortia establish themselves as residents while others are eliminated. Advocated colonization strategies of the microbial community principally embrace theories surrounding biofilm production via mucin attachment and immuno-tolerance by the host.

2.2.3.1 Biofilm and Aggregate Production

Sonnenburg and colleagues suggest that the mucus layer overlying the intestinal epithelium plays a vital role in the maintenance of the structural and functional stability of the microbial community and its tolerance by the host [149]. The interplay between enteric microbes and the mucus layer is generally classified as the “biofilm” and through retention in this polysaccharide-rich mucus gel layer, the microbial community promotes functions necessary, yet foreign, to the host.
The glycan composition of the mucus layer varies along the length of the GIT [22], but the region-specific glycosylation patterns of mucins are highly conserved [150]. The conserved glycan structures may serve to direct members of the microbiota to appropriate host niches by serving as nutrient sources and receptors [151]. For example, *Bacteroides thetaiotaomicron* is characterized with a 4,779-member proteome, none of which are homologues to any known adhesive proteins, although it does contain 163 paralogs of two outer membrane polysaccharide-binding proteins (SusC and SusD) [152, 153], which may aid this particular microbe to attain and utilize a variety of dietary carbohydrates. These proteins have also been suggested to mediate the attachment to mucus glycans, and through DNA microarray gene profiling of the *B. thetaiotaomicron* transcriptome, these genes show notable differences in their expression patterns as a result of the growth phase of the symbiont and nutrient availability [149].

Moreover, aggregates (formed by the microbiota themselves, using undigested food, shed elements of the mucus gel and exfoliated epithelial cells) can serve as scaffolds for the microbial consortia assemblage [149]. The microbiota can assemble on these aggregates via outer membrane polysaccharide-binding proteins, or through other scaffolds such as microbial or host lectins, and host IgA [149]. The aggregates are also characteristic of having dynamic interactions with other aggregates or the mucus gel layer [149].

### 2.2.3.2 Host and Microbial Induced Immuno-tolerance

Early colonizers coordinate with the host immune system ensuring that they are recognized as commensals and thus tolerated as entrenched residents. DCs encompass microbes for several days causing the production and secretion of IgA molecules [154], identified as a prominent molecule involved not only in the regulation of the microbial composition but also the attachment of microbes to the epithelium [155]. A subset of IgA reacts with MAMPs (i.e. LPS) expressed by members of the microbiota [156] and has been proposed to serve as an anchor aiding in the colonization of the enteric bacteria [157]. Exposing core carbohydrate structures that serve as binding site for IgA via the partial deglycosylation of host glycans by commensals, is the proposed mechanism through which IgA acts to anchor microbes and thus contribute to biofilm formation [157].
Furthermore, certain bacteria such as *Bacteroides fragilis*, have the capacity to alter their surface glycan landscape via a serine site-specific type of DNA invertase called Mpi, which targets at least 13 loci within the genome of the symbiont including the promoter regions of seven capsular polysaccharide synthesis loci and polysaccharide biosynthetic locus [158, 159]. The ability of these bacteria to alter their cell surface may be one method of adapting to changes in the glycan landscape of the host and a way to evade detection by host’s immune system [149].

### 2.2.4 Significance of Host-Microbial Interactions

Co-evolution over millions of years has left the host reliant on the metabolic potential of enteric microbes as a result of an inability to execute necessary processes independently [160]. Many studies illustrate the importance of the microbiota and have shown that they necessitate important and specific metabolic, trophic, and protective functions. Considering all functions performed by the gut microbiota, it is not surprising that many gastrointestinal diseases have been associated with an altered microbial composition, commonly referred to as dysbiosis, and are associated with, but not limited to, inflammatory bowel disease [161] and necrotizing enterocolitis [162].

#### 2.2.4.1 Metabolic Functions

The main metabolic function of the colonic microbiota is to increase the efficiency of energy harvest from the diet by fermenting non-digestible polysaccharides including resistant starches, cellulose, hemicelluloses, pectins and gums, as well as endogenous mucus produced by the intestinal epithelia [11, 104, 163]. This is accomplished through a wide repertoire of enzymes including glycosyltransferases and glycosylhydrolases that are not characteristic of the host proteome. The caecum and proximal colon act as the major fermentation vats within the GIT, where 20-60 grams of carbohydrates and 5-20 grams of protein are estimated to represent available substrate for bacterial fermentation per day [164, 165]. Overall, the combined metabolic efforts of the microbial community is the generation of short-chain fatty acids (SCFAs), which are ultimately utilized as absorbable substrate by the host, and as a supply of carbohydrate energy source for bacterial growth and proliferation.
2.2.4.1 Energy Salvage: SCFA Production, Mineral Absorption, and Vitamin Metabolism

The three main SCFAs produced following bacterial fermentation include acetate, propionate, and butyrate and are found within the colon as a molar ratio of 60:20:20, respectively [166]. The majority of SCFAs within the colon are absorbed either by simple diffusion or via anion exchange [167], with only 5-10% excreted in the feces [168]. The three major SCFAs are absorbed at comparable rates in different regions of the colon and are metabolized at three major sites of the body including colonocytes, the liver and peripheral tissue such as muscle cells. Butyrate is the preferential energy substrate for colonocytes (providing 60-70% of their energy requirements) but residual butyrate is also metabolized by the liver. Propionate and acetate are utilized mainly by liver cells for gluconeogenesis and cholesterol synthesis, respectively, with residual acetate also used by peripheral tissue such as muscle cells for energy via oxidation reactions [169]. In fact, commensals, specifically those within the phyla Firmicutes and Bacteroidetes, have a major impact on the phenotypic characterization of the human body due to their ability to differentially ferment indigestible substrates found within the colon, thus providing an alternate source of absorbable energy for the host. For example, higher proportions of Firmicutes versus Bacteroidetes have been associated with obesity [106]. Subsequent to weight loss in obese people, there was a decrease in Firmicutes to Bacteroidetes ratio approaching that found in lean people [170].

In addition to salvaging energy, SCFAs indirectly increase the absorption of minerals such as calcium and magnesium [171]. The commensal microbiota has also been affiliated with vitamin metabolism. Synthesis of some vitamins by commensals (i.e. vitamin B12) is the only source found in nature [172]. Enteric bacteria have also been linked to synthesis of vitamin K [173, 174].

2.2.4.2 Trophic Functions

2.2.4.2.1 Effect on Epithelial Cell Proliferation and Differentiation

Arguably, the most important function of SCFAs is their trophic effects on the intestinal epithelium. Comparisons of conventionalized rats to those germ-free illustrate the importance of
the presence of enteric microbes, where germ-free rats contain a reduced number of total crypt cells compared to their conventionalized counterparts [175]. These findings may be associated with the ability of enteric microbes to produce SCFAs, which have been directly associated with the stimulation of epithelial cell proliferation and differentiation in the colon in vivo [176]. Specifically, butyrate is of interest due to its dual nature with respect to epithelial cell proliferation, generally termed the “butyrate paradox,” which has become increasingly important in cancer research. In the healthy situation, butyrate stimulates proliferation of colonocytes [177, 178]. However, butyrate inhibits cell proliferation and stimulates cell differentiation in neoplastic epithelial cell lines [179], thereby reverting neoplastic cells to normal mature cell phenotypes in vitro [180].

### 2.2.4.2.2 Development and Homeostasis of the Host Immune System

Germ-free models demonstrate the necessity of enteric microbes for the stability and maintenance of the host immune system. For example, animals bred in an environment devoid of bacteria have been shown to have low counts of total lymphoid cells in the GALT, small specialized follicle structures, and low circulating concentrations of immunoglobulins such as IgA and IgG [181-183]. The composition of the GALT is affected by microbial colonization, for instance, after exposure to MAMPs the number of intraepithelial lymphocytes is significantly amplified [184, 185], germinal centers containing Ig producing cells is augmented in follicles and LP [186], and Ig increases considerably in the serum [183].

Furthermore, the microbial community has also been implicated in oral tolerance through their involvement in establishing memory mechanisms for the systemic immune response. For instance, mice with a conventionalized microbial community ensures systemic unresponsiveness to a non-pathogenic antigen after pre-exposure for several months, whereas this insensitivity lasts for only a couple of days in germ-free mice [187]. Although systemic immunoregulatory circuits can be corrected in germ-free mice after reconstitution of a conventionalized enteric microbial composition, these corrective effects are only seen in neonates and not older mice, demonstrating the importance of proper development early in life [188].

Even commensal microbes within the gut try to evade detection by the host immune system, which may be desirable by the host as an exaggerated response to a non-pathogenic organism.
can consume unnecessary energy and damage to colonic tissue. *Bacteroides fragilis* is excellent at circumventing the host immune response by altering its surface antigenicity by producing capsular polysaccharides [159]. In addition, the epithelial cells use PRRs to differentiate between commensals and pathogenic organisms through production and secretion of cytokines, in order to rapidly respond to eventual challenge. By co-incubating probiotic-type bacteria with inflamed human intestinal mucosa, immediate production of different cytokines then those produced under an inflammatory response changes the phenotype of the lamina propria lymphocytes thereby conferring a homeostatic advantage to the host [189].

### 2.2.4.3 Protective Functions

Autochthonous organisms within the GIT are a fundamental component of the natural resistance to PPOs. Several studies show that an associated increased risk of infection is common among germ-free animals introduced into a conventionalized environment [190, 191]. In the healthy situation, there is natural homeostasis between the resident organisms and the host providing stability within the microbial population as a result of colonization resistance. This prevents bacterial overgrowth of opportunistic and commensal bacteria naturally present there. In circumstances that require the use of antibiotics, it has become clear that the ecological balance of the resident organisms is essential in order to maintain this homeostasis within the gut. For example, due to antibiotic usage, overgrowth of toxigenic *Clostridium difficile*, leads to associated pseudomembranous colitis [192]. The protective function of resident bacteria is generally termed the barrier effect.

Resident organisms provide protection from PPOs through several mechanisms. Firstly, by colonizing the GIT, they take up space and consequentially create natural competition for attachment sites. This has been shown to be true *in vitro* as *Lactobacillus* LA 1 binds to cultured human intestinal epithelial cells and as a result prevents the attachment of enterovirulent bacteria [193]. Secondly, commensal microorganisms can also impede the overgrowth of PPOs through competition for nutrients. This is shown using a gnotobiotic mouse model colonized with *Bacteroides thetaiotaomicron*, which utilizes a molecular sensor to control the amount of specific nutrients it requires from the host, thereby preventing overproduction of the nutrient and thus potential intrusion of microbial competitors that require the same nutrients [151]. Lastly,
resident microbes can produce antimicrobial peptides such as bacteriocins to prevent the overgrowth of unwanted bacterial species [194]. The host ensures that antimicrobial peptides such as bacteriocins, are restricted to localized niches via degradation using their digestive proteases [163].

2.3 Probiotics

The World Health Organization and Food and Agriculture Organization defines probiotics as ‘live microorganisms, which when administered in adequate amounts confer a health benefit to the host’ [195]. The rationale for the use of probiotics is twofold. First, ancestral humans consumed a diet with a high constitution of prebiotic-type foods such as plant roots augmenting the growth of lactobacilli and bifidobacteria [196]. Second, given that human fecal matter is composed of 50% bacteria by weight, and given that bacteria have the capacity to confer beneficial health effects to the host, it is reasonable to attempt to ingest beneficial bacterial species in an attempt to increase their proportion within the GIT. Prior to 2002, there were no guidelines on what constitutes a probiotic or what data need to be available to substantiate a health claim. The following sections will focus on the data required for a bacterial strain to be classified as ‘probiotic’, the accepted health claims by Health Canada to date, and clinically proven probiotics sold in Canada.

2.3.1 Guidelines to Acceptable Probiotic Bacteria in Canada

Analysis of the wording used to define probiotics, provided by Health Canada, reveals that strict guidelines need to be met before a bacterial strain can be considered probiotic. From the definition, to be considered a probiotic, the microbe being administered must be alive. This does not imply that it must remain alive throughout its travel in the gastrointestinal tract [195], although it is preferred. The viability at the site of action is important but not necessary given that some desired effects can be reconciled by cell components [197]. The second portion of the definition that states microbes should be administered in adequate amounts (the operative word being ‘adequate’), is difficult to interpret since it is not possible to provide one minimum dose that can be applied to all bacterial strains given that different probiotics are effective at different amounts [197]. Studies have shown that some probiotics are effective at 50 million CFUs/day [198] while others tend to require upwards of 1 trillion CFUs/day to be considered effective.
Thus, it is imperative that the effective dosages prescribed be specific for the microbe of interest and shown to be effective in human clinical trials. Lastly, the probiotic must confer a health benefit, but specific guidelines on what constitutes a health benefit need to be realized. The term ‘health benefit’ can refer to both drug-type effects such as mitigation of diarrhea, or food-type benefits such as supporting a healthy immune system [197]. Physiological effects, correlated with health enhancement usually as a measure of biomarkers opposed to clinical endpoints, may also be considered health benefits (i.e. altering gut microbiota) [197].

Moreover, there exist several regulatory subcategories for the general concept ‘probiotic,’ which include probiotic food, probiotic supplement, probiotic drug, and a designer probiotic (genetically modified microbe), each slightly different from the other in terms of mode of deliverance or genetic manipulation [197]. Counter intuitively, it is not correct to equate a probiotic and native commensal bacteria although both may have similar mechanisms of action resulting in a common health benefit [197]. Furthermore, there is also a distinction between probiotics and a live, active culture. Although active cultures contain live microbes, they are usually only tested for fermentation properties in foods and do not necessarily confer a health benefit [197].

Further concern develops around the common misconception that a probiotic will be effective or safe under all conditions. As a result, the use of probiotics often falls into the category of “alternative” therapies without clearly indicating proper use or the consequential therapeutic effects after ingestion. A critical principle founding the notion of probiotic intervention is the concept that the dysbiosis of the gut microecology may play a pivotal role in human disease. The role probiotics play in the maintenance of gut homeostasis, which will ultimately ensure a healthy environment for the host, is their ability to modulate gut microecology. Some of the proposed mechanisms of action of probiotics are similar to those of the commensal microbial community naturally formed within the GIT. According to Sanders [197], it is difficult to generalize the effect probiotics confer to the host due to differences in strains, dose, subject age (premature infants to elderly), subject health status, and methods used in studies presently conducted, all of which need to be considered before ingesting a probiotic product for an intended purpose.
2.3.2 Clinically Relevant Probiotics in Canada

In an excerpt from the Guide to Food Labelling and Advertising, from the Canadian Food Inspection Agency [200], the eligible probiotic bacterial species include only those from the *Bifidobacteria* and *Lactobacillus* genera due to their long track record of safety (Table 2.4). Although Health Canada has accepted generic probiotic health claims (Table 2.4), no strain-specific health claims have been accepted to date. This is a consequence of a disconnect in observed effects of probiotic bacteria between *in vitro* and *in vivo* studies, and a lack in knowledge of the molecular determinants and mechanisms by which these bacteria confer health benefits to the host. Until these problems are rectified, only general health claims will be available to the general population.

To date, there are several probiotic-containing products with clinically proven beneficial effects (Table 2.5). However, some may be used as preventative measures while others are used to treat disease. For example, VSL#3 is a proven probiotic and available in Canada. It contains *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus plantarum, Lactobacilli delbrueckii* subspecies *bulgaricus, Bifidobacterium longum, Bifidobacterium breve,* and *Streptococcus salivarius* subspecies *thermophilus.* VSL#3 has been proven to aid in the prevention of pouchitis in ulcerative colitis patients and retain remission [201, 202]. In addition, VSL#3 has also been shown to be effective for patients with IBS symptoms by reducing flatulence over the treatment period [203]. Another example of preventative action of a probiotic formula is from Danone’s Activia yogurt, containing *Bifidobacteria animalis* subspecies *lactis DN-173 010.* In a study conducted by Marteau and colleagues in 2002 [204], healthy women provided *B. lactis* were shown to have shortened colonic transit times compared to control.
Table 2.4: Excerpt from the Guide to Food Labelling and Advertising, Canadian Food Inspection Agency

<table>
<thead>
<tr>
<th>Eligible Bacterial Species in Canada</th>
<th>Acceptable (non-strain) Specific Probiotic Health Claims for Food</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bifidobacterium adolescentis</em></td>
<td>1. Probiotic that naturally forms part of the gut flora.</td>
</tr>
<tr>
<td><em>Bifidobacterium animalis</em> subsp.</td>
<td></td>
</tr>
<tr>
<td><em>animalis</em></td>
<td></td>
</tr>
<tr>
<td><em>Bifidobacterium animalis</em> subsp.</td>
<td>2. Provides live microorganisms that naturally form part of the gut flora.</td>
</tr>
<tr>
<td><em>lactis</em> –synonym: <em>B. lactis</em></td>
<td></td>
</tr>
<tr>
<td><em>Bifidobacterium bifidum</em></td>
<td></td>
</tr>
<tr>
<td><em>Bifidobacterium breve</em></td>
<td>3. Probiotic that contributes to healthy gut flora.</td>
</tr>
<tr>
<td><em>Bifidobacterium longum</em> subsp.</td>
<td></td>
</tr>
<tr>
<td><em>infantis</em></td>
<td></td>
</tr>
<tr>
<td><em>Bifidobacterium longum</em> subsp.</td>
<td>4. Provides live microorganisms that contribute to healthy gut flora.</td>
</tr>
<tr>
<td><em>lactum</em></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus casei</em></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus fermentum</em></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus gasseri</em></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus johnsonii</em></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus paracasei</em></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus salivarius</em></td>
<td></td>
</tr>
<tr>
<td>Product</td>
<td>Probiotic</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>Florastor, Medical Futures Inc.</td>
<td><em>Saccharomyces boulardii</em> lyo</td>
</tr>
<tr>
<td>DanActive (Actimel) milk drink, Danone</td>
<td><em>Lactobacillus casei</em> DN 114-001</td>
</tr>
<tr>
<td>Activia yogurt, Danone</td>
<td><em>Bifidobacteria animalis</em> DN 173-010</td>
</tr>
<tr>
<td>VSL#3, Seaford Pharma</td>
<td>8 strains of lactobacilli, bifidobacteria, and streptococcus</td>
</tr>
<tr>
<td>Fem-dophilus, Jarrow Formulas; RepHresh Pro-B--Lil Drug Store</td>
<td><em>Lactobacillus rhamnosus</em> GR-1 and <em>Lactobacillus reuteri</em> RC-14</td>
</tr>
<tr>
<td>Bio K Plus</td>
<td><em>Lactobacillus acidophilus</em> CL1285 and <em>L. casei</em> CL</td>
</tr>
<tr>
<td>Align, P&amp;G</td>
<td><em>Bifidobacteria infantis</em> 35624</td>
</tr>
<tr>
<td>Tu Zen, Ferring Pharmaceuticals</td>
<td><em>Lactobacillus plantarum</em> 299v</td>
</tr>
<tr>
<td>Jamieson Advanced 4-Strain Probiotic</td>
<td><em>Lactobacillus acidophilus</em> LA-5, <em>Bifidobacteria lactis</em> BB-12, <em>Streptococcus thermophilus</em> and <em>Lactobacillus delbrueckii</em> spp. <em>bulgaricus</em></td>
</tr>
</tbody>
</table>

Modified from Reid et al. (2008) [213]
2.4 Taxonomy and General Characteristics of *Bifidobacteria*

Actinobacteria is one of the most prominent phyla in the domain Bacteria, all of which are characteristic of a high-G+C content, except for *Tropheryma whipplei*. One of the six taxonomic grouped as orders within the Actinobacteria phyla is Bifidobacteriales containing the family Bifidobacteriaceae. It is within this family that the genus *Bifidobacteria* is grouped. Today, there are 37 proposed species that have been isolated from the human intestinal tract and dental caries, animals, insects, and raw milk. However, for the purpose of this dissertation, primary interest will be for those isolated from the intestinal tract or feces of healthy humans (Table 2.6).

*Bifidobacteria* are anaerobic, Gram-positive, non-motile, non-spore-forming, non-gas producing bacteria with a high G+C content (55 to 67%) substantiating the stability of their DNA [214]. Their shape often resembles a V- or Y-shape rod resembling branches generally referred to as a bifid or irregular shape [214]. The absence or low concentrations of *N*-acetylamino-sugar [215], Ca$^{2+}$ ions [216], or amino acids (alanine, aspartic acid, glutamic acid, and serine) in growth media [217], have the propensity to induce the characteristic bifid shape.

### Table 2.6: Current bifidobacteria species isolated from human intestine and feces

<table>
<thead>
<tr>
<th>Species Name</th>
<th>Subspecies</th>
<th>Origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. adolescentis</em></td>
<td></td>
<td>Intestine of adult</td>
<td>[218]</td>
</tr>
<tr>
<td><em>B. angulatum</em></td>
<td></td>
<td>Human feces</td>
<td>[219]</td>
</tr>
<tr>
<td><em>B. bifidum</em></td>
<td></td>
<td>Infant and adult feces</td>
<td>[13, 109]</td>
</tr>
<tr>
<td><em>B. breve</em></td>
<td></td>
<td>Intestine of infant</td>
<td>[218]</td>
</tr>
<tr>
<td><em>B. catenulatum</em></td>
<td></td>
<td>Intestine of adult</td>
<td>[219]</td>
</tr>
<tr>
<td><em>B. gallicum</em></td>
<td></td>
<td>Human feces</td>
<td>[220]</td>
</tr>
<tr>
<td><em>B. longum</em></td>
<td><em>infantis</em></td>
<td>Intestine of infant</td>
<td>[218]</td>
</tr>
<tr>
<td></td>
<td><em>longum</em></td>
<td>Intestine of adult</td>
<td>[218]</td>
</tr>
<tr>
<td><em>B. pseudocatenulatum</em></td>
<td></td>
<td>Infant feces</td>
<td>[221]</td>
</tr>
</tbody>
</table>

Modified from Lee et al. (2010) [214]
2.4.1 Early Studies of *Bifidobacteria*

As mentioned previously, Henri Tissier was the first to isolate *Bifidobacteria* from the feces of breast-fed infants in 1899 [222], and inferred that the lower incidence of infantile diarrhea of breast-fed infants was likely due to the large number of *Bifidobacteria* present. Others have supported Tissier’s hypothesis that higher bifidobacteria counts is associated with lower incidences of infantile gastroenteritis when comparing between breast-fed infants and formula-fed infants [223, 224]. Subsequent analysis focused on the composition of human breast milk and found that it contained bifidobacteria growth-promoting factors such as lactulose [225] and N-acetylglucosamine-containing saccharides [226] and other human milk oligosaccharides (HMOs) [227, 228]. It was anticipated that augmenting the pervasiveness of *Bifidobacteria* in the feces of breast-fed babies compared to those bottle-fed, was likely involved in the suppression of undesirable intestinal bacteria [223]. Comparison of feces from breast-fed versus those from bottle-fed infants, supports the hypothesis that an enhanced bifidobacteria proportion follows a concomitant decrease in undesirable microbes such as coliforms and enterobacteria [229]. The most likely explanation for this observation is the capacity for bifidobacteria to lower intestinal pH levels as a result of production of lactic acid and acetic acids [214], which has been supported by fecal pH measurements in infants bottle fed (pH>7) and breast-fed (pH<6) during the first 7 weeks after birth [230].

2.4.2 Evolutionary Mechanisms Established to Deal with Stress Responses

*Bifidobacteria* have developed several mechanisms to deal with stress responses that become essential to ensure survival of the microbe during descent through the human gastrointestinal tract. These mechanisms have evolved to ensure that bifidobacteria can tolerate stressors such as the presence of oxygen and acid.

2.4.2.1 Oxygen Tolerance

*Bifidobacterial* are generally regarded as strict anaerobes, although some have been shown to tolerate oxygen to some degree [231]. While many have variable sensitivity thresholds [232, 233], fermented-milk adapted *B. animalis* subsp. *lactis* has the greatest tolerance to oxygen [234]. The complete genome sequence of *Bifidobacteria* revealed a gene analog for NADH oxidase, which together with oxygen and NADH, generates the accumulation of hydrogen
peroxide, which has been shown to have destructive properties [214]. Further analysis of genome sequences revealed several genes involved with the reduction of \( \text{H}_2\text{O}_2 \) to water, including peroxiredoxin family alkyl hydroperoxide reductase gene analogs, and thioredoxin reductase [214]. Finally, oxygen damage repair genes have also been found in bifidobacterial genomes, including those for nucleoside triphosphate pyrophosphohydrolase and DNA-binding ferritin-like protein [214], both of which have been associated with the removal of hydroxyl radicals [235].

2.4.2.2 Tolerance to pH and Bile Acids

The function of the \( \text{F}_0\text{F}_1 \)-type ATPase system is to pump protons out of the cell thereby providing a mechanism by which bifidobacteria can deal with acidic environments [236]. \textit{Bifidobacterial} genomes that have been sequenced to date all contain a putative \textit{atp} operon containing all genes required for a complete \( \text{F}_0\text{F}_1 \)-type ATPase system [214]. The \( \text{F}_0\text{F}_1 \)-type ATPase system has been shown to have increased activity in related anaerobic bacteria as a consequence of enhanced acidic conditions [237, 238] and have also been shown to be active in different species of \textit{Bifidobacteria} as a result of acidic insult [236, 239]. In addition, all sequenced \textit{Bifidobacteria} contain an extracellular polysaccharide (EPS) or capsular polysaccharide, which may provide resistance to stomach acids and bile salts [240, 241].

2.4.3 Metabolic Potential

The analysis of bifidobacterial genome sequences has imparted a vast collection of information including those related to the metabolic potential of \textit{Bifidobacteria}. It has become apparent that \textit{Bifidobacteria} contain a wide repertoire of genes required for the synthesis of at least 19 amino acids, biosynthesis of pyrimidine and purine nucleotides, and those for the synthesis of certain B vitamins [242], as well as having the capacity to shunt many monosaccharides or disaccharides into the fructose-6-phosphate pathway to produce ATP [243].

2.4.3.1 Vitamin Metabolism

The aptitude for \textit{Bifidobacteria} to synthesize a wide range of B vitamins including thiamine (B1), pyridoxine (B6), folic acid (B11) and nicotinic acid (or niacin; B3), and riboflavin (B2) [243, 244], supports its characterization as a probiotic. For example, ingesting bifidobacterial strains with the ability to produce folate resulted in the restoration of folate in locales of
deficiency, which would otherwise be associated with premalignant changes in colonic epithelia, thereby conferring protection against inflammation and cancer [245].

2.4.3.2 Sugar Metabolism

*Bifidobacteria* also contain a large arsenal of proteins responsible for the catabolism of complex carbohydrates, many of which the hosts cannot breakdown themselves. The recently sequenced *B. dentium* Bd1 (found in the oral cavity) contain proteins involved in carbohydrate metabolism and transport, which is represented by approximately 14% of their genes [243]. The diversity of proteins devoted to the metabolism of carbohydrates provides *Bifidobacteria* the ability to adapt its genome to a competitive habitat rich in complex carbohydrates, such as the human colon, to ensure survival [246]. As a genus, *Bifidobacteria* are considered saccharolytic microbes with the propensity to ferment glucose, galactose and fructose [246]. The bifidobacterial collection of glycosyl hydrolases, although many, are largely unidentifiable. However, their useable substrates are identified and include xylo-oligosaccharides [247], pectin [248], fructo-oligosaccharides [249], plant oligosaccharides [250] and mucins [251]. Over 50 bifidobacterial carbohydrases have been identified to date [252], accentuating the specialized systems the members of this genus have evolved towards carbohydrate metabolism.

2.4.4 Role in the Colon

2.4.4.1 Ensuring Intestinal Microbial Eubiosis

Our knowledge of the role of *Bifidobacteria* in the colon is still in its infancy, but the role is expected to be a beneficial based on studies conducted thus far. In fact, due to a larger proportion of *Bifidobacteria* in unweaned infants compared to adults, it has been suggested that *Bifidobacteria* may play a more significant role in gut microbiota development then in other gut functions [214]. This becomes apparent with respect to the role of *Bifidobacteria* in modulating bacterial groups, which may be considered detrimental to the host. For instance, *Bifidobacteria* have been well documented in supporting the host though competitive hindrance over other enteric microbes including Shiga toxin-producing *Escherichia coli* O157:H7 [253], *Clostridium difficile* [254], and *Clostridium perfringens* [255]. In addition, production of antimicrobial compounds, including bacteriocins by *Bifidobacteria* [256], aid in preventing the overgrowth of PPOs. The role of *Bifidobacteria* in controlling the levels of undesireable microbes have also
been inferred via reverse correlation analysis of bifidobacterial numbers with the numbers of PPOs of interest in the intestine from clinical feeding studies [257-259]. For example, with increasing numbers of *Bifidobacteria* there was a subsequent decrease in coliforms detected in the feces of humans ingesting yogurt [257, 258]. This is further exemplified in an ageing human studies where *Bifidobacteria* numbers decrease significantly and there is a corresponding increase in numbers of *Clostridia* and *E. coli* [60, 260, 261]. Moreover, infants with a *Bifidobacteria* proportion of 96% correlated with lower levels of putrefactive products, including ammonia, indole, p-cresol, and phenol, as well as enzymes involved in their production such as, urease, tryptophanase, and β-glucuronidase, compared to adults with a *Bifidobacterial* proportion of 19% [260].

2.4.4.2 Impact on Immunity

In addition to modulating the enteric microbiota towards a desireable phenotype, *Bifidobacteria* have also been shown to impact on the innate immune response. In a randomized, double blind, placebo controlled clinical trial conducted by Arunachalam and colleagues [262], healthy elderly subjects consuming milk supplemented with *B. lactis* for 6 weeks produced significantly enhanced levels of INF-α and polymorphonuclear cell phagocytic capacity. Furthermore, in a study conducted by O’Hara and colleagues (2006) [12], *B. infantis* was found to attenuate IL-8 secretion at baseline and limit flagellin-induced IL-8 protein secretion in HT-29 cells, while stimulating IL-10 and TNF-α secretion in DCs. Lastly, fermentation products from *B. breve* BbC50sn induces maturation, high IL-10 production, and prolonged survival of DCs via a TLR-2 pathway, and was able to suppress LPS effects on IL-12 secretion and DC apoptosis [263].

2.4.4.3 Synergistic Interactions with Commensal Bacteria

*Bifidobacteria* has also been shown to interact with other enteric microbes, thereby displaying synergistic cooperation within the microbial community of the gut. In a study conducted by Sonnenburg and colleagues (2006), co-colonization of germ-free mice with *Bacteroides thetaiotaomicron* (a dominant species of the human GIT) and *Bifidobacteria longum* NCC2705 (a commensal strain in the human GIT) was analyzed using whole genome transcriptional profiling [264]. This group found that there was an interactive effect between the two microbes commonly found in the mammalian gut measured by the increase in efficiency of mannose
utilization, enhanced synthesis of enzymes utilized in the breakdown of xylose, and impact on host immune responses [264]. However, these effects were not detected when \( B. \) \textit{thetaitaomicron} was co-colonized with \( B. \textit{animalis} \) subsp. \textit{lactis}, and \( L. \textit{casei} \), indicative of the disparity in species-dependent effects microbes impose on the host [264]. Furthermore, \( B. \) \textit{bifidum Z9} has the propensity to inhibit the expression of Th-1 associated genes induced by \( L. \textit{acidophilus} \) NCFM in murine DCs, and instead had an additive effect on genes regulating the innate immune response and moved towards a Th-2 profile [265].

\textbf{2.4.5 \textit{Bifidobacterium bifidum}}

Although \textit{Bifidobacteria} are considered to be health-promoting bacteria, a reoccurring theme common to the probiotic field is every species and strain deriving from the same genus may behave differently. This becomes apparent even within the \textit{Bifidobacteria} genus group where \( B. \textit{dentium} \) Bd1 is considered an opportunistic pathogen due to its ability to cause dental caries [243] while, at least for the time being, the rest are considered beneficial. Several strains of \( B. \textit{bifidum} \) have been isolated from feces of human infants, adults and the elderly (\textbf{Table 2.7}). In addition, two of the eleven \textit{Bifidobacterial} genomes that have been sequenced are from \( B. \textit{bifidum} \) including PRL2010 [251] and S17 [266], providing insightful information about this particular species.
Table 2.7: Isolated *B. bifidum* strains and their source.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIMB75</td>
<td>Adult feces</td>
</tr>
<tr>
<td>DSM20456</td>
<td>Infant feces</td>
</tr>
<tr>
<td>IAB26</td>
<td>Elderly feces</td>
</tr>
<tr>
<td>MPB41</td>
<td>Elderly feces</td>
</tr>
<tr>
<td>MPB34</td>
<td>Elderly feces</td>
</tr>
<tr>
<td>NAB1</td>
<td>Elderly feces</td>
</tr>
<tr>
<td>NCC390</td>
<td>Adult feces</td>
</tr>
<tr>
<td>SAB20</td>
<td>Elderly feces</td>
</tr>
<tr>
<td>SAB32</td>
<td>Elderly feces</td>
</tr>
<tr>
<td>PLR2010</td>
<td>Infant feces</td>
</tr>
<tr>
<td>S17</td>
<td>Infant feces</td>
</tr>
</tbody>
</table>

Modified from Guglielmetti et al. (2008) [13]

2.4.5.1 Health Benefits Associated with *B. bifidum*

*Bifidobacteria bifidum* species are associated with several health benefits including immunomodulation, improvement of the intestinal microbial balance and pathogen exclusion at the mucosal interface in mice [10], reduction of inflammatory symptoms [9], antibacterial activity [256, 267], reducing symptoms of irritable bowel disease [268], and treatment of allergies and shortening rotavirus and diarrhea [269] (these health-promoting benefits are summarized in Table 2.8). However, many of the aforementioned beneficial probiotic effects rely on the ability of the bifidobacteria species to colonize the GIT, maintaining continual dialogue with the host. Colonization of the intestine is regarded as one of the main criteria required before a bacterial species is given probiotic status.
2.4.5.2 Colonization Methods

Currently, the molecular mechanisms through which *Bifidobacteria bifidum* species colonize and confer health benefits to the host are largely unknown. However, the advent of completed genome sequences of *B. bifidum* strains, including S17 [266] and PLR2010 [251], have permitted emerging new insights into the putative pathways through which *B. bifidum* beneficially affects the host. For instance, *B. bifidum* PLR2010 has revealed metabolic pathways for host-derived glycans such as those present on mucins (i.e. O-linked glycans) via glycosyl hydrolases identified from proteome and transcriptome profiling [251]. It has been proposed that through host-derived glycan catabolism, *B. bifidum* species may influence enteric microbial communities ecology via glycan forging [251]. In addition, functional genomic analysis of *B. breve* revealed a IVb tight adherence Tad pili as an essential colonization factor, which is conserved in other *B. breve* strains and other *Bifidobacteria* including *B. bifidum* PLR2010 and S17 [270]. Exopolysaccharides have also been identified in *B. bifidum* and have been proposed to be involved in adherence to host cells [271, 272]

Several strains of *B. bifidum* have been shown previously to adhere tightly to the human intestinal cell lines [271, 273, 274]. In a study by Killer and colleagues (2010) [275], 181 *Bifidobacterial* strains were tested for their ability to adhere to mucins, and nine of the strains with optimal growth on mucins were identified as *B. bifidum*. In fact, *B. bifidum* strains were shown to adhere better than proven probiotic type bacteria such as *L. rhamnosus* GG, which are known to adhere well to mucus [13, 14]. However, none until 2008 have been able to show the specific protein involved in *B. bifidum* adhesion. Dr. Guglielmetti and colleagues were able to isolate a cell-surface lipoprotein, which they called *Bifidobacteria* outer protein A (BopA), that was directly responsible for the adhesive ability of *B. bifidum* species *in vitro* [13, 14]. The BopA adhesive outer protein is only specific to *B. bifidum* species [13]. However, further investigation into the role of BopA *in vivo* is required.
Table 2.8: *B. bifidum* associated effects on the host.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Study Type</th>
<th>Study Design</th>
<th>Conclusions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC11863</td>
<td><em>In vitro</em></td>
<td>Measure bacteriocins production</td>
<td>NCFB 1454 produces novel bacteriocins called bifidocin B; active against <em>Listeria, Enterococcus, Bacillus, Lactobacillus, Leuconostoc,</em> and <em>Pediococcus.</em></td>
<td>[267]</td>
</tr>
<tr>
<td>ATCC29591</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCFB 1453</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NCFB 1454</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NCFB 1455</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17</td>
<td><em>In vitro</em></td>
<td>Caco-2 cells</td>
<td>Strain-specific adhesion depends on pH; ↔ IL-8, TNF-alpha, IL-6.</td>
<td>[276]</td>
</tr>
<tr>
<td>S17</td>
<td><em>In vitro</em></td>
<td>HT-29 cells NF-kB reporter gene system</td>
<td>↓ inflammation induced by LPS; ↓ IL-8 secreted protein, ↓ IL-8 mRNA, TNF-alpha.</td>
<td>[277]</td>
</tr>
<tr>
<td>S17</td>
<td><em>In vitro</em></td>
<td>Caco-2, T84, and HT29; murine models of colitis</td>
<td><em>In vitro</em>: ↑ adhesion IECs, ↑ anti-inflammatory capacity, ↓ IL-8 and NF-kB</td>
<td>[278]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>In vivo</em>: normal weight and colon length and ↓ histology scores</td>
<td></td>
</tr>
<tr>
<td>OLB6378</td>
<td><em>In vitro</em></td>
<td>Rat and IEC-6 NEC model</td>
<td>↑ TLR-2, COX-2, and PGE(2) expression, ↓ apoptosis</td>
<td>[279]</td>
</tr>
<tr>
<td>OLB6378</td>
<td><em>In vivo</em></td>
<td>Preterm rats with NEC</td>
<td>↓ NEC incidence, ↑ IL-6, MUC3</td>
<td>[280]</td>
</tr>
<tr>
<td>DSM 20082</td>
<td><em>Ex vivo</em></td>
<td>Incubation on healthy rat mucosa</td>
<td>Adheres to mucin proteins</td>
<td>[281]</td>
</tr>
</tbody>
</table>
**Unknown** *In vivo*  Broiler chickens with cellulitis (10^7 cells/ml)  ↑ caecal bifidobacteria, no change in clostridia; ↓ the incidence cellulitis  [9]  

**MIMBb75** *In vitro*  Caco-2 cells  *B. bifidum* strains had the greatest adherence, which depends on BopA. BopA and MIMBb75 ↑ IL8.  [13]  

**MIMBb75** *In vitro*  Caco-2 and HT-29 cells  adherence ↑ with mannose and fucose, ↓ with low pH (due to autoaggregation) and in the presence of Oxgall bile salts; mucus as carbon energy source  [14]  

**MIMBb75** *In vivo*  Randomized, double-blind, placebo-controlled clinical trial; n=122 IBS patients  ↓ global IBS and IBS symptoms and improve quality of life  [282]  

**B1** *In vivo*  healthy subjects (n=9)  ↑ β-glucosidase activity, (colonic fermentation of cellulose)  [283]  

**NCIMB 41171** *In vivo*  healthy subjects (n=30); provided novel galactooligosaccharide (7g/day)  ↑ bifidobacteria proportions  [284]  

**Bb11** *Ex vivo*  Co-incubation on mouse spleen B cells with LPS  ↑ total secretion of major Ig isotypes, and IgA secretoy cells. Similar effects on B cells as LPS.  [5]  

**Unknown** *In vivo*  dams and pups given 10^8 CFU/ml with MRV  interferes with replication or attachment and uptake of virus through Peyer's patches.  [285]
Yakult \textit{In vivo} mice given $10^{8.5-9.5}$ cells/ml ↓ \textit{E. coli} infection induced by a large dose of 5-FU, via acetate production

NEC, necrotizing enterocolitis; IECs, intestinal epithelial cells; MRV, murine rotavirus; 5-FU, 5-fluorouracil

2.5 Mouse Model

The mammalian model most commonly used for microbiome analysis are mice [286]. Comparison of human and mouse genomes have identified that both share 99% of their genes and differ by an overall genomic size of only 14% (2.5 gigabases versus 2.9 gigabases, respectively) [287], and therefore mice comprise several of the intestinal barrier genes or their homologues. For example, the protein sequence of human IL-8 shares approximately 60% similarity to that of the rodent KC [288]. Murine KC is mediated by the IL-8 receptor that closely resembles the human IL-8β and IL-8α receptors, with amino acid sequence identity of approximately 71% and 68%, respectively, giving KC the capacity to manipulate neutrophils via attraction and activation [96, 97]. Furthermore, although mice have specific enteric species such as \textit{Lactobacillus murinis} and \textit{Lactobacillus animalis}, it has been shown that these species display high 16S rRNA gene sequence similarity to human \textit{L. salivarius} suggesting they all derive from a single origin [289]. In spite of their deviance in total body size, intestinal physiology (relatively larger caecum found in mice), and diet (mice are coprophagic), both mice and humans maintain the same prominent bacterial phyla present within the distal portions of their intestines including Firmicutes, Bacteroidetes and Actinobacteria [290]. In addition, the bacterial patterning from infancy to old-age in the intestinal tract has been shown to be similar to that in humans [123].

Mouse C57Bl/6J strains are one of the most commonly used strains for microbiome analysis [264, 291-293] for several reasons: the mice are inbred strains and therefore have reduced genetic variability, and they exist as germ-free [291], and therefore can be manipulated for gnotobiotic mouse models [133]. In addition, the C57Bl/6J mice constitutes a large repertoire of knock-out models such as IL-10-knock-out colitis models [292], which can be used for follow-up studies. Lastly, these mice are commercially readily available and easy to handle.
Chapter 3
Rationale, Hypothesis, Objectives

3 Rationale, Hypothesis, Objectives

3.1 Rationale

*B. bifidum* strains have been shown to adhere tightly to human intestinal cell lines [271, 273, 274], including MIMBb75, which has better adhesive capacity than the well-known mucin-adhering bacteria *L. rhamnosus* GG [13, 14]. In addition, 181 *Bifidobacterial* strains were tested for their ability to adhere to mucins, nine of which were characterized as having optimal growth on mucins and were identified as *B. bifidum* species [275]. The ability of *B. bifidum* strains to grow well on mucins could be explained by their recently identified nutrient-acquisition strategy that targets host-derived glycans, such as those present in mucins [251]. Through this nutrient acquisition strategy, it has been proposed that *B. bifidum* species may have a concomitant influence on enteric microbial communities, which has already been shown in terms of actively changing gene expression profile induced by *L. acidophilus* on DCs [265]. However, until 2008, the specific protein involved in *B. bifidum* adhesion was unknown. Dr. Guglielmetti and colleagues were able to isolate a cell-surface lipoprotein, which they called *Bifidobacterial* outer protein A (BopA), that was directly responsible for the adhesive ability of *B. bifidum* species, and shown to have potential immunomodulatory effects via augmentation of IL-8 cytokine expression, *in vitro* [13, 14]. BopA is specific to *B. bifidum* species [13]. Although MIMBb75 has been shown to adhere well to intestinal cell lines and have potential immunomodulatory effects *in vitro*, none to date have been able to show the ability of this novel strain to persist within the gut *in vivo* and determine its associated potential health benefits. In addition, the molecular mechanism by which *B. bifidum* species, including MIMBb75, is able to establish within the GIT and confer its probiotic effect is also largely unknown, although a likely candidate is the BopA cell-surface lipoprotein. The role of BopA in terms of its ability to aid in *B. bifidum* colonization and ensuing beneficial effects *in vivo* merits further investigation.

3.2 Hypothesis and Objectives

The general hypothesis of this research is that *Bifidobacterium bifidum* MIMBb75 colonizes the intestinal tract, resulting in a higher proportion of fecal bifidobacteria and increased expression
of interleukin 8, both depending on its surface protein BopA. The overall objective is to
determine if *B. bifidum* MIMBb75 colonizes the intestine with effects on the barrier gene
expression program and if this depends on BopA.

**Specific objectives:**

(1) To determine if *B. bifidum* MIMBb75 colonizes the intestinal tract *in vivo* with
consequent increase in gut bifidobacteria cell counts and increased mRNA expression of
interleukin 8. (*Chapter 4*).

(2) To establish whether BopA is the molecular determinant responsible for the colonization
of transformed *B. longum* NCC2705 and for the increase in bifidobacteria cell counts,
and expression of mucin genes and interleukin 8 (*Chapter 5*).
Chapter 4

*B. bifidum* MIMBb75 transiently colonizes the large intestine and impacts on endogenous microbiota composition in a region-dependent manner

Contribution of Data

The quantitative data for day 7 and 21 in the results section entitled “Following the absolute cell counts of *Bifidobacteria bifidum* MIMBb75 throughout the study between control and treatment mice for days 0, 7, 14, and 21,” were provided by Angela Wang.

The quantitative data for *B. bifidum* cell counts in the caecum and distal colon contents found in the results section entitled “Ecological niche colonization preference of *B. bifidum* throughout the large intestine and feces of mice on day 14, 24 hours post-gavage,” were provided by Raha Jahani and Ye Seul Song, respectively.

Quantitative data on caecum microbial composition in the results section entitled “Summary of the effects of MIMBb75 on the endogenous microbial composition represented as absolute cell counts (A) and as a percentage of total bacteria (B),” were provided by Natasha Singh.
4.1 Abstract

**Background:** *Bifidobacteria* are common inhabitants of the human gut and comprise several commercially available probiotic species, including *B. bifidum*. Their beneficial health effects are vast and include maintenance of intestinal barrier integrity via immunomodulation and improvement of the intestinal microbiota composition. *B. bifidum* MIMBb75 is a recent isolate and potential probiotic with an exceptional capacity to adhere to intestinal cell lines, mainly through its surface lipoprotein BopA. This strain has also shown potential immunomodulatory control via augmentation of IL-8 expression *in vitro*. The aim of this study was to assess MIMBb75’s ability to colonize the intestinal tract *in vivo* and determine the consequential impact on endogenous microbiota composition and cytokine gene expression.

**Methods:** Twenty-eight C57Bl/6J male mice were daily gavaged with either $10^8$ cells of *B. bifidum* MIMBb75 resuspended in PBS or PBS alone for 14 days. *B. bifidum* and commensals including *C. coccoides*, *C. leptum*, *Bacteroides*, *Bifidobacteria* was enumerated by quantitative PCR in the feces and intestinal contents after 14 days of treatment and one-week after treatment cessation (day 21). Cytokine expression in the caecum and proximal colon was assessed by quantitative PCR.

**Results:** *B. bifidum* MIMBb75 transiently colonized the murine intestine, which was associated with increased counts of *C. coccoides* in the feces, while *C. leptum* and *Bacteroides* remained unchanged. *B. bifidum* uniformly established within the caecum, proximal and distal colon. Compared to all regions of the large intestine, *B. bifidum* was significantly decreased in the feces. Cytokine gene expression in the proximal colon was uneffect.

**Conclusion:** *B. bifidum* MIMBb75 uniformly becomes a transient member of the large intestine *in vivo* and influences *Clostridia* proportions in a region-dependent manner.
4.2 Introduction

The human colon houses a complex microbial consortium that represents approximately 10 times the total number of host somatic and germ cells [105], albeit, only four major phyla are dominant within the human colon including Actinobacteria of which Bifidobacteria comprise a major proportion [107]. Bifidobacteria are a common resident of the human adult colonic microflora population, which remains relatively stable until advanced age [107, 261, 294]. Bifidobacteria have been associated with several health benefits including pathogen exclusion [10] and impacting on the mucosal barrier [264]. In fact, decreases in Bifidobacteria levels in senility in humans, results in a concomitant increase in the levels of detrimental strains of clostridia and enterobacteria [295].

Bifidobacteria comprise several commercially available probiotic species. Their beneficial health effects include immunomodulation [5, 6], improvement of the intestinal microbial balance and pathogen exclusion at the mucosal interface in mice [10], reduction of inflammatory symptoms [9], antibacterial activity [267], reducing symptoms of irritable bowel disease [268], and treatment of allergies and shortening rotavirus and diarrhea [269]. These beneficial attributes are common among several species of bifidobacterial origin.

Specifically, B. bifidum possesses desirable traits favored for probiotic merit including: human origin [296], resistance to gastric acidity and bile toxicity [14, 282], ability to adhere to gut epithelial tissue [14], ability to colonize the host GIT [282], and produce antimicrobial substances such as bifidocin B [256, 267]. The aptitude of B. bifidum to confer health benefits to the host, like many commensals, relies heavily on their capacity to adhere to the mucus gel layer or intestinal epithelium in order to mediate dialogue with the host. Several strains of B. bifidum have been shown previously to adhere tightly to the human intestinal cell lines [271, 273, 274], and were shown to have optimal growth on mucins when compared to other strains of Bifidobacteria [275]. Interestingly, B. bifidum strains were shown to adhere better than proven probiotic type bacteria such as L. rhamnosus GG, notorious for their ability to adhere well to mucus [13, 14].

In addition, dynamic interactions with other commensal bacteria such as those observed with B. bifidum Z9, which actively changes the gene expression profile induced by Lactobacillus
*acidophilus* in dendritic cells *ex vivo* [265], exploit the potential interactive effects probiotic species can have on their environment. *B. bifidum* strains have also been shown to have differential immunomodulatory effects depending on the strain and environmental condition. For example, *B. bifidum* S17 had no effect on cytokine production including IL-6, IL-8, and TNF-α, in a healthy *in vitro* model [276], whereas S17 reduced IL-8 secretion after LPS stimulation *in vitro* [277]. In addition, *B. bifidum* iY inhibited IL-8 secretion in TNF-α stimulated HT29 cells [297], whereas *B. bifidum* strains MIMBb75 and PLR2010 demonstrated up-regulation in IL-8 expression in a healthy *in vitro* model [13, 251], and *B. bifidum* ATCC 86321 decreased pro-inflammatory cytokines IL-6 and TNF-α in aging mice [298]. Furthermore, recent analysis of the genome of *B. bifidum* PLR2010 has revealed innate metabolic pathways conserved to permit foraging of host-derived glycans such as those found in mucins, which has been suggested to have an effect on intestinal microbial ecology [251].

*Bifidobacteria bifidum* MIMBb75 is a recent isolate from healthy adult feces and a potential probiotic with an exceptional capacity to adhere to intestinal cell lines, mainly through its specific surface lipoprotein BopA [13]. This strain has also shown potential immunomodulatory control through increased expression of IL-8 *in vitro* [13]. However, it has not yet been established if this particular strain of *B. bifidum* can survive passage through the intestinal tract *in vivo*, nor has its potential immune-stimulatory effects been examined.

Taken together, the aim of this study was to assess the ability of the *B. bifidum* MIMBb75 strain to colonize the intestinal tract *in vivo*, determine its preferential resident niche(s), and to determine the consequential impact on shifting the endogenous microbiota composition and capacity to modulate the immune-response genes i.e. cytokines and chemokines.
4.3 Materials and Methods

Bacterial strains and culture conditions

*Bifidobacterium bifidum* MIMBb75 was provided by Dr. Guglielmetti from the culture collection at the Department of Microbiology, University of Milan, Italy and was routinely grown overnight under anaerobic conditions at 37°C in MRS broth (Difco, Detroit, MI, USA) supplemented with 0.05% L-cysteine hydrochloride.

Animals and study design

Twenty-eight conventionally raised C57BL/6J male mice, 7 weeks of age, were purchased from Jackson Laboratories and housed in the Division of Comparative Medicine, University of Toronto. Following one-week acclimatization, the mice were randomized to two groups, based on baseline body weight. Four mice were caged together and were provided *ad libitum* access to water and standard chow (Harlan 2018) and were housed under the conventional 12h:12h light-dark cycle until sacrificed. Fourteen mice (seven mice per group) were intragastrically gavaged daily for two weeks with 1x10^8 colony forming units (CFU) of *B. bifidum* MIMBb75 cells resuspended in 0.2ml sterile PBS, or with an equal volume of sterile PBS alone. After fourteen days of treatment, 14 mice (7 mice per group) were kept for an additional week with no gavage. Freshly passed fecal pellets were collected at baseline on day 0, and subsequently thereafter on days 14 and 21 post-gavage and stored at -20°C. Seven mice per group were sacrificed on day 14 and 21 by cervical dislocation following CO2 inhalation (Figure 4.3.1). Upon sacrifice, the entire large intestine was immediately removed. The large intestine, constituting caecum to anus, was divided into three parts including the rectum (distal 1cm) and the proximal and distal colon that correspond to the proximal and distal half, respectively, after rectal excision. Ceacal, proximal and distal colon contents were removed by washing the tissue with ice-cold sterile saline solution (0.9% NaCl), divided into two halves longitudinally, and stored at -80°C until needed. Feces were collected on days 0, 7, 14, and 21 immediately after defecation and put on ice before storing at -20°C until needed. The animal study design and protocol was approved by the Animal Care Committee at the University of Toronto.
Microbiota Composition Analysis of Feces and Intestinal Contents

Gut microbial composition analysis of dominant bacterial groups including those from *Bacteroides*, *Clostridium*, and *Bifidobacterium* genera was assessed in feces and intestinal contents using absolute quantitative Real-Time PCR. DNA was extracted from 16-50mg of wet intestinal contents or feces using E.Z.N.A.™ stool DNA Isolation Kit (Omega, USA), following the manufacturer’s protocol modified to include a lysozyme digestion step (20mg/ml and incubation at 37˚C for 30 minutes). DNA concentration and purity was measured using ThermoScientific’s Nanodrop 1000 Spectrophotometer (ThermoScientific, USA). DNA samples were stored at -20˚C until needed.

Microbiota composition of fecal and proximal colon contents was determined using 50ng of DNA by quantitative Real-Time PCR. *B. bifidum* MIMBb75 was quantified by TaqMan probe-based assay using specific primers designed to anneal to the OppD and BopA gene sequences (Forward: ACCGAATTCGCCTGTCACTT; Reverse: ACGGCACGGATTCTG; Probe: CCGCTGGATGTGAAC). Furthermore, custom-made TaqMan primers targeting variable regions corresponding to 16s rRNA sequences of *Bifidobacteria* [299], *Clostridium coccoides* [299], *Clostridium leptum* [299], *Bacteroides* [299] and total bacteria [300] were used to quantify bacterial groups of interest. To determine absolute bacterial cell counts, 10 µl reactions were run in triplicates in a 384-well optical plate using a 7900 HT Real-Time PCR machine and the TaqMan Universal PCR Master Mix kit using default thermocycler conditions as per the manufacturer’s instructions (Applied Biosystems). Bacteria were enumerated using pre-constructed standard curves and data expressed as log₁₀ cell counts per gram of wet feces or intestinal contents.

Cytokine gene expression analysis

RNA Extraction and Purification: Total RNA was extracted from one-half segment of the proximal colon tissue using the Trizol (Invitrogen) reagent, as per the manufacturer’s instructions. EZNA MicroElute RNA clean-up kit (Omega, Bio-Tek, USA) with on-column DNase treatment, was also employed for the removal of any remaining DNA and other contaminants, eluted in 40µl of RNase-DNase free water, and stored at-80˚C until needed. RNA
quality was assessed using denaturing agarose gel electrophoresis and concentration and purity was measured by spectrophotometry using a Nanodrop (Thermo Scientific, USA) at 230, 260 and 280 nm. Purity ratios above 1.7 were considered good quality and used for qPCR.

Reverse Transcription and Relative Quantitative Polymerase Chain Reaction (qPCR): Using the High Capacity cDNA RT kit (Applied Biosystems), 2µg of purified total RNA was synthesized to cDNA using random hexamers according to the manufacturer’s instructions. Real-time PCR was employed using 100ng of cDNA, TaqMan probe-based mRNA Assays (actin, beta assay ID: Mm02619580_g1, TNF-alpha assay ID: Mm00443258_m1, IL-6 assay ID: Mm00446190_m1, and KC assay ID: Mm04207460_m1), and TaqMan Gene Expression Master Mix (Applied Biosystems) in 10µl PCR reaction. All reactions were run in triplicates in a 384-well optical plate in Applied Biosystems’ 7900 HT Real-Time PCR machine with default thermocycling conditions as per the manufacturer’s instructions. Calculations were made using the ∆∆Ct (comparative threshold cycle) method [301], after normalization to β-actin.

Statistical Analysis

One-way ANOVA followed by the Bonferroni’s Multiple Comparisons Test was employed to determine compositional differences at different time-points and regions, respectively. Bacterial counts were expressed as log_{10}g of feces or intestinal contents with a p-value of p<0.05 to be considered statistically significant. Statistically significant differences in microbial composition and gene expression analysis between groups were assessed using the Mann-Whitney t-test (p<0.05). All statistical analyses were completed using the GraphPad Prism 4 software (La Jolla, CA, USA).
Figure 4.3.1: Study Design

**Two Groups (7 mice/group)**

**Control Group:**
0.2ml sterile PBS

**Treatment Group:**
1x10^8 cells MIMBb75/0.2ml sterile PBS

<table>
<thead>
<tr>
<th>Acclimatization</th>
<th>Treatment</th>
<th>Wash Out</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>-7</td>
<td>0</td>
</tr>
</tbody>
</table>

**Feces**

<table>
<thead>
<tr>
<th>Acclimatization</th>
<th>Treatment</th>
<th>Wash Out</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>-7</td>
<td>0</td>
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</table>

**Sacrifice**

(Initial Predators and Tissue Collection)
4.4 Results

Analysis of the fecal microbial composition prior to treatment

Bacteroides/Prevotella, Bifidobacteria, C. coccoides, C. leptum, and B. bifidum were quantified by quantitative PCR. As shown in Table 4.4.1, there were no statistically significant differences in absolute cell counts between control and treatment groups in any of the bacterial groups of interest (p>0.05, n=7) at baseline. The same was true when counts were expressed as a percentage of total bacteria (Table 4.4.2) for Bifidobacteria and C. leptum, but not for C. coccoides, which was detected in a significantly higher proportion in the treatment group compared to the control group on day 0 (0.0068 ± 0.0015%; p<0.05, n=7; Table 4.4.2). Moreover, no bacteria from the genus cluster Bacteroides-Prevotella or from the species B. bifidum were detectable in the feces of these mice.
Table 4.4.1: Analysis of the microbial composition between control and treatment groups in the feces of mice on day 0.

<table>
<thead>
<tr>
<th>Microbial Group</th>
<th>CFU/gram wet feces (percent)</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>PBS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MIMBb75&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>All Bacteria</td>
<td>4.9 ± 5.9x10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>4.6 ± 1.2x10&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Bifidobacteria</em></td>
<td>2.8 ± 1.2x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>2.5 ± 1.2x10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(0.052 ± 0.029%)</td>
<td>(0.0076 ± 0.0043%)</td>
</tr>
<tr>
<td><em>Clostridium coccoides</em></td>
<td>1.6 ± 6.5x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.3 ± 7.4x10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(0.0019 ± 0.0008%)</td>
<td>(0.0068 ± 0.0015%)</td>
</tr>
<tr>
<td><em>Clostridium leptum</em></td>
<td>2.3 ± 7.5x10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>1.4 ± 4.5x10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(2.6 ± 0.68%)</td>
<td>(3.5 ± 1.1%)</td>
</tr>
<tr>
<td><em>Bacteroides-Prevotella</em></td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td><em>B. bifidum</em></td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values (mean ± SEM for n=7) are represented as absolute cell numbers and percents of total bacteria N.D., not detectable; N/A, not applicable  
<sup>b</sup>Mann-Whitney t-test, * p<0.05
Clearance of *Bifidobacteria bifidum* MIMBb75 during 24 hours post gavage

To assess the ability of MIMBb75 to persist within the gut, DNA extracted from fecal samples collected at 3, 6, 9, 12, 18, 21, and 24 hours after 7 and 14 days of gavage, were examined. As expected, *B. bifidum* cells were not detected in the control group. Feces from rats receiving the treatment contained between $10^7$-$10^{9.5}$ bacterial cells/gram of wet feces depending on the hour (Figure 4.4.1). Moreover, observational differences within the treatment group showed that there was a peak of MIMBb75 cells cleared at 6 hours post treatment. At 12 hours the number of MIMBb75 is significantly reduced. At all other time-points (18h, 21h and 24h), feces from treated mice had significantly lower MIMBb75 cell counts compared to 12 hr (Figure 4.4.1). The number of MIMBb75 cells remains constant from 18h onwards to the 24th hour. The ability of the MIMBb75 cells to remain detectable in an appreciable amount in the treatment group compared to control even after 24 hours post treatment, demonstrates the capacity for which MIMBb75 can colonize the gut *in vivo* (Figure 4.4.1).
Figure 4.4.1: Clearance time of MIMBb75 on day 14 between control and treatment groups.

Fecal samples from control and treatment mice were used for absolute quantification of MIMBb75 cells (black bars). One-way ANOVA, followed by the Bonferroni’s Multiple Comparison Test, was used to determine statistically significant differences in the quantity of MIMBb75 within the treatment group at the different time-points considered. Data are represented as mean ± SEM. There were no *B. bifidum* cells detectable in the control group (data not shown; Ct-values>34). Different letters represent significant differences (p-value<0.05) between different time points of the same group (n=7/group).
Comparison of B. bifidum MIMBb75 enumeration on days 0, 7, 14, and 21

There was no difference in the number of fecal B. bifidum cells species after 7 or 14 days of treatment (cell counts/gram wet feces; p>0.05, n=7). B. bifidum species are not present on day 0, before treatment began, nor are they present on day 21, seven days after treatment ceased (Figure 4.4.2). This implies B. bifidum is not a natural resident of these mice and that fed B. bifidum behaves as a transient member of the intestinal tract and is washed out by seven days after treatment has expired.
Figure 4.4.2: Comparing absolute cell counts of MIMBb75 per gram wet feces between treatment and control groups at 24 hours on day 0, 7, 14, and 21.

Fecal samples from mice provided MIMBb75 or PBS alone were used to quantify absolute numbers of *B. bifidum* cells. N.D. represents *B. bifidum* cells that were not detectable in the feces (Ct-values>34). Data are represented as mean ± SEM and the Mann-Whitney t-test was used to calculate significance, p-value<0.05 (n=7/group).
Regional colonization of B. bifidum

To determine the ecological niche that MIMBb75 preferentially colonizes, contents from the distal portion of the intestinal tract (including caecal contents, proximal colon contents, distal colon contents, and feces) were obtained upon sacrifice of the mice on day 14. DNA extracted from these samples was utilized for absolute quantification using TaqMan specific primers. For all regions considered, there were no B. bifidum cells detected in the control groups. In the treatment groups, B. bifidum could be quantified in all regions tested with no significant difference (caecum, 8.1 ± 0.2 log (cells/gram wet contents); proximal colon, 7.8 ± 0.1 log (cells/gram wet contents); distal colon, 7.7 ± 0.3 log (cells/gram wet contents) as shown in Figure 4.4.3). Intestinal contents from every region in the large intestine had significantly higher number of B. bifidum than feces (6.8 ± 0.1 log (cells/gram wet feces); Figure 4.4.3).
Figure 4.4.3: Regional enumeration of *B. bifidum* species within the large intestine and feces of mice on day 14.

To detect the colonization ability of *B. bifidum* MIMBb75 in different ecological niches, luminal contents originating from the caecum, proximal colon, distal colon, and feces were collected at sacrifice on day 14, 24 hours post-gavage. There were no *B. bifidum* detectable (Ct value >34) in the feces in the control group in any region considered (data not shown). Data are represented as mean ± SEM. One-way ANOVA, followed by the Bonferroni’s Multiple Comparison’s Test to determine significance between different ecological niches of interest. One-way ANOVA and Bonferroni’s Multiple Comparisons Test, * p<0.05, ** p<0.01, (n=7/group).
Analysis of fecal microbial composition on day 14.

To assess the effect of administration of *B. bifidum* MIMBb75 on dominant bacterial groups of interest including total bacteria, *Bifidobacteria*, *C. coccoides*, *C. leptum*, and *Bacteroides-Prevotella* group, DNA extracted from fecal samples on day 14 were used.

The number of total bifidobacteria did not differ between the two groups at day 14 (7.9 ± 0.3 log(cells/gram wet feces), 6.9 ± 0.75 log (cells/gram wet feces), respectively; p>0.05, n=7; Figure 4.4.4). Moreover, total bacteria cell counts were also not affected by gavage with MIMBb75 cells (10.5 ± 0.1 log (cells/gram wet feces), 10.6 ± 0.077 log(cells/gram wet feces), respectively; p>0.05, n=7; Figure 4.4.4).

The number of cells belonging to *C. coccoides* was significantly increased in the treatment group compared to control (6.3 ± 0.21 log (cells/gram wet feces), 5.2 ± 0.26 log (cells/gram wet feces), respectively; p<0.01, n=7; Figure 4.4.4). However, no significant difference in *C. leptum* cell counts were detected between control and treatment groups (8.7 ± 0.17 log (cells/gram wet feces), 8.6 ± 0.19 log (cells/gram wet feces), respectively; p>0.05, n=7; Figure 4.4.4). The *Bacteroides-Prevotella* group was undetectable in the feces of these mice (Figure 4.4.4).

In addition, representing dominant bacterial groups as a percentage of total bacteria revealed a similar pattern demonstrated using absolute cell counts (Figure 4.4.4). As a proportion, there is a significantly higher percentage of *C. coccoides* in the treatment versus control group (0.0079 ± 0.0017%, 0.00080 ± 0.00040%; p<0.01, n=7, Figure 4.5, Panel D), and no change in *C. leptum* percentages in treatment versus control groups (1.8 ± 0.49%, 1.4 ± 0.53%, respectively; p>0.05, n=6-7, Figure 4.5, Panel C). However, after normalizing to total bacteria, there was a statistically significant increase in the percentage of *Bifidobacteria* in the treatment group compared to control (0.21 ± 0.077%, 0.038 ± 0.036%, respectively; p<0.05, n=6-7, Figure 4.5, Panel A).
Figure 4.4.4: Microbial composition analysis in the feces on day 14.

Enumeration of *Bifidobacteria*, *C. coccoides*, *C. leptum*, and *Bacteroides* between control (white bars) and treatment (black bars) groups. N.D. means not detectable (Ct-value>34). Mann-Whitney t-test, *p<0.05, ** p<0.01. Data are represented as mean ± SEM (n=7/group).
Figure 4.4.5: Microbial composition analysis as a percentage of total bacteria in the feces on day 14.

The data were normalized to total bacteria cell counts and are presented as a percentage. Data are represented as mean ± SEM for control (white bars) and treatment (black bars) groups. Mann-Whitney t-test, * p<0.05, ** p<0.01 (n=7/group).
Analysis of colonic microbial composition on day 14

The effect of administration of MIMBb75 on dominant bacterial groups of interest was assessed within the proximal colon. Contrary to the microbial composition analysis in the feces, all bacterial groups of interest shifted upwards in favour of the treatment group compared to control including all bacteria (10 ± 0.10 log (cell counts/gram wet luminal contents), 10 ± 0.089 log (cell counts/gram wet luminal contents), respectively; p<0.01, n=7, Figure 4.4.6), Bifidobacteria (8.1 ± 0.32 log (cell counts/gram wet luminal contents), 4.9 ± 0.12 log (cell counts/gram wet luminal contents), respectively; p<0.01, n=7, Figure 4.4.6), and C. leptum (9.1 ± 0.17 log (cell counts/gram wet luminal contents), 8.1 ± 0.27 log (cell counts/gram wet luminal contents), respectively; p<0.01, n=7, Figure 4.4.6). In addition, C. coccoides remained unaffected by MIMBb75 (4.9 ± 0.27 log (cell counts/gram wet luminal contents), 4.8 ± 0.13 log (cell counts/gram wet luminal contents), respectively; p<0.05, n=7, Figure 4.4.6). B. bifidum species were only detected in the treatment group and not control (7.7 ± 13 log (cell counts/gram wet luminal contents); n=7, Figure 4.4.6), whereas Bacteroides-Prevotella group was not detected in either group (Figure 4.4.6).

In terms of percentages, Bifidobacteria remain significantly higher within the treatment group compared to control (0.65 ± 0.38%, 0.00090 ± 0.00032%, respectively; p<0.01, n=7, Figure 4.4.7 A) similar to that observed with absolute cell counts (Figure 4.4.6).

Parallel to analysis within the feces, C. coccoides remains unchanged between control and treatment groups (0.00068 ± 0.00028%, 0.00030 ± 0.00012%, respectively; p>0.05, n=7, Figure 4.4.7, Panel D), and B. bifidum remains detectable only in the treatment group (0.38 ± 0.10%, n=5, Figure 4.4.7, Panel B). Conversely, C. leptum is not effected by treatment (3.1 ± 0.69%, 1.8 ± 0.36%, respectively; p>0.05, n=7, Figure 4.4.7, Panel C).
Figure 4.4.6: Microbial composition analysis in the proximal colon contents on day 14. Quantitative PCR was used to quantify all bacteria, *Bifidobacteria*, *C. coccoides*, *C. leptum*, *B. bifidum* and *Bacteroides* between control (white bars) and treatment (black bars) groups in the proximal colon contents. N.D. means not detectable (Ct-value>34). Mann-Whitney t-test, *p<0.05, ** p<0.01, Data are represented as the mean of log (cell counts/gram wet luminal contents) ± SEM (n=7/group).
Figure 4.4.7: Microbial composition analysis as a percentage of total bacteria in the proximal colon contents on day 14.

The data were normalized to total bacteria cell counts and are presented as a percentage. Proportions of *Bifidobacteria*, *C. coccoides*, *C. leptum*, *B. bifidum* and *Bacteroides-Prevotella* between control (white bars) and treatment (black bars) groups were evaluated. Data are represented as the mean of bacteria of interest as a percentage to total bacteria ± SEM. Mann-Whitney t-test, * p<0.05, ** p<0.01 (n=7/group). *Bacteroides-Prevotella* group was not detectable.
Impact of B. bifidum MIMBb75 on cytokine gene expression in the proximal colon

There were no significant differences in the mRNA expression of pro-inflammatory cytokines of interest including KC, IL-6, and TNF-α, between the two groups (p>0.05, n=6, Figure 4.4.8).
Figure 4.4.8: *B. bifidum* MIMBb75 effect on proximal colon cytokine mRNA gene expression on day 14.

The effect of MIMBb75 on cytokine gene expression is represented as a fold change in gene expression levels relative to control. Significance was calculated based on the ∆Ct values. Numerical results are given as the mean fold change relative to PBS (control) ± SEM. The Mann-Whitney, * p<0.05.
4.5 Discussion

This study examined the intestinal colonization pattern of the *B. bifidum* strain MIMBb75 (hereafter MIMBb75) in mice. We were able to recover *B. bifidum* in the feces of these mice with cell counts reaching $7.02 \times 10^6$ cell/gram of wet feces, corresponding to 3.75% of the total bifidobacteria. *Bifidobacterium bifidum* was not detected in the feces prior to treatment, which is in line with *B. bifidum* being considered specific to humans [302, 303], and therefore it is assumed that any *B. bifidum* detected in the treatment group originate from MIMBb75 cells provided.

It is generally accepted that consumption of probiotics requires two weeks before any measureable benefits can be detected in both humans and mice, however, it is interesting to note that MIMBb75 could be recovered after 7 and 14 days of treatment ($6.5 \times 10^6$ cells/g wet feces and $2.3 \times 10^7$ cells/g wet feces, respectively). Perhaps future studies of MIMBb75 can determine its effects using a one-week treatment period opposed to two-weeks under the assumption that similar outcomes are ascertained on day 7 compared to day 14. In addition, no MIMBb75 cells were recoverable after one-week of treatment cessation. This indicates that although MIMBb75 had the capacity to colonize, it remained only a transient member of the gastrointestinal tract. This is not surprising given that most probiotic studies show a short-lived recovery once feeding has stopped [304, 305], and therefore there needs to be continuous feeding of the probiotic to ensure its beneficial health effects are conferred to the host. Of further interest would be to analyze fecal samples collected on additional time points within the first week of stopping gavage, to obtain a better approximation of when MIMBb75 is completely washed out.

Subsequent to intra-gastrically gavaging mice with *B. bifidum* MIMBb75 ($10^8$ CFU/0.2ml), feces were collected after 3, 6, 9, 12, 18, 21, and 24 hours and used to determine the clearance time of MIMBb75. Immediately following treatment (3 hours), MIMBb75 were detected in the feces in a relatively high amount, although this is most likely a combination of left over MIMBb75 cells remaining from the previous gavage session and those just provided. A peak of flushed MIMBb75 cells was detected at 6 hours post treatment and steadily declined henceforth until 24 hours post treatment, yet still remained at a relatively high quantity (approximately $10^{6.8}$ cells). Excretion of ingested materials have been shown to be detected as early as 2.6 hours and
have a transit time of approximately 5.8-10 hours in mice [306]. This implies that the MIMBb75 should have been completely eliminated out of the gastrointestinal tract after 10 hours. Recovery of MIMBb75 cells in the feces after 24 hours is suggestive of its ability to survive passage through the gastrointestinal tract and that it remained alive at active site within the alimentary canal [197]. A possible mechanism through which MIMBb75 is capable of survival through the harsh gastric acid conditions and bile secretions of the stomach and upper intestinal tract is through a pH-dependent self-aggregation phenotype characteristic of MIMBb75. Through self-aggregation it has been proposed that MIMBb75 initiates cell-to-cell interactions that hide surface molecular determinants involved in adhesion, and once in the distal portion of the gut, cell clusters disaggregate with increasing pH exposing adhesive determinants to ensure colonization [14].

*Bifidobacteria* are anaerobic species known to be found in high numbers in the large intestine [307]. A recent study from Dr. Guglielmetti and colleagues [14] provided evidence for environmental factors effecting MIMBb75 adhesion including low pH, presence of bile salts, and specific sugars. The conclusions of the study were that distal regions of the intestinal tract provided the most preferential niche for MIMBb75 colonization due to slightly acidic pH, low concentrations of bile acids, and high presence of mannose and fucose. Furthermore, recent analysis of *Bifidobacterium breve* provides evidence that the caecum is the preferential niche for colonization as opposed to the expected proximal colon, at least for *B. breve* within mice [308].

To determine the preferential niche of MIMBb75 within the distal portion of the alimentary canal, caecum, proximal and distal colon contents were collected. This study provides evidence for an indiscriminate colonization strategy of *B. bifidum* in all regions of interest including the caecum, proximal and distal colon. To my knowledge, the regional colonization preference of *B. bifidum* species has not been often tested, although the indiscriminate colonization capacity of caecum, proximal and distal colon has been verified by *B. animalis* in mice [309]. However, *B. bifidum* was not found to be a dominant bifidobacterial species in human caecum biopsies, leading the authors to suggest that they may be restricted to a particular ecological niche [310]. It seems plausible to suggest that, although *B. bifidum* may colonize indiscriminately in mice, this may not apply to humans because there are some anatomical differences between these two
mammals (i.e. larger caecum and no transverse colon in mice), therefore requiring further investigation into the colonization capacity of MIMBb75 in a clinical setting.

In addition, all regions had significantly higher cell counts of MIMBb75 compared to the feces, which is in agreement with other findings showing significant differences in relative distribution of dominant bacteria between feces and colon [293], and feces and caecum [311]. This demonstrates the necessity to investigate regions of interest as opposed to using fecal data as a proxy for estimating bacterial proportions in the colon. The strong capacity for B. bifidum to adhere to intestinal epithelial cell lines [271, 273, 274], and mucins [275], may provide a basis for its ability to indiscriminately colonize all portions of the large intestine, which are characteristically similar in terms of oxygen content, pH, and carbohydrate sources [312].

The dominant bacterial groups have been identified as C. coccoides, C. leptum, Bacteroides-Prevotella, and Bifidobacteria [107]. B. bifidum colonization resulted in a concomitant significant increase in the proportion of Bifidobacteria in all regions analyzed. However, varying effects on the enteric microbial composition were observed depending on the intestinal segment in question. There was a significant increase in total bacterial detected within the proximal colon not observed in the caecum or feces. The increase in total bacteria may have been directly related to a significant increase in the C. leptum, which diminishes once calculated as a percent of total bacteria, perhaps as a consequence of nullified effect of associated increases in Bifidobacteria, B. bifidum and total bacteria.

Significant increases in C. leptum, corresponding to the presence of MIMBb75 was detected in the proximal colon. C. leptum has been shown to decrease with age, which has often been associated with a concomitant increase in diseased states, compared to healthy middle-aged adults [138]. Low numbers of Faecalibacterium prausnitzii, a prominent member of the human microbial gut community belonging to clostridial cluster IV (C. leptum group), is an important butyrate producer. The C. leptum group has been proposed to be crucial in maintaining mucosal homeostasis, given that low counts of F. prausnitzii have been consistently implicated with reduced protection of the gut mucosa often found in active IBD patients [313, 314], often related to the butyrate production characteristic of F. prausnitzii [315]. Butyrate is the preferential energy source of colonocytes [316], and proposed to be an inhibitor of pro-inflammatory
cytokine expression within the intestinal mucosa via hyperacetylation of histones and suppression of NF-κB signalling [317]. In addition, butyrate has the propensity to reinforce the mucosal barrier via mucin and antimicrobial peptide synthesis and secretion, and by directly increasing tight junction protein expression [318]. Therefore, a concomitant increase in *C. leptum* due to the presence of MIMBb75 may provide a potential preventative mechanism, especially for those with increased risk of IBD associated illnesses.

Within the feces there was a significant increase in *C. coccoides* not detected in the proximal colon. However, the caecum reveals a significant decrease in *C. coccoides* levels. Proportionally, however, day 0 fecal sample analysis revealed significantly higher *C. coccoides* in the MIMBb75 group when calculated as a percentage of total bacteria. This may have confounding implications on the findings on day 14 fecal samples. In addition, it would have been interesting to measure *C. coccoides* absolute numbers and proportionally on day 0 in the caecum and proximal colon contents to ensure no differences were detectable before treatment commenced. Although from a different perspective, this may be indicative of clearance of *C. coccoides* into the feces as a result of MIMBb75 colonization for example, through competitive exclusion, often seen between bifidobacteria and *C. difficile* [319]. To our knowledge, there are no studies available in the literature directly comparing the administration of bifidobacteria and direct involvement in microbial compositional shifts, although we show in our study that bifidobacteria do impact on the endogenous microbes. However, other studies show correlative effects of *Bifidobacteria* on the enteric microbial composition. For example, in a study comparing the composition of the human microbiota between infants and adults, high proportions of *Bifidobacteria* in infancy were shown to be associated with lower counts of *C. coccoides*, but with age (adulthood) *Bifidobacteria* numbers significantly declined with simultaneous increases in *C. coccoides* proportions [138], substantiating the inverse relationship detected in the caecum in this study. Moreover, in a study which fed healthy elderly volunteers with a bifidogenic prebiotic, an increase in bifidobacteria was associated with an increase in *C. coccoides* in the feces [320], again concurring with findings from our study, although interactions within the proximal colon or caecum were not measured.

Correlative studies have shown decreases in *C. coccoides* counts in patients with active IBD and intestinal colitis [313], although it is not known if the decreased presence is a causative effect or
consequence of the disease. In addition, Barcenilla and colleagues have demonstrated that isolates of the *C. coccoides-Eubacterium rectale* group are the most prominent butyrate producers [315], which in itself may have therapeutic benefits such as preventing colon cancer [321] or ulcerative colitis [322]. Therefore, at this time it is difficult to establish whether the findings of our study are a desired effect of MIMBb75 administration. In addition, published studies do not usually plan to have a subset of mice to be sacrificed prior to beginning the treatment period, with intestinal contents collected and analyzed for their microbial composition. However, analysis or regions of interest prior to treatment should be taken into consideration as it would provide a direct, region-specific, comparison of microbial shifts due to treatment after a given period to those observed at day 0.

Because *Bifidobacteria* were shown to impact cytokine expression [13], pro-inflammatory cytokine expression was analyzed in the proximal colon. Dr. Guglielmetti and colleagues demonstrated that MIMBb75 induces the selective expression of IL-8 but not IL-6, *in vitro*. The necessity to moderately express IL-8 in the healthy situation is exemplified in a CXCL1-receptor KC (murine homologue for IL-8) knockout mouse study, which demonstrated that these mice have increased susceptibility to dextran sodium sulfate induced colitis [323]. The results of this study conform to the general consensus that a modest expression of pro-inflammatory cytokines in the healthy situation is required to prime the innate immune system for potential pathogenic invasion [15, 324]. As a result, investigation as to whether MIMBb75 initiates the expression of pro-inflammatory cytokines, primarily IL-8, in a healthy mouse model was of interest. The selectivity of increasing expression of IL-8 was assessed via analysis of additional pro-inflammatory cytokines including IL-6 and TNF-α.

In this study, gavage of MIMBb75 to mice for 14 days was shown to have no significant effect on pro-inflammatory cytokine production in the proximal colon at the mRNA level *in vivo*. The effects of *B. bifidum* on innate immunity measured by cytokine expression is heavily reliant on the strain under investigation and model being considered. For example, *B. bifidum* ATCC 86321 decreases IL-6 and TNF-α production in healthy ageing mice [298], whereas intestinal epithelial cell lines displayed unresponsiveness to *B. bifidum* 17 determined via unaltered expression of IL-8 *in vitro* [276]. In addition, decreased IL-8 expression was observed after cells were co-incubated with LPS [277], and in three normal cell lines [278]. Perhaps the reason for no
significant difference in pro-inflammatory cytokine expression, specifically IL-8 expression, may be the result of interacting effects of other bacteria and immune cells, which may have responded promptly to the initial introduction of MIMBb75 to ensure intestinal homeostasis. For example, butyrate has been shown to down-regulate the IL-8 expression through the induced expression of A20, a negative regulator of NF-κB-pathway [325], and therefore an increase in the butyrate-producing *C. leptum* group may have provided the basis for pro-inflammatory cytokine inhibition. Overall, the transient colonization of MIMBb75 and concomitant increase in butyrate-producing bacteria, may prove to have beneficial therapeutic implications, especially in IBS sufferers, which have recently been shown to have been alleviated of symptoms upon ingestion of MIMBb75 [282].
Chapter 5

Genetically engineered *Bifidobacterium longum* NCC2705 expressing the *Bifidobacterium bifidum*-specific cell surface adhesion protein BopA affects gut microbiota composition and impacts colonic intestinal barrier gene expression in mouse

Contribution of Data

Quantitative data for mucin 3, 4, and 13 gene expression on day 14 in the results section entitled “The effect of BopA- and BopA+ transformed bacteria on mucin mRNA gene expression in vivo within the proximal colon on day 14, 24 hours post-gavage” were provided by Sitelle Cheskey.

Quantitative data on caecum microbial composition in table 5.3 in the results section entitled “Summary of the effects of BopA+ on the endogenous microbial composition represented as absolute cell counts (A) and as a percentage of total bacteria (B),” were provided by Natasha Singh.
5.1 Abstract

**Background**: Probiotic bacteria require the capacity to colonize within the intestinal tract to maintain a beneficial dialogue with the host. Molecular determinants underlying the beneficial effects exerted by probiotic bacteria are largely uncharacterized, specifically in *Bifidobacteria*. Recently, a surface lipoprotein named BopA, has been identified as a protein specific to *B. bifidum* species and responsible for its adhesion and immunomodulatory properties, including increased expression of IL-8, *in vitro*. The aim of this study was to determine if BopA is the molecular determinant responsible for the colonization of *B. bifidum* species *in vivo* and to determine if BopA acts as a potential modulator of the fecal microbiota and expression of epithelial barrier genes, including cytokines and mucins, in the proximal colon.

**Methods**: *B. longum* NCC2705 was genetically-engineered to express a chloramphenicol resistance gene and the BopA gene (BopA+) or the empty vector (BopA-). Mice were daily gavaged with $10^8$ bacterial cells/ml resuspended in sterile PBS, for two weeks with either BopA+ or BopA- bacteria. Feces at day 11 and proximal colon contents at day 14 were utilized for microbial composition analysis via qPCR. Proximal colon tissues were also excised at sacrifice after 14 days of treatment for mucin and cytokine mRNA expression analysis via qPCR and KC protein analysis using ELISA.

**Results**: The transformed strains were recovered in the feces and intestinal contents. KC was significantly down-regulated at the mRNA level in the proximal colon of mice gavaged with *B. longum* BopA+ compared to mice gavaged with *B. longum* BopA- (fold-change of 0.77; p-value<0.05) but was not detectable at the protein level. MUC4 was found to be significantly down-regulated in the proximal colon if mice gavaged with BopA+ compared to mice gavaged with BopA- (fold-change of 0.77; p-value<0.05). No differences were detectable for dominant bacterial clusters between groups in the feces or proximal colon contents collected after 11 and 14 days of treatment, respectively.

**Conclusions**: The gavaged transformants are capable of surviving passage through the GI tract without affecting fecal or proximal colon microbial eubiosis. Furthermore, BopA+ may have the capacity to impact the intestinal barrier, at least at the mRNA level, in the proximal colon *in vivo*. 


5.2 Introduction

Eli Metchnikoff was the first to advocate the practice of modifying the endogenous microbiota composition with the use of beneficial microbes to replace potentially harmful bacteria [326]. Around the same time, Henry Tissier observed that his ‘bifid’ bacteria he called *Bacillus bifidus*, currently known as *Bifidobacteria*, was more prevalent in breast-fed infants compared to those formula-fed, which led to infantile diarrhea in the latter. He was the first to propose that administration of bifidobacteria could aid in alleviating symptoms associated with diarrhea via the restoration of a healthy microbial composition [327]. Just over a century later and there is still much to learn about the probiotic concept.

Probiotics, derive from the Greek word meaning 'pro life,' and has been defined as ‘live microorganisms, which when administered in adequate amounts, confer a health benefit to the host’ [195]. Members of the lactic acid-producing bacteria, specifically from the genus *Bifidobacterium*, are the most commonly incorporated microbes in probiotic functional food products because of their positive historical association with foods and their ‘generally regarded as safe’ status [328]. Furthermore, *Bifidobacteria* constitutes approximately 95% of the total gut bacterial population in healthy breast-fed newborns [329], and is considered a prominent bacterial group in the adult gut, remaining fairly stable at 3-6% of the fecal microbial composition until advanced age where they then decline [261, 294]. However, due to a paucity of studies assessing the molecular determinants involved in the ability of these probiotic bacteria to confer beneficial effects to the host, there is a lack of scientific evidence substantiating strain-specific health claims in Canada.

In addition, dynamic interactions with other commensal bacteria such as those observed with *B. bifidum* Z9, which actively changes the gene expression profile induced by *Lactobacillus acidophilus* in dendritic cells *ex vivo* [265], exploit the potential interactive effects probiotic species can have on their environment. *B. bifidum* strains also demonstrate differential immunomodulatory effects depending on the strain and environmental condition. For example, *B. bifidum* S17 reduced IL-8 secretion subsequent to LPS stimulation *in vitro* [277, 278], while the same strain demonstrated no effect on cytokine production including IL-6, IL-8, and TNF-α, in a healthy *in vitro* model [276]. *B. bifidum* iY was shown to inhibit IL-8 secretion in TNF-α stimulated HT29 cells [297]. Moreover, *B. bifidum* strains MIMBb75 and PLR2010
demonstrated up-regulation in IL-8 expression in a healthy in vitro model [13, 251], and finally, 
*B. bifidum* ATCC 86321 decreased pro-inflammatory cytokines IL-6 and TNF-α in aging mice [298]. Furthermore, recent analysis of the genome of *B. bifidum* PLR2010 has revealed innate metabolic pathways conserved to permit foraging of host-derived glycans such as those found in mucins, which has been suggested to have an effect on intestinal microbial ecology [251].

In 2008, Guglielmetti and colleagues identified and isolated the first *bifidobacterial* adhesion protein they called BopA, specific to *B. bifidum* species [13]. The cysteine-anchored, cell-surface lipoprotein, BopA also displayed potential immunomodulatory control in vitro through the selective increased expression of IL-8, but not IL-6 [13]. Thus, BopA may be the very first molecular determinant involved in the mechanism by which the *B. bifidum* species exerts its probiotic properties in the gut.

Therefore, the primary aim of this study was to determine whether the highly adherent properties of *B. bifidum* species are attributable to BopA in vivo. Secondary outcomes of interest include BopA-mediated changes in the microenvironment of the host including intestinal barrier gene expression such as mucins and cytokines that may, in turn, shift the endogenous microbial composition toward a healthy phenotype, where *Bifidobacteria* are found in higher proportions.
5.3 Materials and Methods

Bacterial Strains and Culture Conditions

The transformed *B. longum* NCC2705 containing the BopA gene (BopA+) and the control transformant *B. longum* NCC2705 (BopA-) were obtained by transfection using the *Bifidobacterium* vector pGOSBif33, which contains a strong T5 phage promoter to control the expression of BopA and an antibiotic resistance gene (chloramphenicol acetyltransferase). The strains were obtained from Dr. S. Guglielmetti at the University of Milan, Italy. *B. longum* BopA+ and *B. longum* BopA- were grown anaerobically for 2 days at 37°C in De Man Rogosa and Sharpe (MRS) broth (Oxoid), supplemented with 0.05g/100ml L-cysteine hydrochloride, and 5ug/ml chloramphenicol to obtain a cell density of 10^8 CFU/ml to be used for gavage.

Animals and study design

Fourteen C57Bl/6J male mice, 6 weeks of age, were purchased from Jackson Laboratories and housed in the Division of Comparative Medicine at the University of Toronto. Before the mice were randomly assigned to the treatment groups, they were allotted a one-week period to become accustomed to the animal facility. Randomization of the animals were based on initial bifidobacterial cell counts obtained via quantitative PCR on DNA extracted from fecal pellets, using *Bifidobacterium* genus specific primers [299]. Mice were caged individually and provided *ad libitum* access to water and standard chow (Harlan 2018) and were housed under the conventional 12h:12h light-dark cycle until sacrificed.

Fourteen mice (seven mice per group) were intragastrically gavaged daily for two weeks with 1x10^8 colony forming units (CFU) of either *B. longum* NCC2705 transfected to express BopA (BopA+) or *B. longum* NCC2705 transfected with the empty vector (BopA-) grown as described above, washed and re-suspended in 0.2ml pre-reduced sterile PBS. Freshly passed fecal pellets were collected on days 0, 11, 13, and 14 and stored at -20°C until needed. All mice were sacrificed on day 14 by cervical dislocation following CO\(_2\) inhalation (Figure 5.3.1). Upon sacrifice, the entire large intestine was immediately removed. The large intestine, constituting caecum to anus, was divided into three parts including the rectum (distal 1cm) and the proximal
and distal colon that correspond to the proximal and distal half, respectively, after rectal excision. Proximal colon and caecal contents were removed and the tissues were washed with ice-cold sterile saline solution (0.9% NaCl), divided into two halves longitudinally, and stored at -80˚C until needed. The animal study design and protocol was approved by the Animal Care Committee at the University of Toronto.

**Classical Culturing**

Caecal contents and fecal pellets (20-30 mg) were individually homogenized in sterile PBS supplemented with 0.05% cysteine-HCL and 10x serial dilutions were plated onto MRS agar (Oxoid) containing 5µg/ml chloramphenicol and incubated at 37˚C for 3-4 days. Bacterial counts were expressed as log_{10} CFU per gram of wet feces or caecal contents. The plates were counted by two different individuals and averaged for each mouse.

**Microbiota Composition Analysis of Feces and Intestinal Contents**

Gut microbial composition analysis of dominant bacterial groups including those from *Bacteroides-Prevotella*, *Clostridium*, and *Bifidobacterium* genera was assessed in feces and both proximal colon and caecal contents using absolute quantitative TaqMan Real-Time PCR. DNA was extracted from 16-50 mg of wet intestinal contents or feces using E.Z.N.A.™ stool DNA Isolation Kit (Omega, USA), following the manufacturer’s protocol modified to include a lysozyme digestion step (20mg/ml; 37˚C for 30 minutes). DNA concentration and purity was measured using ThermoScientific’s Nanodrop 1000 Spectrophotometer (ThermoScientific, USA). DNA samples were stored at -20˚C until needed.

Quantitative Real-Time PCR studies using 50ng of DNA for *Bifidobacteria*, *Clostridia coccoides*, *Clostridia leptum*, *Bacteroides-Prevotella* and total bacteria were quantified using 16S rDNA gene specific primers [299] [300], while *B. bifidum* was quantified by TaqMan probe-based assay using specific primers targeting the BopA gene sequence (Forward: 5’ACCGAATTCGCCCTGTCACCTT3’; Reverse: 5’ACGGCGCGGATTGCT3’; Probe: 5’CCGCTGGATGCTGAC3’). To determine absolute bacterial cell counts, reactions were run in triplicates in 10µl reactions using a 384-well optical plate using a 7900 HT Real-Time PCR
machine and the TaqMan Universal PCR Master Mix kit (Applied Biosystems) using default thermocycler conditions as per the manufacturer’s instructions. Bacteria were enumerated using pre-constructed standard curves and data expressed as log_{10} cell counts per gram of wet feces or intestinal contents.

Cytokine and mucin gene expression analysis

Total RNA was extracted from one-half segment of the proximal colon tissue using the miRVANA miRNA Isolation Kit (Ambion, Austin, TX, USA), as per the manufacturer’s instructions, eluted in 100 µl of RNAse-free water, and stored in the -80°C freezer until needed. The concentration and purity was measured by spectrophotometry using a Nanodrop (Thermo Scientific, USA) at 230, 260 and 280 nm. Purity ratios above 1.7 were considered good quality and used for QPCR. Two µg of total RNA were used to generate cDNA with the High Capacity cDNA RT kit (Applied Biosystems), and random hexamers according to the manufacturer’s instructions. Gene expression was assessed by Real-time PCR using 100 ng of cDNA and TaqMan Assays for the genes of interest (actin, beta assay ID: Mm02619580_g1, TNF-alpha assay ID: Mm00443258_m1, IL-6 assay ID: Mm00446190_m1, IL-10 ass ID: Mm00439614_m1, KC assay ID: Mm04207460_m1, MUC1 assay ID: Mm00449599_m1, MUC2 assay ID: Mm00458299_m1, MUC3 assay ID: Mm01207064_m1, MUC4 assay ID: Mm00466896_m1, and MUC13 assay ID: Mm00495397_m1), and TaqMan Gene Expression Master Mix (Applied Biosystems) in 10µl PCR reaction. All reactions were run in triplicates in a 384-well optical plate in Applied Biosystems’ 7900 HT Real-Time PCR machine with default conditions. Relative quantification was assessed using the ΔΔCt (comparative threshold cycle) method [301], after normalization to β-actin.

KC Cytokine protein expression analysis

To quantify KC, one-half segment of colonic tissues were individually homogenized in PBS buffer supplemented with protease inhibitors (pH 7.4), centrifuged at 4°C at 1800 rpm for 10 min and the supernatants were stored at -80°C until needed. To quantify total protein, the Coomassie Plus protein quantification kit (Pierce) was utilized as per the manufacturer’s instructions. The Quantikine Colorimetric Sandwich ELISA kit, specific for KC (R&D Systems), was employed following the manufacturer’s instructions. Protein levels for KC were measured in duplicates and
expressed relative to total protein using a pre-constructed standard curve for albumin. Protein absorbance was measured using a microplate reader set at 450nm and concentrations were calculated from a pre-constructed standard curve.

Detection of \textit{B. longum} in fecal samples

\textit{B. longum} specific primers (\textbf{forward:} 5’ TTCCAGTTGATCGCATGGTC 3’; \textbf{reverse:} 5’GGAAGCCGTATCTCTACGA3’) [330] were purchased from ACGT Corporation and used in a classical PCR reaction at a concentration of 0.5 µM, together with 150 ng of DNA in a final volume of 50 µL; all other reagents were from Fermentas PCR Master Mix (2x) kit. Amplification conditions included an initial denaturation at 95°C for 2 min, followed by 35 cycles at 95°C for 30s, 65°C for 45s and 72°C for 1min, with a final extension cycle at 72°C for 10 min. The amplification products were separated in 1.5% (wt/vol) agarose gel electrophoresis (at 100 V for 30mins).

Statistical Analysis

Region colonization analysis of transformed bacteria was assessed in the feces and intestinal contents on day 13 and 14, respectively, using one-way ANOVA followed by the Bonferroni’s Multiple Comparisons Test. Bacterial counts were expressed as log$_{10}$/g of feces or intestinal contents with a p-value of p<0.05 to be considered statistically significant. Statistically significant differences in microbial composition and gene expression analysis between groups were assessed using the Mann-Whitney t-test (p<0.05). Grubb’s test was performed to determine potential outliers. All statistical analysis were completed using the GraphPad Prism 4 software (La Jolla, CA, USA).
Figure 5.3.1: Study Design

Two Groups of Mice (7 mice/group) Gavaged Daily with 1x10⁶ cells/0.2ml sterile PBS:

*Control*: *B. longum* NCC2705 BopA⁻ [empty vector]
*Treatment*: *B. longum* NCC2705 BopA⁺ [express BopA gene]

<table>
<thead>
<tr>
<th>ACCLIMITIZATION</th>
<th>TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td></td>
</tr>
<tr>
<td>-7</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>13</td>
<td>14</td>
</tr>
</tbody>
</table>

**Fecal Collection**

<table>
<thead>
<tr>
<th>Sacrifice (n=7/group)</th>
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*Intestinal Contents & Tissue Collection X*

Randomization based on initial *Bifidobacteria* cell counts via qPCR
5.4 Results

*Microbial composition analysis in the feces at baseline*

To test the effects of BopA, a *Bifidobacterium* strain was transformed to express BopA. The strain of choice was *B. longum* NCC2705, because its entire genome has been sequenced and no significantly homologous protein to BopA was found [331].

There were no statistically significant differences in absolute cell counts or proportions between BopA- and BopA+ groups detected in the feces for any of the bacterial groups of interest (p>0.05, n=7). Moreover, no bacteria from the genus cluster *Bacteroides-Prevotella* or from the species *B. bifidum* were detectable in the feces of these mice (Table 5.4.1). *B. longum* was detected by classical PCR in both BopA- and BopA+ groups on day -7 (in 6 and 4 mice, respectively) and in all mice in both groups on day 11 (Figure 5.4.8).
Table 5.4.1: Comparison of the microbial composition between BopA- and BopA+ groups on day -7.

<table>
<thead>
<tr>
<th>Microbial Group</th>
<th>CFU/gram wet feces (percent of total bacteria)</th>
<th>BopA-</th>
<th>BopA+</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Bacteria</td>
<td></td>
<td>4.4x10^{10} ± 8.50x10^{9}</td>
<td>6.5x10^{10} ± 1.1x10^{10}</td>
<td>0.1827</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td></td>
<td>2.2x10^{8} ± 2.1x10^{8} (0.03 ± 0.019%)</td>
<td>1.5x10^{8} ± 1.4x10^{8} (0.007 ± 0.0028%)</td>
<td>0.8701</td>
</tr>
<tr>
<td>Clostridium coccoides</td>
<td></td>
<td>4.4x10^{5} ± 3.7x10^{5} (0.0011 ± 0.00035%)</td>
<td>4.2x10^{5} ± 2.8x10^{5} (0.00064 ± 0.00013%)</td>
<td>0.3901</td>
</tr>
<tr>
<td>Clostridium leptum</td>
<td></td>
<td>1.7x10^{9} ± 4.3x10^{8} (4.1 ± 1.1%)</td>
<td>2.2x10^{9} ± 7.1x10^{8} (2.7 ± 0.56%)</td>
<td>0.4686</td>
</tr>
<tr>
<td>Bacteroides-</td>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
<td>N/A</td>
</tr>
<tr>
<td>Prevotella</td>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
<td>N/A</td>
</tr>
<tr>
<td>B. bifidum</td>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
<td>N/A</td>
</tr>
</tbody>
</table>

\[ a \] Values (mean ± SEM for n=7) are represented as absolute cell numbers
N.D., not detectable; N/A, not applicable

\[ b \] Mann-Whitney t-test, *p<0.05
Presence of BopA- and BopA+ in the feces and caecal contents

No statistically significant difference was found between the colonization ability of BopA+ versus BopA- in both the caecum contents and feces (8.5±0.2 CFU [caecum BopA-] versus 8.2±0.08 CFU [caecum BopA+], p>0.05, n=3-5; 7.6±0.2 CFU [feces BopA-] versus 8.2±0.18 CFU [feces BopA+], p>0.05, n=3-5; Figure 5.4.1). The BopA- group contained significantly more colonies in the caecal contents compared to feces (8.5±0.2 CFU in caecum versus 7.7 ± 0.2 CFU in feces; p<0.05, n=3-5; Figure 5.4.1). Similar patterns were not observed for the BopA+ group (8.2 ± 0.08 CFU [caecum] versus 8.3 ± 0.2 CFU; p>0.05, n=3-5; Figure 5.4.1).

Furthermore, B. longum was detected by PCR in the feces from most mice on day -7 and all mice on day 11, although their presence were in qualitatively higher amounts on day 11 compared to day -7 (baseline values; Figure 5.4.8).
Figure 5.4.1: Enumeration of BopA+ and BopA- bacterial colonies grown on selective medium containing chloramphenicol in the feces and caecal contents on day 13 and day 14, respectively.

Plates with 50-150 colonies were used to estimate the total number of transformants present. The plates were counted by two different individuals and averaged for each mouse. Black bars and white bars represent the BopA+ and BopA- groups, respectively. The data are presented as means of the log$_{10}$ CFU/gram of wet intestinal contents or feces ± SEM. Mann-Whitney t-test was employed to test statistical significance. * p-value<0.05 (n=3-5/group).
The absolute cell counts of endogenous microbes indicate that BopA does not significantly impact the number of total bacteria, nor the number of \textit{Bifidobacteria} cells in the feces of mice receiving BopA+ compared to BopA- (\(p>0.05\), \(n=7\); \textbf{Figure 5.4.2}). There were no significant differences in cell counts of \textit{C. coccoides} and \textit{C. leptum} groups between BopA+ versus BopA- groups (\textbf{Figure 5.4.2}). The \textit{Bacteroides-Prevotella} group and \textit{B. bifidum} species were undetectable in the feces of all mice. With consideration into the relative abundance of bacterial groups of interest as a percentage of total bacteria, only \textit{Bifidobacteria} was found to be significantly decreased in the feces of the BopA+ group versus BopA- (0.04 ± 0.02\% [BopA+, \(n=7\) versus 1.4 ± 0.7\% [BopA-, \(n=7\)], respectively, \(p<0.05\), \textbf{Figure 5.4.3, Panel A}). No other significant differences were detected for other bacterial groups analyzed (\(p>0.05\), \textbf{Figure 5.4.3, Panel B and C}). However, mice gavaged with transformed bacteria expressing the BopA gene significantly increased \textit{Bifidobacteria} numbers in the feces on day 11 compared to day -7 (8.2 ± 0.5 log (cell counts/gram wet feces) versus 6.5 ± 0.3 log (cell counts/gram wet feces), respectively; \(p<0.05\), \(n=7\), \textbf{Figure 5.4.4}). The same is not evident for the BopA- group (\(p>0.05\), \(n=7-8\), \textbf{Figure 5.4.4}).

Similarly to fecal analysis, there was no statistically significant difference detected in the proximal colon contents between BopA- and BopA+ groups for all bacterial groups of interest (\(p>0.05\), \(n=6-7\), \textbf{Figure 5.4.5}). The \textit{Bacteroides-Prevotella} group and \textit{B. bifidum} species were not detected in either group. In terms of percentages, there was no statistically significant difference between BopA- and BopA+ groups for any of the bacterial groups analyzed (\(p>0.05\), \(n=6-7\), \textbf{Figure 5.4.6}).

Furthermore, caecal versus proximal colon contents versus feces revealed no statistically significant difference in \textit{Bifidobacteria} cell counts for both BopA- and BopA+ groups (\textbf{Figure 5.4.7}).
Figure 5.4.2: Microbial composition analysis in the feces on day 11.

Absolute cells counts of *Bifidobacteria*, *C. coccoides*, *C. leptum*, and *Bacteroides-Prevotella*. The data are presented as means of log (cell counts/gram wet feces) ± SEM. White bars represent the BopA- group and black bars represent the BopA+ group. The Mann-Whitney t-test was used to determine statistical significance, *p*<0.05 (n=7/group). There were no detectable *Bacteroides-Prevotella* or *B. bifidum* in the feces of these mice (data not shown).
Figure 5.4.3: Microbial composition analysis in the feces as a percentage of total bacteria on day 11.

The data presented corresponds to those from Figure 5.4.2, now represented as a percentage of total bacteria for *Bifidobacteria* [A], *C. coccoides* [B], and *C. leptum* [C]. Mann-Whitney t-test, * p-value<0.05 (n=7/group).
Figure 5.4.4: Detection of *Bifidobacteria* in the feces of mice on day -7 versus day 11 for both BopA- and BopA+ groups.

White bars represent the BopA- group and black bars represent the BopA+ group. The Mann-Whitney t-test, p-value < 0.05. Groups with different letters are significantly different (n=7/group).
Proximal colon contents were used to quantify all bacteria, *Bifidobacteria*, *C. coccoides*, *C. leptum*, *B. bifidum* and *Bacteroides-Prevotella* between BopA- (white bars) and BopA+ (black bars) groups. *Bacteroides-Prevotella* and *B. bifidum* were not detectable (Ct-value>34). Data are represented as the mean of log (cell counts/gram wet luminal contents) ± SEM. Mann-Whitney t-test, *p<0.05* (n=7/group).
Figure 5.4.6: Microbial composition analysis as a percentage of total bacteria in the proximal colon contents on day 14.

The data were normalized to total bacteria cell counts and are presented as a percentage. Absolute quantification of Bifidobacteria [A], C. coccoides [B], C. leptum [C] between BopA- (white bars) and BopA+ (black bars) groups are represented as mean percentage of total bacteria ± SEM. Mann-Whitney t-test, * p<0.05 (n=7/group).
Figure 5.4.7: Comparison of the regional enumeration of *Bifidobacteria* within the feces and large intestine of mice on day 11 and 14, respectively, between BopA- and BopA+ groups.

BopA- groups are represented by white bars and BopA+ groups are represented by black bars. Data are represented as mean log (cell counts/gram wet feces) ± SEM. One-way ANOVA, followed by the Bonferroni’s Multiple Comparison’s Test was utilized * p<0.05 (n=7/group). C.C., caecal contents; P.C.C., proximal colon contents.
Figure 5.4.8: Detection of *B. longum* species in the feces at day -7 versus day 11.

NTC, no template control; PC, positive control (*B. longum* NCC2705).
Impact of BopA on intestinal barrier mRNA gene expression

BopA did not significantly affect the expression of any of the cytokines tested except for KC, which had a significantly lower expression in the BopA+ group (0.8±0.1 fold relative to BopA-; p<0.05, n=7, Figure 5.4.9). KC was not detectable at the protein level KC in the proximal colon of these mice (data not shown).

In regards to mucins, there were no significant differences in the colonic expression of mucin 1-3, and 13 between the two groups, except for MUC4, which was significantly down-regulated in mice fed B. longum BopA+ (0.8±0.1 fold relative to BopA-; p<0.05, n=7, Figure 5.4.10).
Figure 5.4.9: Cytokines gene expression in the proximal colon of mice receiving *B. longum* BopA+ (black bars) or *B. longum* BopA- (white bars).

Fold change were calculated versus BopA- (=1) using the delta delta Ct method after normalization to beta actin. Data are given as the mean fold change relative to BopA- ± SEM (n = 7/group). The Mann-Whitney t-test was used to determine statistical significance on delta Ct arithmetic means between groups,* p-value<0.05.
Figure 5.4.10: Mucin gene expression in the proximal colon of mice receiving *B. longum* BopA+ (black bars) or *B. longum* BopA- (white bars).

Fold change were calculated versus BopA- (=1) using the delta delta Ct method after normalization to beta actin. Numerical results are given as the mean fold change relative to BopA- ± SEM (n=7/group). The Mann-Whitney t-test, * p-value<0.05.
5.4 Discussion

In this study, we evaluated the ability of transformed *B. longum*, expressing or not, the *B. bifidum* surface protein BopA, to colonize the murine intestinal tract and to impact its endogenous microbiota composition and barrier gene expression. The transformants were capable of surviving passage through the gastrointestinal tract, since they were recovered alive in the feces and caecum contents. A higher proportion of bifidobacteria was found in the caecum compared to the feces, which had been shown in another study for *B. breve* UCC2003 [308], and *B. bifidum* MIMBb75 (Chapter 4). To our knowledge, only one other study tested the successful recovery of live transformed bifidobacteria cells after passage through the murine intestinal tract, which was the *B. breve* species found to preferentially colonize the caecum [308].

To assess the capacity of BopA to modulate the endogenous microbial community, TaqMan primers specific for several species that cluster together to represent the dominant bacterial groups found within the human intestine and qPCR were utilized. BopA did not affect the composition of the endogenous microbiota within the feces or proximal colon contents, although the proportion of *Bifidobacteria* within the feces was significantly decreased in the feces from the BopA+ treated mice compared to mice given BopA-. This suggests that BopA may be impacting on other microbes found within the feces that were not the focus of this study. These bacteria could include those from the novel operational taxonomic unit the authors named ‘mouse intestinal bacteria,’ belonging to *Cytophaga-Flavobacter-Bacteroides-Prevotella* phylum [332], which would escape detection by our *Bacteroides-Prevotella* primers. However, this decrease in the proportion of *Bifidobacteria* was not observed in the proximal colon contents, demonstrating the reoccurring theme that microbial functions including adhesion is highly dependent on region.

*Bifidobacterium longum* NCC2705 is itself a probiotic strain capable of establishing in the intestinal tract [333], for example through exopolysaccharides [334] and fimbriae-like structures [331]. Assuming that BopA provided an adherent advantage to the transformant, we would expect to see significantly more *Bifidobacteria* in the BopA+ group compared to BopA-, which was not the case in this study. It is likely, that the adhesion capacity of *B. longum* was already maximized by its endogenous mechanisms, and the presence of BopA did not confer an
ecological advantage to this strain. Furthermore, *C. coccoides* and *C. leptum* significantly increase in the caecum contents from mice treated with the BopA+ transformant compared to mice treated with the BopA- transformant (data not shown), which was not shown in either the proximal colon contents or the feces, highlighting that BopA has a region-specific capacity of modulating the commensal microbial composition. Favouring increases in *C. coccoides* and *C. leptum* groups may have a beneficial impact on the host considering that these are butyrate-producing bacteria [315, 321] and have been found to be less represented in the feces of active IBS and infectious colitis patients compared to healthy counterparts [313]. Given the changes in *Clostridia* in the caecum it would be interesting to evaluate how BopA impacts the Firmicutes to Bacteroides-Prevotella ratio, since this is altered in IBD [313]. Unfortunately, this was not possible in this study, since the mice employed had no quantifiable Bacteroidetes with the conditions used.

Furthermore, *B. longum* species are a component of the endogenous microbial community in these mice, given that at day-7, most have some detectable levels of *B. longum*. This was another unexpected observation given that *B. longum* species are thought to be strictly of human origin [310]. However, Lamendella and colleagues (2008) observed that *B. longum* species do form part of the endogenous microbial ecology in some animals including sheep and pigs [302], albeit mice were not tested, it provides some literature support for their presence.

Another indirect, but more quantitative measure of vector recovery and persistence of the transformed bacteria within the gut, was to compare the number of *Bifidobacteria* present in the feces at day -7 versus day 11, 24 hours post-treatment. Only the BopA+ group had significantly more bifidobacteria in the feces compared to their day -7 counterparts indicating a role for BopA+ in the adhesion of the transformed strains.

Lastly, we analyzed the bifidobacteria cell numbers regionally to determine whether there was preferential regional colonization. From the results it can be deduced that no regional preference for colonization for both BopA- and BopA+ groups was evident. However, we are limited given that bifidobacteria counts are being assessed as opposed to BopA- or BopA+ transformed bacteria themselves. This is limiting because it is possible that although transformed bacteria may be significantly increased in a particular region, it is shadowed by the potential decrease in other bifidobacteria species, therefore remaining unchanged at the genus level. For future studies
it would be interesting to have day -7 caecum and proximal colon contents to have baseline samples to compare to.

Probiotic type bacteria have the propensity to induce secretion of pro-inflammatory cytokines, including IL-8 [335], which has been said to have a protective role in inflammation [324]. However, several in vitro studies have shown that these effects depend on the species (or strain) and condition investigated. For instance, no difference in IL-8, IL-6, or TNF-α cytokine expression was measured in the presence of B. bifidum S17 using Caco-2 cells [276], whereas another study reported that both B. bifidum S17 and B. longum NCC2705 reduced IL-8 secretion in LPS induced inflammation in vitro [277]. In addition, B. bifidum S17 was found to reduced expression of IL-8 in Caco-2, HT29, and T84 cells [278], and IL-8 secretion was shown to be inhibited by B. bifidum iY in TNF-α stimulated HT29 cells [297]. However, most concerning are the effects demonstrated by B. bifidum MIMBb75 and isolated BopA protein in vitro, which were shown to increase IL-8 secretion [13].

This contradicts the results of our study, which revealed that BopA has an inhibitory effect on IL-8 expression at mRNA level, which could be explained by differences between the simplistic in vitro model versus the more complex in vivo environment. For example, in the in vitro study, a high concentration of isolated BopA (10-20µg) were in direct contact with receptors found on the intestinal cell lines in vitro [13]. However, in an in vivo setting there are several physical barriers including the mucus layer, characterized by an inner firmly attached sterile layer devoid of bacteria [27], thereby preventing the receptor-mediated interaction presumably required to initiate IL-8 up-regulation. Furthermore, B. longum NCC2705 has been shown to significantly up-regulate IL-8 expression in the same in vitro study [13], and perhaps the presence of BopA somehow interrupts the innate mechanism by which NCC2705 regulates IL-8. Interestingly, although BopA down-regulates KC at the mRNA level, no KC protein expression was detected in both the BopA+ and BopA- groups, which may allude to the absence of a real physiological effect.

In this study, BopA decreased the expression of MUC4 in the proximal colon at the mRNA level. This may be a result of KC suppression, since it has been shown that mucin secretion can occur via an IL-8 dependent pathway [336], although this has been shown only for MUC5AC and not MUC4, it may be an interesting pathway to investigate for future studies. In addition, B. longum
NCC2705 has been shown to significantly increase the expression of MUC4 [264], therefore, it may not be that BopA down-regulates MUC4, rather the presence of BopA may be inhibiting this function of \textit{B. longum}. To determine whether this is in fact true future studies should advocate the use of a wild-type \textit{B. longum} NCC2705 and negative control group (i.e. PBS). It may not be surprising to find mucin genes regulated by \textit{B. longum} given that it has the capacity to release galactosyl \( \beta \)-1-3 \( N \)-acetylglactosamine extracellularly from mucin glycoconjugates by EngBF, which could then be transported into the cell by a putative ABC transporter [331]. As a result, the substrate could then potentially enter the galacto-\( N \)-biose metabolic pathway described in \textit{B. longum} [337, 338], where it is metabolized through the glycolytic pathway or by amino sugar metabolism, thus serving as a carbon energy source for this species. Mucin degradation has been considered an undesirable characteristic of probiotics [339], since it is believed that it could favour alteration of the intestinal mucosal barrier. This may provide evidence for a putative receptor by which BopA uses to attach thereby initiating host down-regulation of mucin genes, specifically mucin 4 (a possible receptor), to prevent it from residing in the gut and ensuring it remains only as a transient member.

In conclusion, the expression of BopA increases \textit{C. coccoides} and \textit{C. leptum} cell counts solely in the caecal region of the intestinal tract, which may have potential beneficial implications linked to butyrate production. Furthermore, augmentation of IL-8 and MUC4 expression have been implicated in diseased states such as IBD [340, 341]. Therefore, assuming BopA is responsible for the decrease in expression of IL-8 and MUC4, these data may provide evidence for a BopA anti-inflammatory, preventative mechanism through which \textit{B. bifidum} species maintain intestinal homeostasis.
Chapter 6
Concluding Remarks

6 Concluding Remarks

6.1 Major Overall Findings from the *B. bifidum* and BopA Studies

The first study assessed the probiotic characteristics associated with *B. bifidum* MIMBb75 by investigating its ability to survive passage through and persist within the GIT, and the impact it had on the dominant enteric microbial community and on cytokine expression. In this study, MIMBb75 did survive passage through the GIT of mice, and persisted, within all regions of the distal portion of the alimentary canal and in the feces for at least 24 hours post-gavage, after 14 days of treatment. MIMBb75 displayed no ecological niche colonization preference comparatively between the caecum, proximal, or distal colon. However, it was also determined that this strain is a transient member, demonstrated by wash-out after one week of treatment cessation. This study also discovered the potential of this strain to shift the endogenous microbial community toward a healthy phenotype, by increasing *Bifidobacteria* cell counts, albeit solely in the proximal colon. In addition, *Clostridia* were identified as the dominant bacterial genus susceptible to modulation by MIMBb75, although this change did depend on species (i.e. *C. leptum* versus *C. coccoides*) and region of the intestine considered. This study also found that ingestion of MIMBb75 at the levels and length of time tested did not impact cytokine gene expression, at least at the mRNA level.

Next, the second study demonstrated that colonization of transfected *B. longum* NCC2705 is not dependent on the *B. bifidum* surface protein. However, BopA does increase *Clostridium* clusters including both *C. coccoides* and *C. leptum*, although only observed in the caecum. Moreover, BopA had the propensity to modulate intestinal barrier genes via down-regulation of both IL-8 and MUC4 mRNA expression in the proximal colon. Taken together, MIMBb75 demonstrated several probiotic characteristics, mainly its ability to transiently colonize the intestinal tract of mice. The molecular determinant responsible for its colonization cannot be univocally ascribed to BopA.
6.2 General Discussion

This dissertation reports on two studies: the first study was conducted to investigate the potential probiotic nature of the novel B. bifidum strain MIMBb75 in a healthy in vivo model. The second study focused primarily on the role of BopA, identified as the molecular determinant of B. bifidum species, responsible for its adhesive capacity in vitro [13].

Several strains of B. bifidum have been shown previously to adhere tightly to the human intestinal cell lines [271, 273, 274], but only one has shown that BopA may be the putative surface protein enabling the strong adhesive attribute of B. bifidum species. The first study revealed the capacity of B. bifidum MIMBb75 to remain a transient colonizer within the distal regions of the GIT. Although the second study also revealed the persistent ability of transformed bacteria after 24 hours post-gavage, after 14 days of treatment in the caecum and feces, it did not depend on the presence of the BopA protein. The presence of BopA probably did not confer an ecological advantage to this strain given that the adhesive capacity of B. longum was already maximized by its endogenous mechanisms such as the presence of exopolysaccharides [334] and fimbriae-like structures [331], which have been shown to mediate microbial adhesion of epithelial, mucosal, or other host cell surfaces. However, the presence of both MIMBb75 and BopA stimulated the growth of C. leptum and C. coccoides, both of which are the most prominent butyrate producers [315], which in itself may have therapeutic benefits such as preventing colon cancer [321] or ulcerative colitis [322].

Furthermore, MIMBb75 and BopA have immunomodulatory potential as a result of colonization previously demonstrated in vitro [13]. Although MIMBb75 and BopA have been shown to selectively increase IL-8 protein expression in vitro, others have shown that stimulation of cytokines by B. bifidum is highly dependent on the environmental surrounding, which has been discussed in detail in chapters 4 and 5. Therefore, the findings of the in vitro study may not account for interactive effects commonly found in an in vivo setting. Interactions with other endogenous bacteria may help to explain the inhibitory effect on IL-8 expression in the MIMBb75 study, and the down-regulation in the presence of BopA. For instance, B. bifidum Z9 actively changes the gene expression profile induced by Lactobacillus acidophilus in dendritic cells ex vivo [265], or the potential interaction with B. thetaiotaomicro, which has the propensity to attenuate inflammation by promoting PPARγ-dependent export of the NF-κB.
subunit RelA [342]. In addition, the C. leptum cluster was demonstrated to significantly increase as a consequence of both MIMBb75 and BopA administration therefore, the increased cell counts of C. leptum, including the identified anti-inflammatory F. prausnitzii commensal [314], could explain the maintenance of an anti-inflammatory milieu by ensuring levels of IL-8 remain static (MIMBb75) or reducing its expression (BopA).

Furthermore, the absence of immune cells in vitro does not take into consideration the homeostatic mechanisms involved to maintain a balance between Th-1 and Th-2 responses in the healthy situation. It has been shown that in the presence of lymphocytes, the IkBa-induced degradation and NF-kB transcriptional activity by commensal B. vulgatus was inhibited [324]. In chapter 4 of this dissertation, B. bifidum MIMBb75 demonstrated no effect on the expression of IL-8 in the proximal colon in vivo; therefore, the reduction in IL-8 expression shown in the BopA study may be a communal effect by both the presence of BopA and B. longum NCC2705. In addition, there are several other B. bifidum species that also encompass the BopA surface protein, which have also shown inhibitory effects on IL-8 expression [277].

The observations of the in vitro study conducted in 2008 [13], the MIMBb75 study (chapter 4), and BopA study (chapter 5), present some inconsistencies. The limitations of the in vitro study have been briefly discussed in this section however, to further reconcile inconsistencies an in depth analysis of potential limitations of the two studies presented in this dissertation is essential.

6.3 Limitations and Future Directions

One of the limitations of this study is that the mice employed had no detectable Bacteroidetes, one of the two main phyla within the gut microbiota. While the primers that we employed span both the Bacteroides and Prevotella genera which together represent most of the Bacteroidetes, a future study could also employ primers specific for additional murine Bacteroides species. In addition, a subset of mice should be sacrificed prior to beginning the treatment period, with intestinal contents collected and analyzed for their microbial composition. This would provide a direct, region-specific, comparison of microbial shifts due to treatment after a given period to those observed at day 0.
Cytokines gene and protein expression were measured 24 hours after the last gavage on day 14, which may have been a too delayed time point to assess their expression. In a healthy model provided with a non-pathogenic type bacteria, it is likely that shifts in cytokine gene expression may only be detectable immediately after ingestion, but then return to basal levels once homeostatic mechanisms of the host have taken effect. Future experiments should consider measuring cytokine expression immediately after exposure to the probiotic and address kinetics of cytokine expression in the intestinal region of interest.

Another limitation of both studies is that the primers used target large groups of bacteria; albeit species or strain shifts may have occurred as a result of treatment, these were not detected overall at the genus level. Future studies could consider designing primers targeting specific species, for example within the \textit{C. coccoides} and \textit{C. leptum} groups which were found to be affected by treatment.

Furthermore, \textit{B. bifidum} MIMBb75 passage and survival in the intestinal tract of the mouse does not necessarily suggest survival through the upper portion of the alimentary canal in humans as a result in natural differences in physiology of the GIT (i.e. stomach pH < 3 in humans versus stomach pH 4 in mice). However, this has been addressed in a recent study conducted by Guglielmetti and colleagues this year, which provided MIMBb75 capsules to patients suffering from symptoms associated with IBS, and found improvement in global IBS and symptom scores, indicative of its survival [282].

Classical cultivation techniques were used to determine the recovery of transformed bacteria in the feces. However, the results may not be justified due to a reduced sample size corresponding to a reduced power of analysis to less than the desired 80%. A future study could utilize specific primers targeting the engineered strain to assess their colonization via molecular techniques.

This study employed the probiotic \textit{B. longum} NCC2705 as a recipient strain for expressing BopA. However, it is likely that the adhesion capacity of \textit{B. longum} was already maximized by its own endogenous mechanisms therefore, the presence of BopA did not provide an ecological advantage to this strain, demonstrated by the absence of a measurable increase of bifidobacteria cell counts. As a result, we were ultimately unable to ascribe BopA as the major adhesive protein responsible for the colonization capacity of the transformed \textit{B. longum} strain \textit{in vivo}. Future
studies could consider transforming a strain with less adhesive capabilities such as *B. animalis* subsp. *lactis* Bb-12, which has been shown to have poor adherent capacity *in vitro* [13], and has its entire genome sequenced [343]. In addition, the appreciation of shortcomings identified in the BopA study provides future studies with the advantage of setting up a more comprehensive study design, where additional positive control (i.e. wild-type *B. longum* NCC2705 and wild-type MIMBB75) and negative control (PBS) groups are included.

6.4 Implications

The aptitude of a probiotic bacterium to confer health benefits to the host relies heavily on their ability to colonize within the gut. Bacteria that are simply washed out quickly, typically cannot be considered ‘probiotics’ since they do not maintain dialogue with the host long enough to provide a health benefit. Moreover, it has become evident that not all bacteria may operate in the same manner even if the bacteria are closely related at the genus (95% 16S rRNA sequence identity) or even at the species level (98% 16S rRNA sequence identity) [104]. In other words, a given bacterial strain that may be potentially beneficial due to a high degree of similarity to bacteria of a certain genus/species, previously shown to be beneficial to the host, does not endow that specific strain with ‘probiotic’ status until it has been shown to confer health benefits of its own in a clinical setting. This notion necessitated experimental validation of the probiotic effect of *B. bifidum* MIMBB75, which had previously only been tested *in vitro*.

The results of our study reveal the aptitude of MIMBB75 to survive passage through GIT of the mouse, persist within the colon after 14 days of treatment, wash-out after one week of treatment cessation, and increase proportions of butyrate producing bacteria, without resulting in any detectable detrimental side effects. This provides substantial evidence for the probiotic nature of this strain, which can now be provided as a potential probiotic in a clinical setting. In fact, a clinical trial providing MIMBB75 to IBS patients has already been completed by Dr. Guglielmetti and colleagues this year [282]. The results demonstrated the probiotic nature of MIMBB75 by alleviating symptoms associated with IBS. Together with our findings, MIMBB75 has the potential to be an effective alternative to drug therapy for IBS. Future studies may favour the use of a healthy subject clinical trials, where clinical endpoints of interest may be the colonization capacity and persistence of MIMBB75 and the impact on the endogenous microbial
composition. Comparing these results with those observed in the C57Bl/6J mice would be of interest.

Recent reports suggest that an imbalance of intestinal microbiota with significant reduction of bifidobacteria may contribute to the development of IBS [344, 345]. Dysbiosis of endogenous microbiota composition has also been implicated in IBD [314, 346]. Therefore, the findings from our study are particularly important for Canadians given that Canada has the highest incidence of IBD in the world, with nearly 30,000 Canadians dying of diseases associated with the digestive system annually [347]. In addition, health care costs and lost productivity as a consequence of digestive disorders accounts for approximately $18 billion annually (reported in 2000), with $71 million in hospitalization costs for treating severe diarrhea alone (reported in 2005) [347].

Moreover, BopA is the first bifidobacterial adhesion protein discovered to date. The BopA study is the first to investigate the molecular determinant by which B. bifidum is able to colonize the GIT and consequentially shift the endogenous microbial community and impact the intestinal barrier. Although, the role of BopA in assisting the colonization of B. bifidum was not completely elucidated, the effects of BopA on down-regulating IL-8 and MUC4 gene expression may have beneficial therapeutic implications for diseased states such as IBD where these genes have been shown to be up-regulated [340, 341]. Furthermore, the results of the BopA study may be relevant for future studies that hope to investigate pathologies related to dysfunction of the intestinal epithelium, such as IBD. Further investigation into the role of BopA may provide direct evidence for the efficacy of B. bifidum species and therefore, provide support for the formulation of strain-specific probiotic-related health claims. Furthermore, future studies may help contribute to the development of dietary guidelines with respect to the consumption of probiotic-containing dairy products in order to maintain a healthy gut environment.
Chapter 7
Conclusions

7 Conclusions

The major conclusions of this research are:

1. *B. bifidum* MIMBb75 colonizes the mouse large intestine resulting in augmented bifidobacteria cell counts in the caecum and proximal colon but does not affect cytokine gene expression.

2. *B. longum* NCC2705 BopA+ administration did not result in augmented bifidobacteria cell counts in the caecum and proximal colon compared to *B. longum* NCC2705 BopA-, but resulted in the down-regulation of interleukin 8 and mucin 4 gene expression in the proximal colon.

3. A secondary outcome of this study was that the administration of both *B. bifidum* MIMBb75 and *B. longum* NCC2705 BopA+ resulted in augmentation of *C. coccoides* and *C. leptum* proportions in the gut.
References


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