Isomaltodextrin dose-dependently reduces colitis development in HLA-B27 rat colitis model with associated changes to gut microbiota composition and short chain fatty acid production

by

Nilanjani Premraj

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Department of Medicine
University of Alberta

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Abstract

Prevalence rates for inflammatory bowel diseases (IBD) worldwide have been drastically increasing over recent decades. IBD is characterized by altered intestinal microbiome (i.e. dysbiosis), described by reduced strict butyrate-producing anaerobes versus increased facultative anaerobes in comparison to healthy individuals. Data from preclinical studies and clinical trials show prebiotic inulin-type fibers can prevent/reduce colitis; however, other dietary fibers (e.g. alpha-glucan, α-glucan) are understudied and it is unknown if fiber-induced microbial shifts are protective in experimental colitis. The purpose of this thesis was to assess the efficacy of isomaltodextrin (IMD), a novel highly branched α-glucan, in reducing intestinal inflammation in HLA-B27 transgenic (TG) rat colitis model and to identify the protective mechanisms associated with gut microbial composition and function. HLA-B27 TG rats, 4 weeks of age, were fed standard chow diet supplemented with 7.5% IMD, 15% IMD, 15% cellulose (negative control chow), or 15% fructooligosaccharides (FOS, positive control) for 12 weeks. Body weight and food intake were measured. Cecal and colonic inflammation was assessed by macroscopic scoring and mucosal cytokine (IL-1β and IFNγ) concentrations. Changes in microbial metabolism was evaluated by measuring short chain fatty acid (SCFA) proportions in stool and cecal contents. Endpoint fecal and cecal microbiota composition differences were assessed by 16S rRNA gene sequencing (Illumina MiSeq platform). IMD showed a dose-dependent effect on cecal inflammation. The inflammation reducing effects by IMD was more commonly seen in males but were not universal for either sex, showing individual-specific benefits for the fiber. IMD demonstrated a dose dependent reduction in cecal inflammation, where IMD 15% was more effective than IMD 7.5%, which was confirmed by both gross gut score (GGS) and mucosal IL-1β concentration analyses. Both IMD groups were associated with reduced cecal

concentrations of isobutyrate, valerate and isovalerate, with IMD 15% being more effective. Only IMD 15% reduced cecal propionic acid proportions, similar to that of FOS. These specific SCFA also showed a significant positive correlation with cecal IL-1β concentrations, suggesting their use as chronic inflammation markers in HLA-B27 TG rat model. Significant shifts in gut microbial composition were seen, including a decrease in alpha diversity in IMD 15% and FOS groups. Key changes in IMD groups included increased *Bifidobacterium*, which has previously been shown to have colitis reducing effects, as well as decreased *Alistipes*, which has previously been associated with colitis. *Bifidobacterium* and *Alistipes* were associated negatively and positively with cecal IL-1β concentrations, respectively, suggesting their use as microbial indicators for inflammation in HLA-B27 TG rat model. In conclusion, IMD was dose-dependently effective in reducing chronic cecal inflammation in experimental colitis. Benefits were associated with specific shifts in gut microbiota composition and SCFA production. Results from this preclinical study warrant future microbiota-altering intervention trials using IMD in clinical IBD.

Preface

This thesis is an original work by Nilanjani Premraj. The research project, of which this thesis is a part, received ethics approval from the University of Alberta Research Ethics Board, under the project name " Effect of two different doses of isomaltodextrin (IMD) on colitis development and gut microbiome in HLA-B27 transgenic rats" AUP00002722, date 09/18/2018

Acknowledgements

Pursuing a Master's degree was possible for me due to the encouragement, guidance and support from my supervisor, mentors, colleagues, family and friends. I would like to express my gratitude to my supervisor, Dr. Levinus A. Dieleman, for accepting me as a Master's student in his lab. I am thankful for all the support he has provided throughout my program, and for his guidance in writing this thesis.

I would also like to express my deepest thanks to Dr. Rosica Valcheva for her encouragement of my research goals and for all the personal hours she has sacrificed to mentor me through the pursuit of these goals. I am thankful for the current and former members of our research group for their assistance and friendship throughout my program: Ognjen Kovic, Simona Veniamin and Ammar Hassanzadeh Keshteli.

I wish to extend my thanks to my supervisory committee member, Dr. Karen Madsen, and the arm's length examiner, Dr. Leluo Guan, for their advice and support. A special thanks to Dr. Maria Elisa Perez-Munoz for her assistance with bioinformatics. I am also grateful for my graduate coordinator, Barb Thomson, for her guidance throughout the program.

Importantly, I would like to thank my parents, brother and friends for their love, patience, encouragement, and endless support.

I would like to acknowledge the financial support provided by Hayashibara Co, Ltd. (Okayama, Japan), The Centre of Excellence for Gastrointestinal Inflammation and Immunity Research (CEGIIR), The University of Alberta and Faculty of Medicine and Dentistry.

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Abbreviations:

AMP – anti-microbial peptide

ANOVA – one-way analysis of variance

CD – Crohn's disease

DP – degrees of polymerization

DSS – dextran sodium sulfate

FMT – fecal microbial transplantation

FOS – fructo-oligosaccharides

GGS – gross gut score

GOS – galacto-oligosaccharides

IBD – inflammatory Bowel Disease

IEC – intestinal epithelial cell

IFNγ – interferon gamma

Ig – immunoglobulin

IL – interleukin

IMD – isomaltodextrin

IMO – isomalto-oligosaccharides

LAB – lactic acid bacteria

LPS – lipopolysaccharide

MD – Mediterranean diet

NSAID – non-steroidal anti-inflammatory drug

OTU – operational taxonomic unit

PBS - phosphate-buffered saline

PCR – polymerase chain reaction

PLS-DA – partial least square discriminant analysis

PRR – pattern recognition receptor

RIPA - radioimmunoprecipitation assay

SCD – specific carbohydrate diet

SCFA – short chain fatty acids

SD – standard deviation

SPF – specific pathogen free

TLR – toll-like receptor

TNBS – trinitrobenzenesulphonic acid

TNF – tumor necrosis factor

UC – ulcerative colitis

VIP – variable importance in projection

Chapter 1: Introduction

1.1 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD), consisting of Crohn's disease (CD) and ulcerative colitis (UC), are characterized by chronic relapsing inflammation of the gastrointestinal (GI) tract. In UC, inflammation is continuous and limited to the mucosa of the colon, while CD is characterized by discontinuous, transmural inflammation anywhere in the GI tract. Symptoms for these incurable diseases may include fatigue, weight loss, abdominal pain, diarrhea, and rectal bleeding. Extra-intestinal manifestations of disease are commonly experienced in the joints, skin, eyes, and bile ducts. As a result, these symptoms can impact quality of life such as short- or long-term work losses, social stigma, and diminished employment prospects.

IBD prevalence is highest in developed nations, especially Europe and North America, although incidence rates have been on the rise in newly industrialized countries in Africa, Asia and South America [1]. Diagnosis of IBD generally requires endoscopic procedures and imaging to reveal intestinal damage, such as colonoscopy, flexible sigmoidoscopy and upper endoscopy and lately small bowel enteroscopy. Disease onset occurs most commonly during teens and twenties and is lifelong [2,3]; IBD affects individuals throughout entire period of study and employment. IBD also significantly elevates the risk of colorectal cancer development when it involves the colon [3-5]. This immune-mediated inflammation is driven by genetic predisposition, an abnormal immune response, and exposure to environmental triggers that will be discussed in further detail below.

1.2 IBD Significance in Canada

A systematic literature review of 262 worldwide articles spanning from 1950-2010 revealed Canada as the nation with the highest reported IBD incidence rates (19.2 per 100,000 for UC and 20.2 per 100,000 for CD) and second highest IBD prevalence rates (248 per 100,000 for UC and 319 per 100,000 for CD), in the world [2].

According to a 2012 report commissioned by the Crohn's and Colitis Foundation of Canada, at that time, there was approximately 233,000 Canadians living with IBD, and over 10,200 new cases being diagnosed every year [3]. More recent numbers show that, as of 2018, this number has increased to approximately 270,000 Canadians that suffer from IBD [6]. Due to the incidence of IBD being much higher than the risk of death from IBD, the prevalence of IBD is estimated to continue to increase by 2.9% per year to a forecasted estimate of 403,000 cases by 2030 [6]. Economic impacts of IBD to Canadians is estimated at a cost of over $2.7 billion annually - over $11,900 per person with IBD every year [3,6]. Direct medical costs (~$1.2 billion) were dominated by medications (~$521 million), while indirect costs (~$1.6 billion) were even more costly and were dominated by long-term work losses ($979 million) [3]. Targeting the two dominant factors of economic stress, a potential strategy in reducing costs may be to find a more cost-effective alternative therapy to reduce symptoms and, in turn, reduce long-term work losses. Alarmingly, the trend shows that incidence rates of IBD has been on the rise, particularly in children under the age of 10 years [3]. With these rising incidence rates, the costs to Canadians will only continue to rise.

1.3 Etiology of IBD

The Etio-pathogenesis of IBD is very complex and remains unknown. Currently, the most widely accepted hypothesis is that pathogenesis is caused by a combination of factors; an abnormal immunological response mediated by commensal gut microbes and environmental factors in a genetically susceptible host [7-10]. However, the exact nature of these complex interactions remains limitedly understood.

The genetic contribution to IBD etiopathogenesis is apparent, as the greatest risk for IBD development is having a relative with the disease, and this risk increases drastically if both parents suffer from IBD [11]. However, studies in monozygotic twins show that concordance rates for CD is only 30-35% and only 10-15% for UC, suggesting additional complexity than genetics to the disease mechanisms [12]; individuals presenting the susceptibility genes require other non-genetic influences to manifest disease. This suggests that IBD is not only a disorder affected by genetics and immune dysfunction, but also by the web of complex interactions with environmental factors.

There are multiple known environmental risk factors for IBD such as: early life exposures such as mode of birth, breastfeeding and antibiotic exposure in infancy; later life exposures such as, smoking, stress, infections, diet, oral contraceptives, and the use of non-steroidal anti-inflammatory drugs (NSAIDs) [13,14,16]. Western nations have a higher incidence and prevalence rate of IBD than developing nations; however, an apparent pattern can be seen in that, as industrialization increases in these areas, so does the incidence rate of IBD [3]. This increase in disease rates may be explained by the hygiene hypothesis, stating overprotection from exposure to microbials in early childhood could limit immunological development so that

exposure later in life could trigger an inappropriate immunologic response [9,15,16]. Another potential explanation for the increase in disease rates may be industrialized food processing and the introduction of fast foods or convenient processed foods, making their diets resemble the more inflammatory "Western diet", that is higher in processed carbohydrates, saturated fats, and refined sugars, while being lower in fibers. Diet and IBD has been extensively studied, and the "Western diet" has been shown to increase gut inflammation, which could be associated with its effect on compositional changes of the gut microbiota [9,16,17].

1.4 IBD Treatment: Medical Therapies

IBD is a lifelong disease with no known cure. Both CD and UC involve an overly aggressive adaptive immune response from the host. As such, current therapies aim to improve quality of life by symptom reduction through the use of pharmacologic treatments that modulate the immune system; common medications include anti-inflammatory drugs (commonly 5-aminosalicylic acid compounds), corticosteroids, immune suppressors and biologics. Majority of patients require ongoing treatment, and, in severe cases, surgery may be required. Surgeries can include removing diseased portions of the intestines or even colectomy. Although patients respond well to these lifelong standard therapies, there is considerable costs to patients and some patients may find them inadequate and/or inducing of adverse effects. Serious side effects of these treatments can include increased rates of infections and malignancies [18,19].

1.5.1 The Gut Microbiota: Introduction

The human body maintains a mutually beneficial symbiotic relationship with the abundant and diverse microbiota occupying its gastrointestinal tract. Approximately 100-trillion microbes occupy the human gastrointestinal tract and produce thousands of metabolites that support functions of the host [20]. Their combined genome, also referred to as the microbiome, contains ~150-fold greater number of genes than the host's human genome [21]. Their density gradient increases from the acidic environment of the stomach to the colon. Resident microorganisms consist primarily of bacteria and the ubiquitous, but significantly less abundant fungi. Due to the high inter-individual variation in the gut microbiota based on factors such as geographic origin, antibiotic uptake, and genetics, the core gut microbiota composition currently remains undefined [22,23]. Twin studies show heritability of the gut microbiota; however, environmental factors appear to be the larger determinants of the microbiota composition. Extensive analyses have produced the current broad consensus on microbial diversity; the dominant bacterial phyla in the human gut microbiota during a healthy state are majority Firmicutes, Actinobacteria and Bacteroidetes, which make up approximately 90% of the fecal microbiota, with the less abundant Proteobacteria, Fusobacteria and Verrucomicrobia [22,24,25]. In 2011, Peer Bork and associates suggested classifying individuals based on variations in the gut bacterial composition using three human enterotypes, specifically identifiable by the changes in the level of one of the three robust genera – *Bacteroides* (enterotype 1)*, Prevotella* (enterotype 2)*,* and *Ruminococcus* (enterotype 3) – that are not nation or continent-specific [26]. Enterotypes are stable, and properties such as body mass index, sex and age cannot explain their observation [26]. Enterotypes have however been strongly associated with long-term diets; enterotype 1, the *Bacteroides*-dominated enterotype, has been associated with a protein and animal fat-rich diet,

while enterotype 2, the *Prevotella*-dominated enterotype, has been linked to a carbohydrate-rich diet [26]. Majority of commensal microbes are obligate anaerobes that confer benefits to the host through a range of functions including supporting gut barrier protection, fermentation of macronutrients, protection from pathogens and stimulation of local and systemic immunity [27-30]. Encoding over 3-million genes, compared to the 23 000 genes of the human genome, and with its own set of functions for the host, the microbiome is thought of as a virtual organ of the body [31].

Throughout an average life span, approximately 60 tonnes of food and copious microorganisms from the environment pass through the digestive tract, imposing an enormous threat to gut integrity [32]. Alterations to the microbial composition, termed "dysbiosis", can occur due to the gain or loss of commensal members or changes to relative abundances, and has been associated with negative effects on host health. Dysbiosis will be discussed in further details below.

1.5.2 The Gut Microbiota: Functions

The gut microbial composition undergoes dramatic changes during early life, but from approximately 2.5 years of age, the composition, diversity and functional capabilities resemble that of an adult microbiota [32-34]. This process of maturation occurs from ecological succession in which the community undergoes continuous compositional and functional alterations until a climax community is established. The majority of these microbes are either harmless or beneficial to the host; they extract nutrients from diet, contribute to normal immune functions, and protect against pathogens [31,33].

Various colonic bacteria express the enzymes required to ferment complex carbohydrates that are indigestible by humans, to generate metabolites such as short chain fatty acids (SCFA) that are absorbed by the epithelial cells of the GI tract [32-34]. The major SCFAs (>95%) produced are acetate, propionate, and butyrate. In the human gut, Bacteroidetes are the main acetate and propionate producers, while Firmicutes (e.g., *Faecalibacterium prausnitzii* and *Eubacterium rectale*) are the main butyrate producers [35,36]. The main butyrate producers are anaerobes, which thrive in an environment with low oxygen concentrations like that of a healthy colon. SCFAs have been shown to provide numerous benefits to the host including: Butyrate serves as a fuel source for colonocytes, reduction of inflammation, activation of intestinal gluconeogenesis, generation of a hypoxic state to maintain gut oxygen balance and prevent dysbiosis, and enhancement of gut barrier function through regulating assembly of tight junction which provides increased protection against pathogens; Propionate's stimulation of gluconeogenesis in the liver; Acetate's role as an essential metabolite for growth of other intestinal bacteria, and use for lipogenesis in peripheral tissues [31-33,37-42]. Other minor SCFAs (<5%) produced by the colonic microbiota are branched SCFAs such as isovalerate, valerate, isobutyrate - by-products of protein fermentation by mainly the *Bacteroides* genera in the human microbiota [22,23,43]. Fermentation of amino acids as an energy source occurs mostly in the distal colon, where carbohydrate sources are already depleted, and is seen more frequently in individuals with a high protein/low fiber diet [23,44]. Although research into the direct effects of branched SCFAs is limited, there are potentially toxic or carcinogenic products in protein fermentation (such as ammonia and hydrogen sulphide), and branched SCFAs are often used as fecal markers for protein fermentation [22,23].

Gut microbial research has been nearly exclusive to bacterial species and contributions of other commensal microbes remains poorly defined. In comparison to bacteria, the mycobiota, primarily yeasts, retain far less diversity in the gut (<1% of the microbiota), and is therefore less commonly studied [45]. However, yeasts are >100-times larger than bacteria and, though a defined group of conserved phyla remains undetermined, the kingdom Fungi is ubiquitous to the mammalian microbiota [45,46]. Two commonly commensal yeast genera, *Saccharomyces* and *Candida*, have been shown in broad-spectrum antibiotic-treated mice with mono-colonization to: a. overturn mortality due to DSS-induced colitis; b. protect against colonic shortening, intestinal inflammation and permeability resulting from DSS-induced colitis; c. play an immunomodulatory role in extra-intestinal tissues, specifically the respiratory tract, in response to influenza [45,46]. The hypothesized mechanisms behind these effects are persistent, immune stimulation by a cell wall component mannans, similar to stimulation by bacterial lipopolysaccharides [45,46].

Through its collective metabolic activities and host interactions, the gut microbiota may be influential to both physiology and disease susceptibility of the host. This brief overview presented focused mostly on the benefits of a microbiota in homeostasis, however the microbiota can also induce negative repercussions to the host when in dysbiosis [32,39,47]

1.5.3 The Gut Microbiota: Immune Interactions

IBD pathogenesis has been strongly associated with immune response dysregulation. A major characteristic of IBD is chronic inflammation of the gut with repeated cycles of relapse and remission. In a healthy gut, homeostasis is maintained by suppressing excessive immune

response to foreign antigens, thus preventing inflammation. The gastrointestinal tract is constantly exposed to microbes; however, the immune system maintains minimal responses to these commensal microbes. Inflammation can occur when a link in the immune system is abnormal. Associations have been found between IBD and mutations causing dysregulation of the immune system.

An enormous portion of the immune system's function is aimed at controlling its relationship with the microbiota, as seen by the highest number of immune cells in the body residing at sites colonized by commensals, including locations such as the skin and GI tract [48]. The primary strategy utilized by the host is to minimize contact between microorganisms and the host, thereby limiting tissue inflammation and microbial translocation [48]. This segregation is accomplished by the combined action of mucous production, intestinal epithelial cells (IECs), antimicrobial peptides (AMPs), immunoglobulin A (IgA) and immune cells [48].

In the gut, the mucus produced by goblet cells is the first shield limiting contact between the microbiota and the intestinal epithelial cells (IECs). The constant production and movement of the mucus layer, as well as the accumulation of AMPs and immunoglobulins in the inner layers of the mucus, contribute to the physical separation from IECs [48]. Healthy individuals have a nearly impenetrable inner mucous layer in the colon, while IBD patients have been shown to have bacteria present [49].

In IBD, when pattern recognition receptors (PRRs) on IECs recognize bacterial antigens, inflammatory factors such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFNy) are released, which induce expression of epithelial cell apoptosis related proteins, such as Caspase-1, while inhibiting the expression of anti-apoptotic proteins [50]. This results in induced

IEC apoptosis, weakened function of IEC's resistance to pathogens and increased permeability of intestinal mucosa [50]. Further affecting intestinal permeability, the tight junction proteins that bind adjacent IECs and regulate intestinal permeability in a healthy gut are significantly decreased in active IBD [51]. In clinical and animal models of IBD, levels of the pro-inflammatory cytokine, IL-1β, are significantly higher and an association has been seen with increased disease severity as well as increased intestinal epithelial tight junction permeability [52-55]. A hypothesized mechanism is that during active stages of IBD, bacteria shift from within the enteric cavity to the mucosa lamina propria, activate immune cells to produce inflammatory cytokines, which then regulate expression of tight junction proteins, impairing intestinal mucosal barrier function and further increasing epithelial permeability [51]. Another possible mechanism is via interactions with the gut microbiota; IBD has been associated with a dysbiosis of the gut microbiota, such as decreased number of SCFA producing bacteria (Bacteroidetes and Firmicutes), as well as decreased absorption of SCFAs by IECs, denying the host of the numerous anti-inflammatory benefits associated with SCFAs described above [56]. SCFAs, in particular, butyrate, provides an energy substrate for IECs and regulates the assembly of tight junction proteins, limiting bacterial translocation [37,38,40,57,58].

1.5.4 The Gut Microbiota: Dysbiosis in IBD

Despite the normal symbiotic relationship between the host and gut microbiota being mutualistic, imbalances due to compositional and metabolic changes, or dysbiosis, may be associated with prolonged chronic inflammation such as in IBD. Disruption of the normal balance of the microbiota, termed dysbiosis, has also been associated with obesity, colorectal cancer, and infections [20,32,33,47]. The immense degree of inter-individual variation in gut

environment, microbe-microbe interactions, as well as microbiota composition and function, has made the state of homeostasis and dysbiosis nearly impossible to define universally. The alterations to the gut microbial profile have been associated with the proliferation of pro-inflammatory microbes and/or the diminishment of anti-inflammatory microbes. Lower bacterial diversity has also been observed in patients with IBD [60]. The role of the microbiota in IBD is further complicated by the lack of definitive conclusion whether dysbiosis is truly causative, or merely a consequence, of inflammation.

Multiple studies have analyzed differences in the gut microbial composition between patients with IBD and healthy individuals, particularly with respect to microbial diversity and the relative abundance of specific bacterial taxa. In a healthy intestine, there are normally low oxygen levels and thus a relatively large obligate anaerobe population. Chronic intestinal inflammation increases blood flow to the intestines resulting in increased oxygen levels that severely drop species richness and alters abundance of several taxa, including selection for facultative anaerobes, such as many Proteobacteria, and against obligate anaerobes such as many Firmicutes [40]. The phylum Firmicutes – especially *F. prausnitzii* – was often reduced in the stool of patients with CD, while members of *Enterobacteriaceae* were increased in comparison to healthy individuals [60,61]. Abnormalities in the gut microbial composition have also been detected in UC, although to a lesser degree than CD, such as a decrease in butyrate-producing bacteria such as *F. prausnitzii* and *Roseburia hominis* (82-84). Many members of the Firmicutes play an important role in butyrate production, which serves as an energy source for colonic epithelial cells, which oxidize butyrate to CO_2 [37,38,40]. This decreased abundance of colonic butyrate producers in IBD results in an oxygenated epithelium which, combined with the impaired colonization resistance from depleted microbial diversity, creates an environment that

can allow expansion of pathobionts [40]. Pathobionts are symbionts in the gut microbial community that expands as a result of dysbiosis and exert pathogenic effects on the host. The enriched pathobionts are better equipped for competition in an inflamed gut than healthy and may instigate chronic inflammation in susceptible hosts by shedding pro-inflammatory effectors and activating innate immunity, which worsens inflammation and interferes with its resolution [40]. For example, Proteobacteria are found in increased amounts in patients with CD and/or UC: Adherent-invasive *Escherichia coli* strains are able to adhere to cultured intestinal epithelial cells, a property that could allow for colonization of the mucosa, and were specifically associated with ileal mucosa in CD patients [63]; *Desulfovibrio* spp. are sulphate-reducing bacterial species that have evolved the ability to persist in oxygen-rich environments and were shown to be specifically increased in the colons of acute and chronic UC patients [64]; *Campylobacter concisus* are significantly more prevalent in patients with CD and have been observed to have flagellum with the ability to attach to tips of microvilli, a potential process required for colonization and penetration into host intestinal epithelial cells, and significantly elevated levels of IL-8 in epithelial HT-29 cells as well as IL-8 and TNF-α in monocytic THP-1 cells [65,66].

The significant impact on the gut environment from the inflammation induced by the IBD disease state, as well as the participation of pathobionts in the inflammatory process exacerbates the destructive inflammatory spiral characteristic of IBD. Whether dysbiosis is a result of IBD or vice versa is a question that remains unanswered. This would require a longitudinal study such as following healthy first-degree relatives of IBD patients, who have a genetic predisposition towards developing the disease, to determine if dysbiosis occurs before or after disease development.

1.6 IBD Treatment: Non-Medical Therapies

Conventional therapies for IBD consist of pharmacologic treatments that modulate inflammation through targeting immune dysregulation. As mentioned above, the options given to IBD patients are commonly limited to strong pharmaceuticals and/or surgeries that come with serious adverse effects. As a result, many IBD patients turn to non-allopathic therapies such as dietary changes, spiritual healing, natural products, meditation, homeopathy, acupuncture, Chiropractic therapy, as well as mind-body exercises such as yoga, Tai-Chi and Qi Gong [62]. While some patients use these non-allopathic therapies in conjunction with conventional medicine, termed "complementary therapies", others choose to use these practices in place of conventional medicine, termed "alternative therapies".

Of these non-allopathic therapies, the influence of diet has been rapidly picking up interest. As mentioned previously, the "Western diet" that is known to be high in processed carbohydrates, refined sugars, and saturated fats while being low in fibers has been shown to increase inflammation that could be associated with dysbiosis of the gut microbiota [9,16]. Westernization, an influence that is becoming a global phenomenon, may also offer a possible explanation as to why there are rising incidence rates in countries where IBD was preciously rare [2,67]. Some IBD patients choose dietary changes such as complete alterations to concordance with popular IBD diets: the Specific Carbohydrate Diet (SCD), which limits carbohydrates to predominantly monosaccharides and selective polysaccharides and includes supplementation with homemade fermented yogurt [68]; the Mediterranean Diet (MD), higher intake of fiber-rich plant-based foods, moderate intake of fish/seafoods and low consumption of red meats, with olive oil as the main source of added fats [69].

Dietary changes to macronutrients can induce significant shifts to the gut microbiota. Human intervention studies have shown that these shifts can occur very rapidly [70,71]. Only four days of extreme dietary change to either an entirely animal-based or plant-based diet showed significant changes in both microbial composition as well as microbial metabolism; animal-based diet had the greater impact on microbiota composition, which included increases in bile-tolerant microbes (*Alistipes, Bilophila* and *Bacteroides*) and reduction in Firmicutes that metabolize dietary plant polysaccharides (*Roseburia, Eubacterium rectale* and *Ruminococcus bromii*), and shifted microbial metabolism towards protein fermentation [70]. A less extreme example of a two-week diet swap study, investigating the high rates of colon cancer in African Americans, fed African American's a high-fiber, low-fat African-style diet and gave rural Africans a low-fiber, high-fat western-style diet showed significant reciprocal changes in mucosal biomarkers for colon cancer risk as well as aspects of the gut microbiota and metabolome associated with cancer risk [71]. A study of UC patients comparing a low-fat, high-fiber diet to an improved standard American diet (increased fruits, vegetables and fiber than typical American diet) showed the low-fat, higher-fiber group presented decreased inflammatory markers and showed an anti-inflammatory shift in the microbiota with increased *F. prausnitzii* and *Prevotella* counts in stool [72]. In pediatric Crohn's disease, a comparison between exclusive enteral nutrition, a recommended beneficial diet, and a CD exclusion diet consisting of a whole-food diet with partial enteral nutrition, designed to reduce exposure to dietary components that may have negative effects on the microbiota and gut barrier, showed that the exclusion diet induced sustained remission in more patients and also induced shifts to the fecal microbiome associated with remission, showing the benefits of using diet in conjunction with drugs for optimal treatment of CD [73].

While some patients make the lifestyle choice of altering their diet, others may choose less drastic changes such as supplementing their current diets with probiotics and prebiotics. With evidence for the multitude of roles of the gut microbiota in IBD expanding rapidly, focus has begun shifting towards methods to restore or enhance the beneficial species of the microbiota. Proposed methods to favorably alter the gut microbiota is through the use of probiotics and prebiotics. These food supplements, termed as "functional foods", have shown numerous and complex effects on the gut microbiota as well as the host immune system [74,75].

Probiotics are defined as live microorganisms that, when administered in adequate amounts, exert a beneficial effect to host health [74,75,85]. When these microorganisms are ingested, they must be able to resist the physiochemical conditions of the digestive tract. The most frequently used probiotic strains are of the genera *Lactobacillus* and *Bifidobacterium* [74,75]. Probiotic products may contain either a single strain or a mixture of multiple strains. Probiotic effects are very strain specific and cannot be generalized, such as the immunomodulatory effects of *Lactobacillus spp., Bifidobacterium longum* and *Bifidobacterium bifidum;* inhibitory effects on pathogen colonization by attaching to epithelial cells and physically blocking pathogen adherence as seen in *Lactobacillus acidophilus* LAP5 and *Lactobacillus fermentum* LF3, or by the production of anti-microbials as seen in *Bifidobacterium spp.* and several lactic acid bacteria; barrier function improving effects of *Lactobacillus rhamnosus* GG and *Lactobacillus plantarum* [32,76-84].

Prebiotics are defined as non-digestible dietary compounds that confer physiological benefits to the host by imposing specific changes, both in composition and/or activity, to the gut microbiota [36,74,75,85]. Supplementation with prebiotics may then restore the gut microbial diversity and activity. Prebiotics will be explained in greater depth below. The combination of

the two, termed synbiotics, is a mixture of probiotics and prebiotics that in combination exert a beneficial effect on the host by improving the survival and implantation of the live microbial dietary supplements in the gastrointestinal tract [74,75,85]. Currently, research into synbiotics is preliminary and very limited.

Another method of favorably altering the gut microbiota is through the use of fecal microbial transplantation (FMT). FMT is the transferring of a fecal matter solution from a healthy donor into a recipient to change the gut microbiota of the recipient and confer a health benefit [86-88]. FMT has been most commonly used in treatment of recurrent or refractory *Clostridioides difficile* infection [86]. However, evidence of FMT's beneficial effects on dysbiosis had led to its exploration as a possible therapy in IBD. A systematic review and meta-analysis spanning 18 studies, which included 122 IBD patients treated with FMT, revealed remission rates of 22% and 60.5% for UC and CD patients, respectively [89]. Interestingly, younger UC patients (age 7-20) responded better with 64.1% remission rates [89]. This suggests that FMT is more beneficial in CD as well as in a younger UC population. Reports of FMT treatment in active UC also show mixed results; one study with 75 patients given weekly FMT for 6 weeks found remission rates of 24% compared to 5% in water control, while another study with 50 patients given FMT at baseline and 3 weeks showed no difference in clinical remission compared to negative control [90,91]. In addition, response of UC patients to FMT treatment is donor-dependent; the abovementioned study by Moayyedi *et al.* initially recruited two donors and found that remission was not experienced by any patients receiving FMT from donor A, however, some patients did achieve remission when swapped to FMT with donor B [90]. Further analyses revealed notable differences between the two donors, including enrichment for the genera *Escherichia* and *Streptococcus* in donor A, while donor B displayed significant

enrichment for the family Lachnospiraceae and the genera *Ruminococcus* [90]. Taxonomic profile comparison of responsive vs non-responsive UC patients receiving donor B FMT also revealed that, at end of therapy, responders showed more similarity with donor B than non-responders [90]. Though uncommon, serious adverse events such as death have been recently reported, highlighting the need of standardization in the donor screening process [92,93]. More commonly, patients have reported the development of fevers, bloating, flatulence, diarrhea, and vomiting, as well as a few reports of worsening symptoms following FMT [94-96]. Therefore, more high-quality and well-powered clinical trials with selective donor criteria are required to further assess the efficacy of FMT in IBD treatment.

1.7 Prebiotics

The definition of prebiotics was first introduced in 1995 by Gibson and Roberfroid as "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health" [97]. This definition has been discussed and refined over the years and at the 6th Meeting of the International Scientific Association of Probiotics and Prebiotics (ISAPP) in 2008, the term dietary prebiotic was updated to "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" [98]. Most definitions at this point required prebiotics to be 'specific' or 'selective' towards certain health-promoting taxonomic groups and/or beneficial metabolic activities; however, the introduction of affordable high throughput sequencing in intestinal microbiota research made it evident that no carbohydrate is likely fermented by only one microbial group in the gut and there are none that

are fermentable by all microbial groups [24,99]. In an effort to provide a clearer identification for prebiotic compounds, Bindels et al. proposed a broader revision of the definition to shift the focus towards ecological and functional characteristics of the microbiota that are more likely to be relevant for host physiology [99]. As such, a prebiotic is defined as a non-digestible compound that is metabolized by the intestinal microbiota and, through its modulatory effect on the composition and/or activity of the gut microbiota, confers a beneficial physiological effect on the host.

An ideal prebiotic should meet the following criteria: 1) resistant to digestion by the acids in the stomach, bile salts and other hydrolyzing enzymes of the intestine, 2) should not be absorbed in the upper gastrointestinal tract, 3) be easily fermentable by the intestinal microbiota, 4) exert benefits locally in the gut or elsewhere on the host. The most widely studied, and commercially available, prebiotics are inulin, galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) [75]. Chicory is the primary industrial source of inulin, from which FOS are obtained through partial enzymatic hydrolysis, and GOS are produced from lactose using β-galactosidase [75]. The exact mechanisms of the protective effects of prebiotics still remain relatively unknown; however, current hypotheses include alteration of the resident gastrointestinal microbiota composition and/or metabolism leading to improved intestinal barrier function, and regulation of the mucosal and systemic immune response.

Prebiotics cannot be digested in the upper gastrointestinal tract and reach the colon unchanged, where resident microorganisms selectively ferment them. Their principal effect is related to the metabolism of the microbiota, and without the presence of dietary fiber in the colon, anaerobic bacteria resort to drawing energy through protein fermentation which can lead to the production of toxic and potentially carcinogenic compounds as discussed above. Prebiotics

affect the resident microbial composition by stimulating the growth of selective saccharolytic commensal protective microorganisms, which can provide resistance to colonization by disease-inducing microbes and thus, contributing to colitis reduction [100-104]. Some prebiotics such as GOS can also exert a direct antimicrobial effect through adherence to the bacterial binding sites on the enterocyte surface, and thus, blocking the adhesion of pathogenic bacteria to IECs [75]. The major products of fermentation of prebiotics are SCFAs, such as acetate, propionate, and butyrate. The amount produced is dependent on the composition of the microbiota, the substrate used and its dosage and the gut transit time. As discussed above, SCFAs have a multitude of benefits to host health.

1.8 Prebiotics in IBD Research

Research into the management of IBD through prebiotic usage have shown intriguing results. A few clinical studies on the usage of prebiotics in IBD management are presented below in Table 1. A study by Welters *et al.* testing dietary supplementation with inulin (24g/day for 3 weeks) on patients with pouchitis revealed an increased stool butyrate concentration, decreased stool pH, decreased numbers of *Bacteroides fragilis* in the stool and diminished concentrations of secondary bile acids in the stool [105]. The study also showed that these results of inulin supplementation were endoscopically and histologically accompanied by a reduction in the inflammation of the mucosa in the ileal pouch reservoir [105].

A study by Lindsay *et al.* testing fructooligosaccharide supplementation (15g/day for 3 weeks) on active ileocolonic Crohn's disease patients showed increased fecal bifidobacteria, decrease in Crohn's disease activity and modified dendritic cell function; Increased percentage of IL-10-positive dendritic cells and increased percentage of dendritic cells expressing Toll-like

receptor (TLR) 2 and TLR-4 [106]. However, a subsequent randomized placebo controlled study by Benjamin *et al.* testing dietary supplementation with the same fructooligosaccharides (15g/day) for 4 weeks on patients with active Crohn's disease revealed no clinical benefits and no significant changes to fecal amounts of bifidobacteria and *F. prausnitzii*, despite the immune impact seen on dendritic cell function, a reduction in IL-6-positive lamina propria dendritic cells, and increased dendritic cell staining of immunoregulatory IL-10 [107]. In contrast, a study by De Preter *et al.* on oligofructose-enriched inulin in inactive or mild CD (10g once daily for 4 weeks) showed improvement in disease activity and microbial shifts of increased *B. longum* and decreased in *Ruminococcus gnavus* counts, and also found an association with increased carbohydrate fermentation metabolites, butyrate and acetaldehyde [108].These results suggest that prebiotics may be beneficial in a subgroup of CD patients with inactive or mild disease, hence the exact therapeutic conditions remain to be studied.

A study by Casellas *et al.* testing supplementation with oligofructose-enriched inulin (12g/day for 2 weeks) on patients with active UC revealed a decrease in dyspeptic symptoms scale as well as an early (day 7) significant reduction of fecal calprotectin [109]. An exploratory study by Valcheva *et al.* on inulin-type fructans (15g daily for 9 weeks) in active mild-to-moderate UC patients showed improvements in clinical disease activity and fecal calprotectin, which was associated with compositional changes to the fecal microbiota (increased *Bifidobacteriaceae* and *Lachnospiraceae* relative abundances) and changes to SCFA pattern in responders (increased total SCFA production and butyrate compared to baseline, reduced isobutyrate and isovalerate) [110]. Interestingly, specific bacterial taxa at mucosal sites, but not in fecal samples, were associated with colonic inflammation, supporting the hypothesis that mucosal microbes have greater impact on the host immune response than fecal microbiome

[111]. These results suggest that the inulin-type fructans' prebiotic activity may be attributed to changes in microbial metabolism rather than compositional changes.

In summary, these studies show the various effects of different prebiotics; however, well-powered, placebo-controlled clinical studies will be required to further validate the efficacy of prebiotics as a therapeutic for IBD management.

Table 1. Clinical intervention studies of prebiotics in managing inflammatory bowel disease.

Prebiotic Treatment	Disease	Study Design	Duration	Microbial changes	Clinical Outcomes	Reference
Inulin (24g/day)	Pouchitis	Placebo controlled double-blind	3 weeks	↓ *Bacteroides fragilis*	No change of clinical symptoms	Welters et al., 2002 [105].
FOS (15g/day)	CD	Open label trial	3 weeks	↑ Fecal bifidobacteria	Improvement in disease activity	Lindsay et al., 2006 [106]
FOS (15g/day)	CD	Placebo controlled double-blind	4 weeks	No significant differences	No clinical benefit	Benjamin et al., 2011 [107]
FOS + Inulin (10g/day)	CD	Placebo controlled double-blind	4 weeks	↑ *Bifidobacterium longum* ↓ *Ruminococcus gnavus*	Improvement in disease activity	De Preter et al., 2013 [108]
FOS + Inulin (12g/day)	UC	Placebo controlled double-blind	2 weeks	N/A	Reduction of dyspepsia symptoms	Casellas et al., 2007 [109]
FOS + Inulin (7.5g or 15g daily)	UC	Open label trial	9 weeks	↑ *Bifidobacteriaceae* ↑ *Lachnospiraceae*	Improvement in disease activity	Valcheva et al., 2019 [110]

The efficacy of prebiotics as a tool for management of IBD is more extensively studied in experimental colitis models, which are useful tools to explore the associated mechanisms (Table 2). In a study by Hoentjen *et al,* HLA-B27 TG rat colitis models, which genetically develop chronic colitis, were given Synergy 1 (combination of inulin and oligofructose 1:1) supplementation (5g/day/kg body weight) for 7 weeks and showed a reduction in colonic inflammation [112]. This reduction was associated with alterations to the gut microbiota including increased cecal *Lactobacillus* and *Bifidobacterium,* a decrease in cecal proinflammatory cytokine IL-1β and increase cecal TGF-1β [112]. Another study on HLA-B27 TG rats by Schultz *et al*. supplementing with a synbiotic containing inulin and probiotic strains (*L. acidophilus* La-5 and *Bifidobacterium lactis* Bb-12) showed a reduction in colonic inflammation as well as an increase in *Bifidobacterium animalis* numbers [113]. Interestingly, the probiotic strains in the synbiotic supplement were below the detection levels, suggesting that the prebiotic inulin is the main effective compound in this supplement [113]. A study by Koleva *et al.* showed that HLA-B27 TG rats fed either a rat chow diet or a chemically defined diet (AIN-76A), supplemented with FOS or isomalto-oligosaccharides (IMO) (8g/kg body weight/day for 12 weeks) had reduced colitis in rats fed chow diet but produced no anti-inflammatory effect when supplemented to the chemically defined diet [114]. This reduction in colitis was also associated with a decrease of fecal *Clostridium cluster* XI and increased concentrations of total SCFAs in cecal contents of rats on chow diet in comparison to the chemically defined diet [114]. The chemically defined diet increased proportions of specific branched SCFAs (iso-butyrate, valerate and isovalerate) for both fibers which was associated with increased colitis and IL-1β concentration in the cecal mucosa [114]. These interesting findings indicate that the protective effects of fiber supplementation are also dependent on the background diet [114].

A study on dextran sodium sulfate (DSS)-induced colitis rats fed goat's milk oligosaccharides by Lara-Villoslada *et al.* showed decreased DSS-induced weight loss, a decreased acute colonic inflammatory process, less severe colonic lesions, increased expression of genes involved in intestinal function such as mucine-3 and an increase in colonic lactobacilli and bifidobacteria [115]. Another study in DSS-induced colitis rats by Videla *et al.* showed that orally administered inulin prevented inflammation associated with increased counts of lactobacilli, while enemas of both inulin (400mg/day in 5ml saline and rectally administered) and butyrate (40mmol/L or 80mmol/L in 5ml saline and rectally administered) had no effect [116].

A study by Camuesco *et al*. into the preventative effects of lactulose investigated a 2-week feeding of lactulose prior to a trinitrobenzenesulphonic acid (TNBS) instillation, with sample collections 1 week after colitis induction [117]. The study showed that lactulose exerted a preventative anti-inflammatory effect on colitis, which was associated with increased colonic lactobacilli and bifidobacteria species [117]. Another study in TNBS-induced colitis rats by Cherbut *et al.* tested twice daily intragastric infusions of FOS for 2 weeks [118]. Intragastric infusions with FOS reduced the gross score for inflammation, myeloperoxidase activity and pH, while increasing butyrate and lactate concentrations and lactic acid bacteria (LAB) counts in the cecum [118]. The same study also tested intragastric infusions of LAB (10^{11} cfu/d), and intracolonic infusions of butyrate and lactate and interestingly found that the significant FOS-induced benefits were only reproducible by the intragastric LAB infusions; the organic acid infusions required the addition of LAB ($10^{9.5}$ cfu/d) in order to reproduce the FOS effects [118]. It seems that FOS was able to reduce the intestinal inflammatory activity mainly through increasing intestinal LAB counts [118]. Another study by Shingo *et al.* showed that supplementation with short-chain inulin-like fructans with 4 degrees of polymerization (DP4)

accelerated the healing process post-instillation, which was associated with a higher cecal numbers of lactobacilli and bifidobacteria, as well as an increase in fecal SCFA concentrations, particularly propionate in TNBS-induced colitis rats [119].

Table 2. Animal model studies of prebiotic in the management of inflammatory bowel diseases.

Prebiotic Treatment	**Animal model**	**Duration**	**Microbial changes**	**Outcomes**	**Reference**
Synergy 1* (5g/day/kg body weight)	HLA-B27 TG rats	7 weeks	↑ cecal *Lactobacillus* and *Bifidobacterium*	↓ Colonic inflammation	Hoentjen et al., 2005 [112]
Synbiotic with inulin and probiotics**	HLA-B27 TG rats	8 weeks	↑ *Bifidobacterium animalis*	↓ colonic inflammation	Schultz et al., 2004 [113]
FOS or IMO (8g/kg body weight/day)	HLA-B27 TG rats	12 weeks	FOS: ↑ Bifidobacteria and *Enterobacteriaceae* Both: ↓*Clostridium* cluster IV	↓ colitis in chow diet group but not AIN-76A diet	Koleva et al., 2014 [114]
Goat's milk oligosaccharide (20g/kg diet)	DSS-induced colitis rats	20 days	↑ colonic *Lactobacilli* and *Bifidobacteria*	↓ colonic inflammation	Lara-Villoslada et al., 2005 [115]
Inulin (400mg/day)	DSS-induced colitis rats	14 days	↑ *Lactobacilli*	Prevented inflammation	Videla et al., 2001 [116]
Lactulose (25% wt/vol water)	TNBS-induced colitis rats	2 weeks	↑ colonic *Lactobacilli* and *Bifidobacteria*	Preventative anti-inflammatory effect.	Camuesco et al., 2005 [117]
Intragastric infusion of FOS (1g/day)	TNBS-induced colitis rats	2 weeks	↑ cecal lactic acid bacteria	↓ Gross score for inflammation	Cherbut et al. 2003 [118]
Short-chain inulin-like fructans DP4*** (60g/kg)	TNBS-induced colitis rats	24 days	↑ *Lactobacillus* and *Bifidobacteria*	↓ colonic inflammation	Shingo et al. 2011 [119]

*Synergy 1 is prebiotic combination of long-chain inulin and oligofructose 1:1.
** *L. acidophilis* La-5 and *Bifidobacterium lactis* Bb-12
***Short-chain inulin-like fructans with 4 degrees of polymerization

1.9 Conclusion

IBD is a life-time disorder involving complex interactions between host genetics and environmental factors. Dysbiosis of the gastrointestinal microbiota has been shown in numerous studies to have a possible role in the etiology of IBD. The microbiota has been shown to be strongly influenced by diet. Prebiotics have been shown to have various beneficial effects on the host, especially through its effects on the gut microbiota. The end-products of prebiotic fermentation by the microbiota are beneficial in both healthy individuals as well as IBD patients. In contrast to conventional IBD treatments, prebiotics are inexpensive, easy to administer and do not have significant side effects. As such, prebiotics are emerging as promising alternative therapies for management of IBD. Clinical studies on prebiotic supplementation in IBD is limited, making the exact protective mechanisms of prebiotics as well as their benefits to compositional shifts and function of the microbiota difficult to understand. Further studies are required to determine the precise interactions between prebiotics and the host physiology, immune system, and the resident microbiota.

1.10 Overview of Master's Work

There is growing interest in studying prebiotics and their beneficial effects on the microbiota to prevent and/or alleviate symptoms of IBD. Data from preclinical studies and clinical trials show prebiotic inulin-type fibers can prevent/reduce colitis; however, other dietary

fibers, such as highly branched α-glucans, are understudied and it is unknown if microbial shifts induced by these α-glucans can be protective in experimental colitis.

The focus of my thesis work was to investigate the effectiveness of isomaltodextrin (IMD), a novel highly branched α-glucan product made by Hayashibara Co, Ltd. (Okayama, Japan), in reducing colitis development. IMD is a highly branched α-glucan that cannot be metabolized by host enzymes [120]. It is a soluble dietary fiber produced from maltodextrin using *Paenibacillus alginolyticus* PP710-derived *α-glucosyltransferase* and *α-amylase* [120]. A previous study in DSS-induced colitis mouse models reported IMD's ability to reduce inflammatory cytokines TNF-α and IL-6 in colonic tissues as well as its ability to inhibit expression of TLR-4, a receptor well known for recognizing bacterial lipopolysaccharides, suggesting that IMD's mode of action may involve changes in the microbiota derived antigens [121]. A study in IL-10 knockout mice reported IMD's sex-specific response, showing positive benefits to colitis in males only, that included decreased inflammatory cytokines IL-12p70 concentration in the colon, enhanced microbial richness and evenness of the microbiota and lesser reduction in relative abundance of *Coprococcus* compared to females, which was a bacterial group negatively associated with IL-12p70 concentration [122].

As experimental colitis models only partly represent the pathophysiology of human IBD, as well as the conflicting data reported in DSS and IL-10 knockout mice models, the current study intended to explore the efficacy of IMD on colitis reduction, tackle previously suggested mechanisms of protections and further investigate the sex-driven anti-inflammatory response in a transgenic animal model.

The animal model used in this project are HLA-B27 TG rats, which is a validated IBD model that have been used in previous IBD research as discussed above [112-114]. HLA-B27

TG rats are genetically engineered Fisher rats with overexpressed HLA-B27 and human β2-microglobulin resulting in immune dysregulation [123]. This dysregulation leads to the development of several chronic conditions including: 1) chronic gastrointestinal inflammation developed around 6-8 weeks of age, 2) arthritis developed around 5-6 months of age, 3) psoriasis vulgaris (skin and nail lesions) developed around 5-6 months of age, and 4) genital inflammation leading to infertility in males developed around 2-3 months of age. HLA-B27 TG rats are genetically susceptible to spontaneously developing immunologically mediated chronic intestinal inflammation when raised in normal specific pathogen-free (SPF) environmental conditions in the presence of commensal intestinal microbiota, thus mimicking human IBD. Rodent and human intestinal microbiota are different; however, our lab identified that the prebiotic FOS stimulated similar protective bacterial groups that reduced experimental colitis in HLA-B27 TG rats as well as human UC (bifidobacteria and butyrate producing Firmicutes) and that other specific bacterial groups were associated with colitis (Enterobacteriaceae) [106]. These results show the translational potential and validation of the HLA-B27 TG rat colitis models to study the mechanisms and efficacy of IMD in IBD. Treatment with IMD in this study will begin at 4 weeks of age, prior to the common age of disease onset in HLA-B27 rats, to determine its effect on the development of colitis.

Based on this information, this thesis hypothesizes that:

1) IMD treatment will reduce colitis development in HLA-B27 TG rat colitis model.
2) High dose IMD will be more beneficial in reducing colitis than a low dose of IMD.

3) IMD treatment will reduce development of colitis by increasing SCFA levels and/or changes in gut microbiome composition.

To fulfill these hypotheses the aims of the study are to:

1) Assess if IMD is effective in reducing intestinal inflammation in HLA-B27 TG rat colitis model and at what dosage IMD will show the strongest efficacy.
2) Identify shifts in gut microbiome composition and fermentation products and their association with colitis development.

Study parameters:

1) Chronic intestinal inflammation marker, IL-1β, will be measured in colonic and cecal tissue.
2) Shifts in gut microbiome composition as a result of IMD treatment and colitis development will be determined for endpoint.
3) Changes in microbial fermentation products will be evaluated by measuring SCFA composition of stool and cecal content samples at endpoint.

Chapter 2: Isomaltodextrin Dose-Dependently Reduces Colitis Development in HLA-B27 Rat Colitis Model with Associated Changes to Gut Microbiome Composition and Short Chain Fatty Acid Production.

2.1 Introduction

Inflammatory bowel diseases (IBD), consisting of Crohn's disease (CD) and ulcerative colitis (UC), are characterized by chronic relapsing inflammation of the gastrointestinal tract. Canada has the highest reported IBD incidence rates and second highest prevalence rates in the world, with over 270,000 Canadians suffering from IBD as of 2018 [2,6]. Alarmingly, these rates have been increasing, particularly in children under the age of 10, and are estimated to continue increasing [3,6]. Estimated costs to Canadians are over $2.7 billion annually, with dominant factors being medications and work loss [3]. A potential strategy in reducing total costs is finding a more cost-effective alternative therapy to conventional medical therapies for reducing IBD symptoms and, as a result, long-term work losses.

IBD's etiopathogenesis is very complex and remains unclear; however, the hypothesis that is currently widely accepted is that pathogenesis is caused by a combination of factors including abnormal immune responses mediated by the commensal gut microbiota and environmental factors in a genetically susceptible host [7-10]. It is apparent that genetics contribute to the pathogenesis of IBD, as the risk for IBD development increases in individuals that have a relative with IBD, and this risk is further increased if both parents suffer from IBD [11]. Monozygotic twin studies show that concordance rates for CD is only 30-35% and 10-15% for UC, suggesting additional complexity than genetics alone to the disease mechanisms [12]. Western nations have higher incidence and prevalence rates of IBD compared to developing

nations and an apparent pattern can be seen between industrialization and incidence of IBD [3]. A possible explanation may be industrialized food processing and introduction of the 'Western diet' that is high in processed foods and lower in healthy dietary fibers, which has been shown to increase gut inflammation [9,16,17].

Microbial contributions to IBD are more complex due to the lack of definitive conclusion as to whether the dysbiosis seen in IBD is truly causative, or merely a consequence, of inflammation. Lower bacterial diversity has been observed in patients with IBD [60]. The phylum Firmicutes, specifically *Faecalibacterium prausnitzii*, is often reduced in the stool of patients with CD compared to healthy controls, while members of *Enterobacteriaceae* have been shown in higher amounts [60,61]. In patients with UC, decreased butyrate-producing bacteria such as *F. prausnitzii* and *Roseburia hominis* has been seen [82-84]. The reduction seen in these known producers of short chain fatty acids (SCFA) may also play a role in chronic intestinal inflammation. SCFAs are microbial metabolites that can be absorbed by the host's gastrointestinal tract and provide numerous benefits to the host. Thus, butyrate essentially acts in the bowel as a fuel source for colonic enterocytes, reduction of inflammation, activation of intestinal gluconeogenesis, generation of a hypoxic state to maintain gut oxygen balance and prevent dysbiosis, and enhancement of gut barrier function through regulating assembly of tight junction which provides increased protection against pathogens. Propionate is mainly metabolized in the liver and stimulates liver's gluconeogenesis. Finally, acetate is metabolized mostly in peripheral tissue, capturing one H^+ and forming acetyl-coenzyme A as an intermediate product, which can then enter several metabolic pathways (Krebs cycle, ketone body formation, fatty acids synthesis, gluconeogenesis) [31-33,37-42]. Targeting these host-microbial interactions through stimulating the growth of gut microbes may benefit IBD patients. Dietary

fibers which, as previously mentioned, are reduced in the 'Western diet' may be a potential tool for this.

A prebiotic is defined as a non-digestible compound that is metabolized by the intestinal microbiota and, through its modulatory effect on the composition and/or activity of the gut microbiota, confers a beneficial physiological effect on the host [99]. The most widely studied, and commercially available, prebiotics are inulin, galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) [75]. Data from preclinical studies and clinical trials show the beneficial effects of these prebiotics, including inulin and FOS, in reducing and/or preventing experimental and human ulcerative colitis [106-110, 112-119]; however, other dietary fibers, such as α-glucans, are understudied and it is unknown if microbial shifts induced by them can be protective in experimental colitis.

This study will examine isomaltodextrin (IMD), a resistant α-glucan produce made by Hayashibara Co, Ltd. (Okayama, Japan), at two doses to assess what IMD dosage is effective in reducing colitis development. IMD is a highly branched α-glucan that is not be metabolized by host enzymes [120]. It is a soluble dietary fiber produced from maltodextrin using *α-glucosyltransferase* and *α-amylase*, derived from *Paenibacillus alginolyticus* PP710 [120]. A previous study in DSS-induced colitis mouse models reported anti-inflammatory effects of IMD including: reduction of inflammatory cytokines, TNF-α and IL-6, in colonic tissues; and inhibiting expression of Toll-like receptor 4 (TLR-4), a receptor known for recognizing bacterial lipopolysaccharides (LPS), suggesting that IMD's mode of action may involve changes in the microbiota derived antigens [121]. Another study tested IMD on IL-10 knockout mice and reported sex-specific responses, with benefits only for males, including decreased inflammatory cytokine IL-12p70 concentration in the colon, enhanced richness and evenness of the microbiota

and lesser reduction in relative abundance of *Coprococcus* compared to females, which was a bacterial group negatively associated with IL-12p70 concentration [122]. These rather conflicting data require further validation of IMD anti-inflammatory effect on chronic intestinal inflammation as well as the precise dosage with maximum effect. HLA-B27 TG rats are previously validated IBD models that are genetically engineered Fisher rats with added HLA-B27 and human β2-microglobulin resulting in a dysregulated immune system [112-114,123]. HLA-B27 TG rats are genetically susceptible to spontaneously developing immunologically mediated chronic intestinal inflammation when raised in normal specific pathogen-free (SPF) environmental conditions in the presence of commensal intestinal microbiota, thus mimicking human IBD. We hypothesize that IMD treatment will reduce colitis development in HLA-B27 TG rat colitis models associated with increased SCFA production and/or changes in the gut microbial composition, and that this effect will be more significant in a higher dose of IMD. We aim to assess the efficacy of IMD in reducing colitis development in HLA-B27 TG rats as well as the associated shifts in microbial composition and fermentation products.

2.2 Methods

2.2.1 Animals

Animal use was approved by the Animal Care and Use Committee of the University of Alberta (animal protocol: AUP000002722) and conducted in accordance with the Canadian Council on Animal Care Guidelines. HLA-B27 TG rats, a validated animal model genetically susceptible to spontaneously develop immunologically mediated chronic intestinal inflammation mimicking human IBD, was used for this study [112-114, 121]. HLA-B27 TG rats originally purchased from Charles River (Wilmington, MA, USA), were bred, and maintained in SPF housing conditions at the University of Alberta's (Edmonton, Canada) Health Sciences Laboratory Animal Services. Presence or absence of the HLA-B27 transgene was assessed by PCR using DNA isolated from tail clippings. Experimental TG animals were housed two per cage in a temperature (22ºC) and light-controlled (12 h light/dark cycle) environment; food and water were provided *ad libitum* for consumption. Rats were fed the commercial rat chow standard diet (5053 PicoLab® Rodent Diet 20, Lab Diet Inc., Leduc, AB, Canada).

2.2.2 Experimental Design and Animal Handling

Layout of the experimental design is shown in Figure 1. Food and water were provided *ad libitum* throughout study duration. Prior to entering the nutritional study (4 weeks of age), all rats were fed standard chow PicoLab® Rodent Diet 20 5030 (Lab Diet Inc., Leduc, AB, Canada) (21% proteins, 11% fats, 54% carbohydrates and 4.5% fibers), a commercial standard rat chow. Four week old HLA-B27 TG rats, prior to developing colitis, were randomly assigned into one of the 4 isocaloric dietary groups: 1. Negative Control group given standard chow diet

supplemented with cellulose, a fiber that is not fermented by the human or rodent gut microbiota (15% *w/w*), hereinafter referred to as Chow group, with males (*n*=6) and females (*n*=8); 2. Positive Control group given standard diet supplemented with fructooligosaccharides, which has been shown to be beneficial in reducing colitis in HLA-B27 rats (FOS, Orafti P-95, Raffinerie Tirlemontoise, Tienen, Belgium) (15% *w/w*), hereinafter referred to as FOS group, with males (*n*=6) and females (*n*=8) [114]; 3. Experimental group 1 given standard chow diet supplemented with IMD, a novel highly branched α-glucan product by Hayashibara Co, Ltd. (Okayama, Japan) (7.5% *w/w*) and cellulose (7.5% *w/w*), hereinafter referred to as IMD 7.5% group, with males (*n*=8) and females (*n*=8); and 4. Experimental group 2 fed standard diet supplemented with IMD (15% *w/w*), hereinafter referred to as IMD 15% group, with males (*n*=7) and females (*n*=8). Upon entering the nutritional study, rats were given half doses of fiber supplementations (7.5% w/w fiber) for the first 2 weeks for acclimation where: Chow group's diet consisted of standard chow supplemented with 7.5% *w/w* cellulose; FOS group's diet consisted of standard chow supplemented with 7.5% *w/w* FOS; IMD 7.5% group's diet consisted of standard chow supplemented with 3.75% *w/w* IMD and 3.75% *w/w* cellulose; and IMD 15% group's diet consisted of standard chow supplemented with 7.5% *w/w* IMD. After the two-week adjustment period, rats were supplemented with the full experimental doses of their assigned dietary group. Diets were prepared by pulverizing the standard chow and mixing thoroughly with the corresponding powdered fiber blends. Diets were fed in powdered form. Treatment continued for 12 weeks until rats reached 16 weeks of age. Since the diet intervention was introduced at an early stage of rat's development (childhood) and was carried on into animal's adulthood it was important to assess if the diet changes had an effect on the rat's growth curve. Food consumption was monitored twice weekly and individual rat's diet intake was calculated by dividing each

cage food consumption by number of rats. Rats' body weights were recorded at weeks 4, 5, 6, 7, 8, 10, 12, 14 and 15/16, of age. Fecal samples were collected immediately after defecation at weeks 4, 8, 12, and 16 weeks of age and stored at −80°C. Animals were euthanized by CO_2 asphyxia at 16 weeks of age. At necropsy, digesta was collected prior to cecal and colonic tissue measurement for weight and length. Samples were immediately frozen and stored at −80°C.

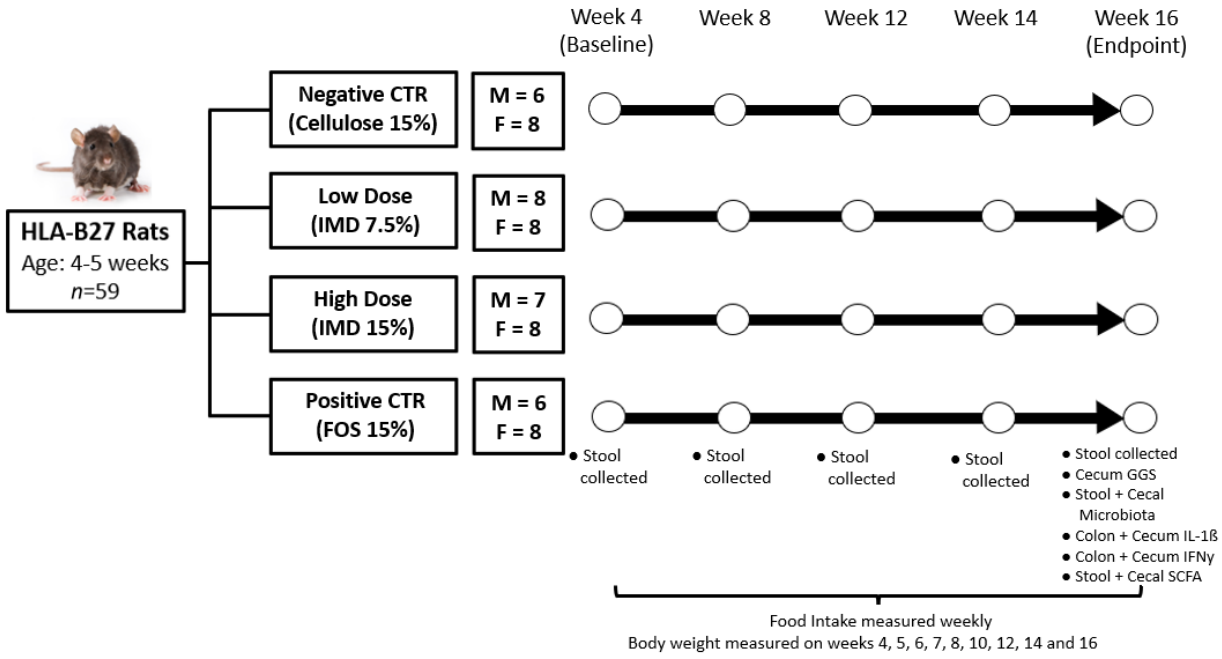


Figure 1. Experimental design. Abbreviations used: CTR, control; IMD, Isomaltodextrin; M, Male; F, Female; GGS, Gross Gut Score; IL-1β, interleukin 1-beta; SCFA, Short Chain Fatty Acid.

2.2.3 Gross Gut Scores

Macroscopic inflammation was graded using a previously validated method of scoring called Gross Gut Score (GGS), where cecal inflammation evaluation included the following criteria: 1) the number of cecal nodules, 2) the severity of mesenteric contractions, 3) the severity of adhesions, and 4) the extent of cecal wall thickening [124,125]. At necropsy, each rat's cecum was inspected grossly by two individuals in a blinded fashion and scored on a scale of 0 to 4 GGS, where 0 is no signs of macroscopic inflammation and 4 is severe signs of macroscopic inflammation. Samples for scoring were taken from the widest portion of the cecum.

2.2.4 Tissue Homogenization

Cecum and colon tissue samples taken at necropsy were thawed on ice and approximately 0.05- 0.1g was sliced off and added to 1.5mL of a solution prepared with 500:1 radioimmunoprecipitation assay (RIPA) buffer to proteinase inhibitor cocktail solution (P8340, Sigma-Aldrich). RIPA buffer was prepared by mixing 1mL of 1% Nonidet P-40 (0.1% final concentration), 0.5g of 0.5% sodium deoxychalate (0.005g/mL final concentration), 1mL of 10% sodium dodecyl sulphate (0.1% final concentration), and 100mL phosphate-buffered saline (PBS), and filter sterilizing through a 0.45μm filter. Samples were homogenized using Fisherbrand™ 150 Homogenizer. Samples were then centrifuged at 10,000g for 10 minutes at 4◦C to pellet the cell debris. Supernatant was collected and stored at -80◦C until further analysis.

2.2.5 Cecum and Colon Protein Measurement

Homogenized cecum and colon tissues were thawed on ice and protein concentrations were measured using a Bradford Protein Assay kit (Bio-Rad Laboratories Inc., California, USA) as per the manufacturer's protocol. Bovine serum albumin was used for the standard curve at an initial concentration of 0.2mg/mL, followed by 0.5mg/mL, 1.0mg/mL, 1.5mg/mL and maximum concentration of 2.0mg/mL.

2.2.6 Cecum and Colon Cytokine Analyses

The mucosal interleukin-1β (IL-1β) and interferon-γ (IFNγ) were quantified in cecal and colonic tissue homogenates using a commercial IL-1β rat ELISA DuoSet ELISA Development System kit (R&D Systems, Inc., Minneapolis, MN, U.S.A.) and IFNγ rat ELISA kit (ThermoFisher Scientific Inc., Waltham, MA, U.S.A). Recombinant rat IL-1β and IFNγ with known concentration was used as standard for the ELISA assay and 2-fold dilutions of the standard were run on each plate. Results were calculated using total protein for normalization of the targeted cytokine measurement and presented as μg IL-1β/g protein and μg IFNγ/g protein. IL-1β and IFNγ concentrations in cecum and colon tissues normalized to protein concentrations were used for comparison between diet groups.

2.2.7 Short Chain Fatty Acid (SCFA) Measurements

SCFAs in cecal contents and stool were quantified using gas chromatography. Approximately 200mg of sample was homogenized in 1000μL of hydrochloric acid (0.1N) by

vortexing for 10-30 seconds or until totally disrupted. 250µL of phosphoric acid (25% v/v) was then added and again homogenized by vortexing for 10-30 seconds. Samples were then centrifuged for 15 minutes at 10000 rpm until supernatant appeared clear and clarified liquid was removed into a fresh tube. Sample extracts were then sent to Agriculture Food and Nutritional Sciences Chromatography Core laboratory (University of Alberta, Edmonton, Canada) for quantification of SCFAs. Briefly, internal standard solution (150 mg of 4-methyl-valeric acid, S381810, Sigma-Aldrich) was added to supernatant and 5% phosphoric acid in a glass chromatography tube, thoroughly mixed, and kept for 30 minutes at room temperature. SCFAs in supernatant were analyzed using a Varian model 3400 Gas Chromatograph (Walnut Creek, CA, USA) with a Stabilwax-DA column (30-m × 0.25-mm i.d.; Restek, Bellefonte, PA, USA). SCFAs quantities were normalized to weight of stool or cecal content used and concentrations of individuals SCFAs were calculated as a proportion (%) of total SCFA concentration, prior to statistical analyses.

2.2.8 Isolation of Genomic DNA

Approximately 0.1g of cecal content or stool was thawed on ice and DNA was extracted using the Qiagen QIAamp DNA stool kit following the International Human Microbiome Standards' "IHMS SIO 06 V1: Standard Operating Protocol For Fecal Samples DNA Extraction, Protocol Q", with a few alterations [118]. Briefly, frozen samples (0.1g) mixed with sterile zirconia beads (0.1 mm, 0.3 g) were initially lyzed in 1mL stool lysis buffer (ASL) for 15 min at 95°C followed by mechanical cell disruption in the Fastprep™ Instrument (5 cycles of 1 min beadbeating at 6 m/s and 5 minutes resting on ice). Samples were then centrifuged (16000 x g

and 4°C for 5min) and supernatant was transferred to a fresh tube. Proteins were precipitated with 10M ammonium acetate (260µL) followed by incubation on ice for 5min and centrifugation (16000g at 4°C for 10 min). The supernatant was then mixed with equal volume of isopropanol and incubated on ice for 30 min. Following a centrifugation (16000g at 4°C for 15min) the resultant pellet was washed with 0.5mL EtOH (70% v/v), spin down and dried at room temperature. The pellet was then dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and used in Qiagen spin column extraction using QIAmp DNA Stool Mini kit.

2.2.9 16S rDNA Sequencing

16S rRNA libraries were prepared following the "16S Metagenomic Sequencing Library Protocol Preparation – Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System" Protocol [127]. This protocol is designed to amplify ~460 bp from the V3 and V4 region. The final library check was done using QIAxcel (screening cartridge, method AM420, alignment marker 15 bp-3Kb, size marker 50-800 bp) and size of final library was calculated as the average of the detected bands (~590 bp); each final library was quantified by Qubit and the molarity was calculated using the formula "((concentration in ng/uL)/(660 g/mol x average library size)) x 10^6 = concentration in nM"; each final library was diluted to 4nM before all libraries were pooled and the final pool was run on Bioanalyzer to confirm size (621bp) and on Qubit to determine concentration (4.3nM). The library pool was denatured and diluted to 8pM before combining with 8pM PhiX control to get the final 25% PhiX, which was loaded on a MiSeq and run using a V3 (600 cycle kit) to get pair end reads of 300bp for each sample.

Sequencing was performed by The Applied Genomic Core at the University of Alberta. Number of reads per sample were between 14,000 and 170,000.

2.2.10 Bioinfomatics Analysis

Bioinformatics analysis of the sequenced 16S rDNA libraries was performed by Dr. Maria Elisa Perez-Muñoz (University of Alberta, Edmonton, Canada). Raw paired-end reads from the 59 cecal and 59 stool endpoint samples (n=118) were trimmed to 270bp using the FASTX-toolkit [128]. Reads were then quality-filtered and paired using the merge-Illumina-pairs application from Illumina utils. Quality criteria included: p-value ≤ 0.03, enforced Q30 check, perfect matching to primers and no ambiguous nucleotides. Reads that did not meet quality criteria were discarded. After trimming and quality filtering, 2,362,428 total paired sequences were obtained (average 20,021±5,983; minimum=4,166; maximum=42,637). Usearch v.10 was then used to compile and dereplicate sequences, discard singletons, remove chimeras, cluster OTUs at 98% identity, identify representative sequences for OTUs and generate a final OTU table. Same number of non-chimeric sequences were binned by sample/subject and submitted to the Ribosomal Database Project Classified (RDP; http://rdp.cme.msu.edu/) for taxonomic assignment from phyla to genera. OTUs were assigned identity by submitting representative sequences to the Silva ACT database, and taxonomic assignments were confirmed using NCBI BlastN (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch), EzBioCloud (https://www.ezbiocloud.net/), and Ribosomal Database Project Seqmatch (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) (Michigan State University, 2016). Taxa with <4 counts in the entire dataset were discarded. Raw sequence counts were calculated as

relative abundances (%) and taxa with a mean relative abundance of ≤0.01% in the entire dataset were then removed. Alpha diversity metrics were computed (Simpson Index, Pielou Evenness Index), as well as beta diversity metrics (Bray-Curtis) and one-way ANOVA with Kruskal-Wallis test and two-stage step-up method of Benjamini, Krieger and Yekutieli test to correct for multiple comparisons by controlling the False Discovery Rate was used to determine significant differences between groups.

2.2.11 Statistical Analysis

The following statistical analyses were performed using GraphPad Prism v.8., differences in growth curves, food intakes, cecal GGS, cecal/colon IL-1β and IFNγ concentrations, cecum/colon weight:length ratios, relative abundances of bacterial groups and SCFAs between diet groups. Statistical analyses for growth curves, food intakes, cecal GGS and cecal/colon IL-1β and IFNγ concentrations and weight:length ratios were calculated using unpaired T-test or ordinary one-way ANOVA with two-stage step-up method of Benjamini, Krieger and Yekutieli test to correct for multiple comparisons by controlling the False Discovery Rate. Statistical analyses for relative abundances of bacterial groups and SCFAs between diet groups were calculated using non-parametric Kruskal-Wallis test and two-stage step-up method of Benjamini, Krieger and Yekutieli test to correct for multiple comparisons by controlling the False Discovery Rate. Results of ANOVA tests are presented as q values, where $q \leq 0.05$ was set as significant and $q \leq 0.1$ was set as approaching significance or tentatively significant. Results of T-tests are presented as p values, where $p \leq 0.05$ was set as significant.

The following analyses were performed using MetaboAnalyst 5.0. (https://www.metaboanalyst.ca/home.xhtml). Correlation between IL-1β concentrations and SCFA proportions, IL-1β concentrations and relative abundances of bacterial groups, as well as SCFA proportions and relative abundances of bacterial groups, was determined using Spearman non-parametric correlation with adjusted P value for multiple comparisons. Results for correlation analyses are presented as R values, where $0.3 \leq R \leq 0.6$ was set as moderately correlated and $0.6 < R$ was set as strongly correlated, and adjusted p values where $p \leq 0.05$ was set as significant. To determine alterations to cecal and colon microbial groups by IMD supplementation, we performed supervised multivariate analyses using partial least square discriminant analysis (PLS-DA), where data sets were normalized by log transformation and auto-scaling (mean-centered and divided by the standard deviation of each value). Results for PLS-DA analyses are presented as 2D scores plot and variable importance in projection (VIP) scores.

2.3. Results

2.3.1. Effect of fiber treatments on relative body weight gain and diet intake

As this nutritional study was conducted for 12 weeks, beginning at late childhood to adulthood age in rats and introduced significant alterations to the dietary macronutrient ratios through the addition of undigestible fibers, it was important to assess if animal's food intake and body weight gains were affected by treatment groups. Starting weight (mean ± SEM) for males, Chow = 69.5g ± 2.4, IMD 7.5% = 55g ± 3.1, IMD 15% = 64.3g ± 4.2, FOS 15% = 71.0g ± 6.4. Endpoint weight for males, Chow = 240.7g ± 11.2, IMD 7.5% = 249.1g ± 11.6, IMD 15% = 269.1g ± 6.8, FOS 15% = 265.2g ± 11.4. Starting weight (g ± SD) for females, Chow = 57.9g ± 3.1, IMD 7.5% = 55.8g ± 4.9, IMD 15% = 65.3g ± 6.0, FOS 15% = 61.6g ± 7.1. Endpoint weight for females, Chow = 151.5g ± 3.6, IMD 7.5% = 160g ± 4.4, IMD 15% = 154.4g ± 5.3, FOS 15% = 151.9g ± 3.6. Starting weight for IMD 7.5% males was tentatively lower than Chow males ($q=0.0853$), which was not seen in IMD 15% or FOS males. No difference was seen between groups for females' starting weights, males' endpoint weights or females' endpoint weights. At endpoint, males were approximately 60% heavier than the females of same diet group. As such, growth curves and diet intakes were separated for the sexes prior to analysis.

Figure 2 shows average diet intake (g/day/rat) for each sex measured throughout the study. No significant difference in diet intake was seen in male (Figure 2A) across all diet groups and females as well (Figure 2B). Figure 2 also shows changes in body weight throughout the study for each sex calculated as % weight gain relative to baseline weight. For males at week 5, average relative weight gain (mean ± SEM) was statistically higher in IMD 7.5% group (71.80% ± 13.06) compared to all other groups, Chow (41.09% ± 3.56, $q=0.0019$), IMD 15% (57.85% ± 9.98, $q=0.0713$), and FOS 15% (49.63% ± 10.5, $q=0.0270$) (Figure 2C). Similarly, IMD 7.5%

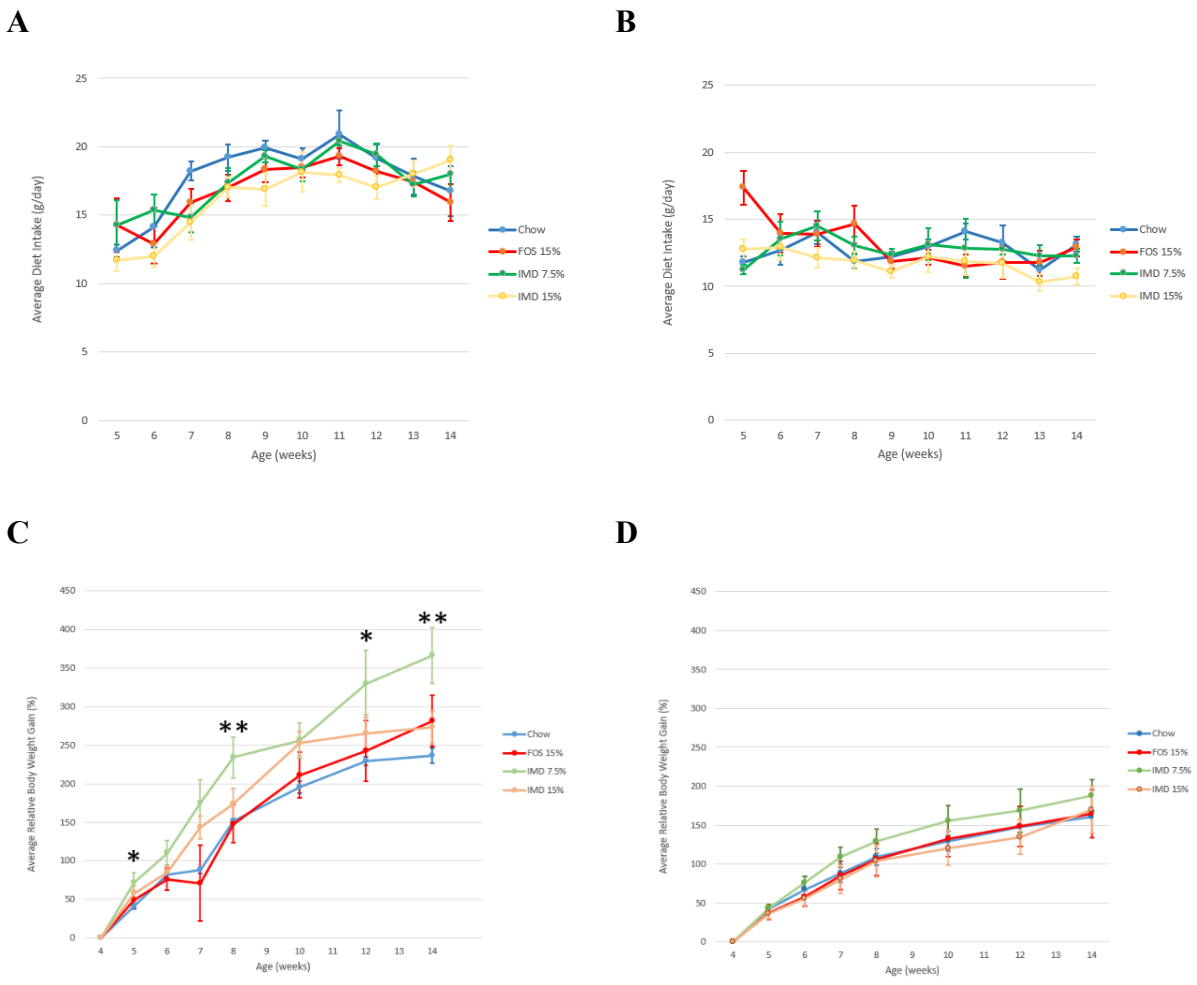


Figure 2. Effects of fiber treatments on average diet intake of males (A) and females (B) as well as average relative body weight gain of male (C) and female (D) HLA-B27 transgenic rats. Blue = Chow. Red = FOS 15%. Green = IMD 7.5%. Orange = IMD 15%. For males (A) and (C), Chow n=6, FOS 15% n=6, IMD 7.5% n=7 and IMD 15% n=8. For females (B) and (C), Chow n=8, FOS 15% n=8, IMD 7.5% n=8 and IMD 15% n=8. Diet intake was calculated as an average between the 2 rats in each cage. Data are presented as mean ± SEM. Statistical significance was calculated between diet groups for each week using one-way ANOVA and two-stage Benjamini, Krieger and Yekutieli test. *$q \leq 0.1$. **$q \leq 0.05$.

fed male rats showed higher average relative weight gain at week 8 when compared to the control Chow (234.2% ± 26.5 versus 151.2% ± 3.4, q=0.0270) (Figure 2C). IMD 7.5% fed male rats also demonstrated significant relative body weight gain when compared to their age mates assigned to IMD 15% or FOS (174.6% ± 19.2, q= 0.066; 147.7% ± 24.6, q=0.027, respectively) (Figure 2C). At week 14, average relative weight gain for male rats on IMD 7.5% (366.16% ± 36.11) was higher than Chow (236.73% ± 9.80) (q=0.0198), IMD 15% (273.41% ± 20.61) (q=0.0641), and FOS 15% (281.15% ± 33.43) (q=0.0795) (Figure 2C). Relative body weight gains of males were similar across all diet groups except for specific timepoints mentioned where IMD 7.5% group had higher weight gains. No significant weight gain differences were seen in female rats across all diet groups (Figure 2D).

2.3.2. Effect of fiber treatments on cecal macroscopic inflammation

Figure 3A shows the average GGS per treatment in cecum, scored on a scale of 0-4 at necropsy, with 0 being the least and 4 being the highest, for signs of visual macroscopic cecal inflammation. The highest average GGS (GGS ± SD) was seen in Chow group (3.3 ± 0.9), which was significantly higher than IMD 15% (2.1 ± 0.9, q=0.0002) and FOS group (0.9 ± 0.8, q<0.0001) (Figure 3A). Chow group was only had tentatively higher GGS than IMD 7.5% (3 ± 0.7, q=0.0539). Interestingly, IMD 15% had significantly lower macroscopic inflammation than IMD 7.5% (q=0.0013). A previously published study of IMD in IL-10 knockout mice found that the studied resistant starch had sex-specific benefits only for males [122]. Additionally, females had different diet intake and relative body weight gain compared to males (Figure 2). As such, GGS were also assessed for each of the sexes separately.

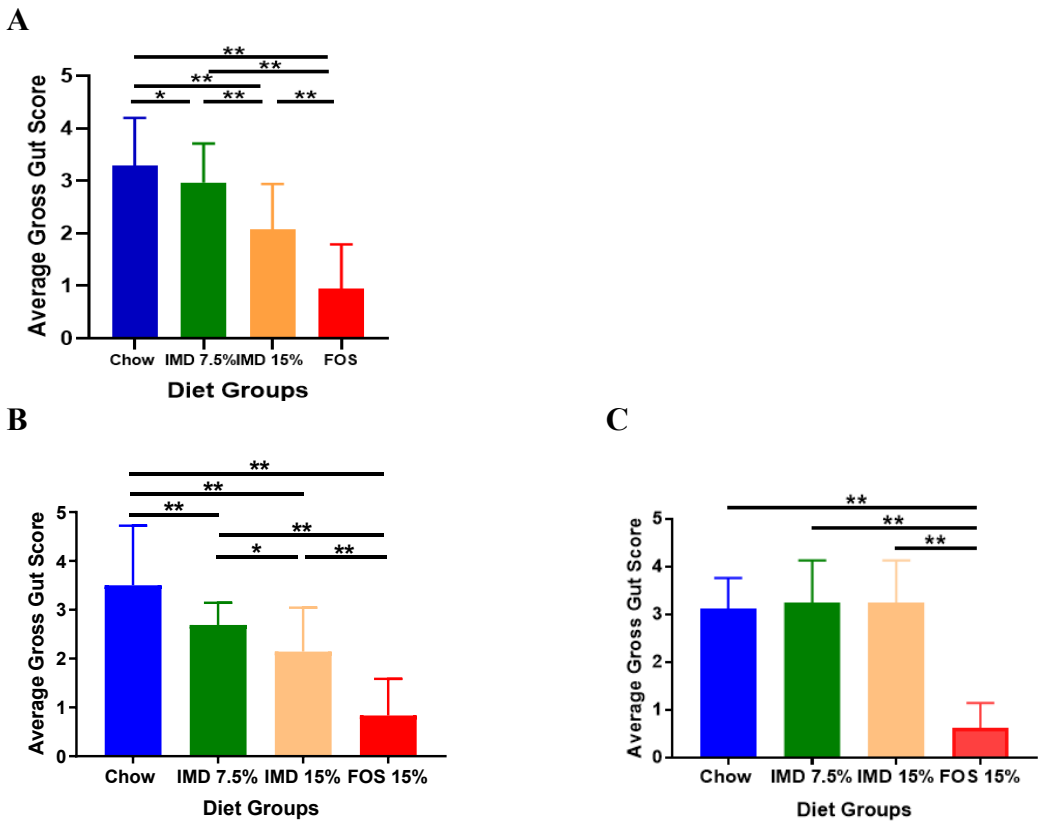


Figure 3. Effect of fiber treatments on cecal gross gut score (GGS) for (A) combined sexes (B) males and (C) females. Blue = Chow. Red = FOS 15%. Green = IMD 7.5%. Orange = IMD 15%. For Males, Chow n=6, IMD 7.5% n=8 and IMD 15% n=7, FOS 15% n=6. For females, Chow n=8, IMD 7.5% n=8 and IMD 15% n=8, FOS 15% n=8. Each rat's cecum was inspected grossly at necropsy by two individuals in a blinded fashion and scored on a scale of 0-4, with 4 being highest macroscopic inflammation. Data is presented as mean ± SD. Statistical significance was calculated between groups using ordinary one-way ANOVA and two-stage Benjamini, Krieger and Yekutieli test. *$q \leq 0.1$. ** $q \leq 0.05$

Figure 3B shows the comparison of average GGS across diet groups for males. Male rats in Chow group showed the highest average GGS (3.5 ± 0.5). Animals assigned to IMD 7.5% had significantly lower macroscopic inflammation when compared to the Chow control (2.7 ± 0.5, q=0.036). The higher dose of IMD 15% further reduced the GGS in males (2.1 ± 0.9, q=0.006).

ANOVA analysis also determined that IMD 15% diet was tentatively more effective in comparison to IMD 7.5% ($q=0.08$). Animals provided with FOS supplemented diet demonstrated very mild macroscopic cecal inflammation (0.8 ± 0.8, $q<0.0001$) in comparison to Chow control.

Figure 3C shows GGS for females compared across diet groups. For the females, no statistical differences for GGS were seen between Chow group (3.1 ± 0.6), IMD 7.5% (3.3 ± 0.9) and IMD 15% (3 ± 0.9) (Figure 2.3B). Only females randomized to FOS 15% group (1.1 ± 1) had statistically lower mean GGS than Chow ($q<0.0001$) (Figure 3C).

2.3.3. Effect of fiber treatments on cecum and colon IL-1β concentration

As GGS is a subjective measure of inflammation, concentrations of the inflammatory cytokine IL-1β and IFNγ in the tissues were measured as an additional objective marker of inflammation to validate the GGS findings. Figure 4A shows a comparison of cecal IL-1β concentrations (μg/g protein) for all animals assigned to the four diet groups. Cecal IL-1β concentrations for the combined sexes was highest in Chow group in comparison to IMD 7.5% group ($q=0.0853$) and IMD 15% ($q=0.0146$) (Figure 4A). Rats randomized to FOS 15% showed the lowest cecal IL-1β concentration versus the Chow control ($q=0.0005$) (Figure 4A).

Due to the sex-specific findings of a previously published study on the benefits of IMD in IL-10 knockout mice [122], as well as the differences seen between males and females' diet intakes and relative body weight gains (Figure 2), colon and cecum IL-1β concentrations were also assessed separately for each sex. Figure 4B shows cecal IL-1β concentrations in the male animals assigned to the four diet groups. Male rats randomized to Chow control demonstrated the highest IL-1β levels in cecum versus IMD 7.5% ($q=0.0650$) and IMD 15% ($q=0.0207$).

These findings support the GGS data observed for males showing the IMD anti-inflammatory effect in cecum. In contrast, females showed no significant differences in their cecal IL-1β concentrations between Chow and either IMD groups (Figure 4C). Only FOS was lower in comparison to Chow, though these differences were only tentatively significance

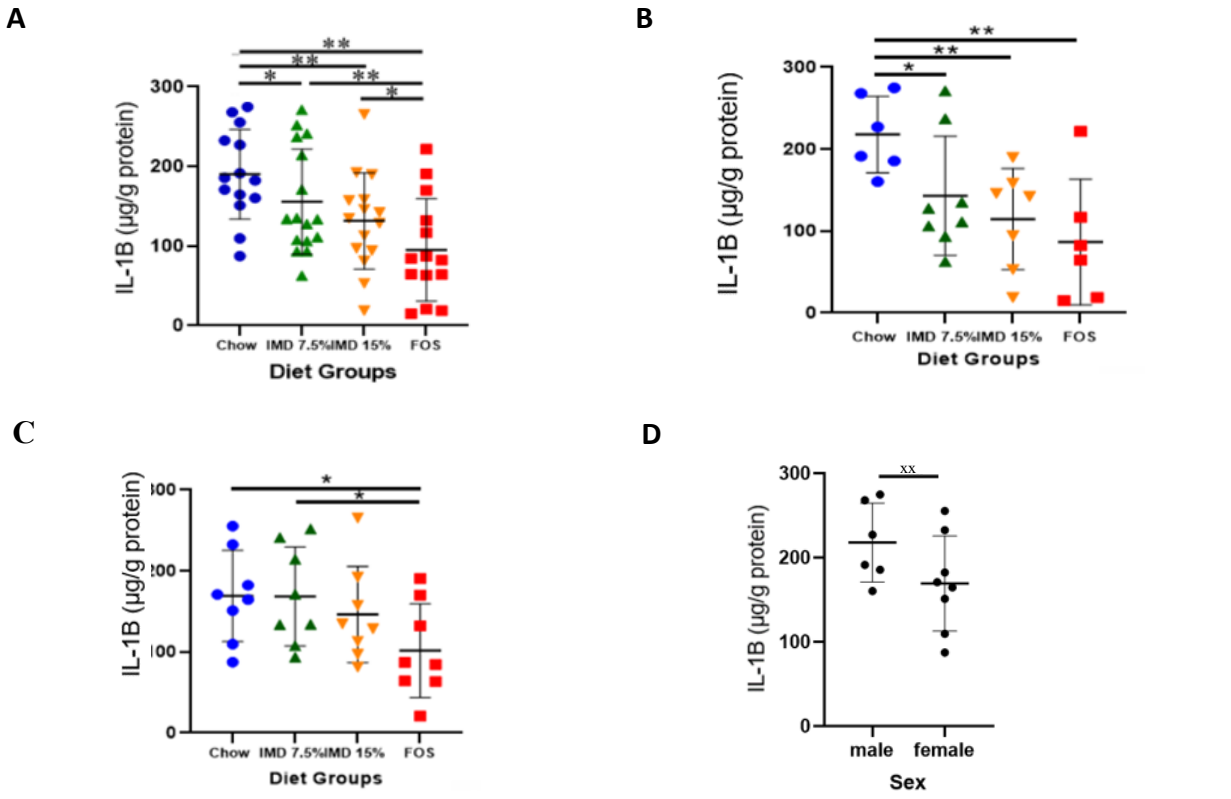


Figure 4. Effect of fiber treatments on mucosal IL-1β concentrations in the cecum for (A) combined sexes, (B) males, and (C) females, as well as (D) comparison of mucosal IL-1β concentrations in the cecum of chow males versus females. For Males, Chow n=6, FOS 15% n=6, IMD 7.5% n=8 and IMD 15% n=7. For females, Chow n=8, FOS 15% n=8, IMD 7.5% n=8 and IMD 15% n=8. IL-1β was assessed in tissue collected at 16 weeks (endpoint) using rat-specific IL-1β ELISA. Concentrations were normalized to total protein concentration. Data is presented as mean ± SD. Statistical significance was calculated between groups using ordinary one-way ANOVA or unpaired T-test and two-stage Benjamini, Krieger and Yekutieli test. *q ≤ 0.1 **q ≤0.05 ^{xx}p≤0.05

(q=0.0958) (Figure 4C). Males of Chow group appeared to have higher IL-1β concentrations than females and statistical analyses of the two groups revealed the mean IL-1β values for males fed Chow diet was significantly higher than females on the same diet (p=0.0313) (Figure 4D).

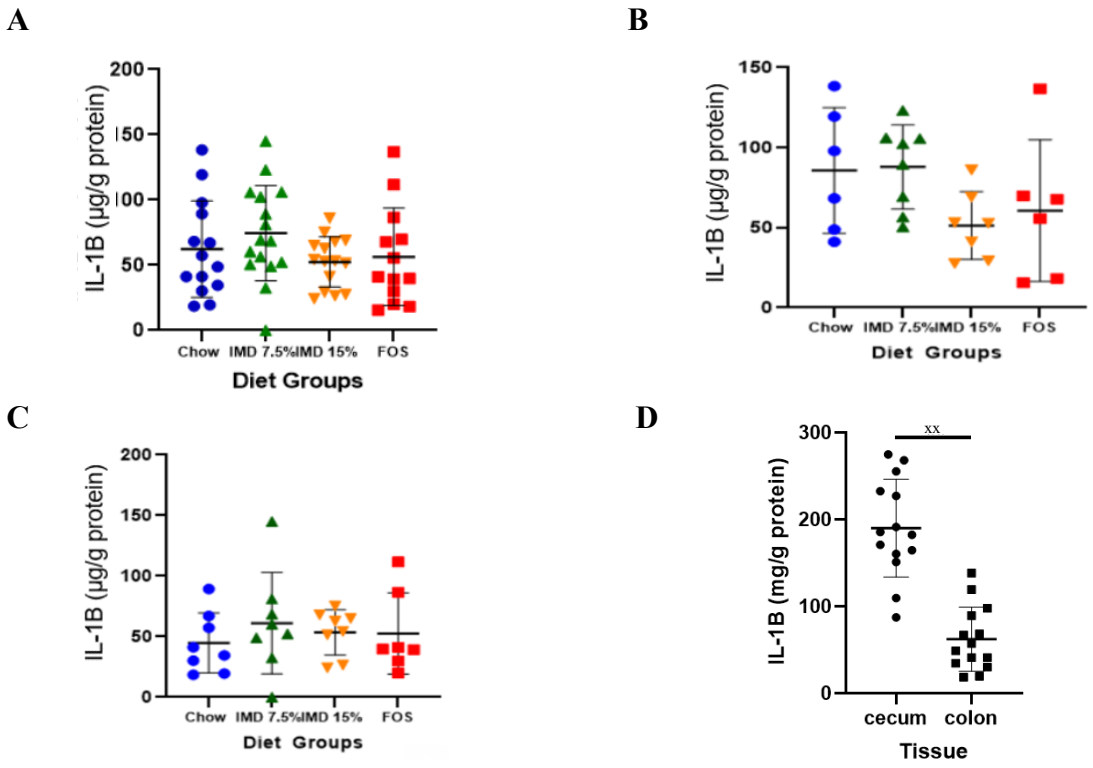


Figure 5. Effect of fiber treatments on mucosal IL-1β concentrations in the colon for (A) combined sexes, (B) males, and (C) females, as well as a (D) comparison of mucosal IL-1β concentrations in all chow cecum versus colon samples. For Males, Chow n=6, FOS 15% n=6, IMD 7.5% n=8 and IMD 15% n=7. For females, Chow n=8, FOS 15% n=8, IMD 7.5% n=8 and IMD 15% n=8. IL-1β was assessed in tissue collected at 16 weeks (endpoint) using rat-specific IL-1β ELISA. Concentrations were normalized to total protein concentration. Statistical significance was calculated between groups using unpaired T-test or ordinary one-way ANOVA and two-stage Benjamini, Krieger and Yekutieli test. *$q \leq 0.1$. ** $q \leq 0.05$ $^{xx}p \leq 0.05$

For females, 5 out of the 8 (62.5%) in the IMD 15% group had cecal IL-1β concentrations below that of the mean for males in IMD 15% group (109.97 µg/g protein). For the IMD 7.5% group, 4 out of the 8 females (50%) were below the mean cecal IL-1β concentrations of the males in IMD 7.5% (143.02 µg/g protein). Of the 4 females, 2 were cage-mates from the same little, while the other two were not. In the males, 2 out of 8 (25%) rats on IMD 7.5%, which were cage-mates, had higher cecal IL-1β concentration than the mean of the Chow group (217.91 µg/g protein). Considering the individual specific response, all following tests for the study combined the sexes prior to statistical analyses.

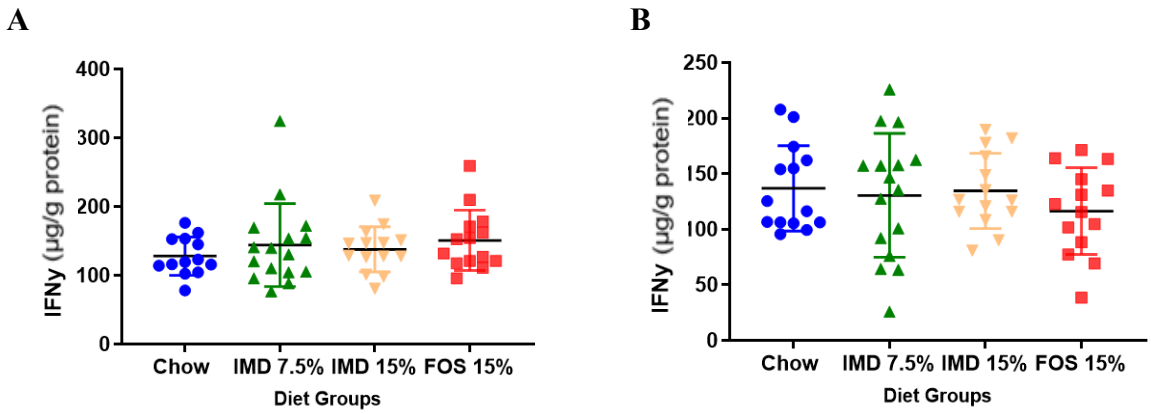


Figure 6. Effect of fiber treatments on mucosal IFNγ concentrations in the cecum (A) and colon (B) of combined sexes. For both graphs, Chow n=14, FOS 15% n=14, IMD 7.5% n=16 and IMD 15% n=15. IFNγ was assessed in tissue collected at 16 weeks (endpoint) using rat specific IFNγ ELISA. Concentrations were normalized to total protein concentration. Data is presented as mean ± SD. Statistical significance was calculated between groups using ordinary one-way ANOVA or unpaired T-test and two-stage Benjamini, Krieger and Yekutieli test. *$q \leq 0.1$. ** $q \leq 0.05$

No statistical significance was seen between diet groups for colonic IL-1β concentrations for either sex or when both sexes data was combined (Figure 5A-C). Paired-wise Wilcoxon test comparison of IL-1β concentrations in cecum versus colon tissue from Chow control animals revealed significantly less inflammation in colonic tissue in comparison to the matching cecal tissue ($p<0.0001$) (Figure 5D). Additionally, statistical significance was not seen across diet groups for cecal or colonic IFNγ concentrations (Figure 6).

2.3.4. Effect of fiber treatments on cecum and colon weight:length ratio

Figure 7A shows cecal weight (mg) to length (mm) ratio (mean ± SD). The lowest weight:length ratio was detected in IMD 15% group (3.20 ± 0.53), which was tentatively lower

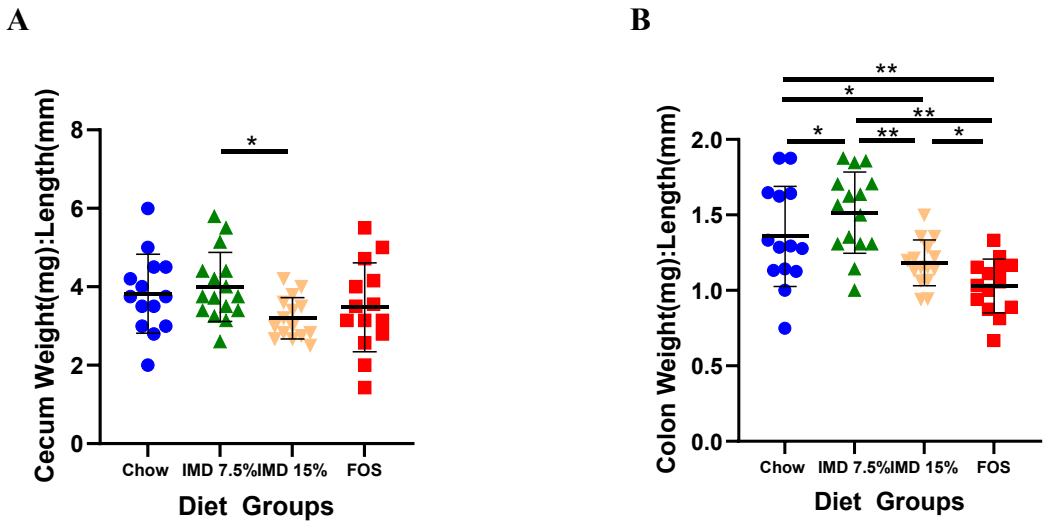


Figure 7. Effect of fiber treatments on weight(mg) and length(mm) ratio of cecum (A) and colon (B) of combined sexes. For both graphs, Chow n=14, FOS 15% n=14, IMD 7.5% n=16 and IMD 15% n=15. Data is presented as mean ± SD. Statistical significance was calculated between groups using ordinary one-way ANOVA and two-stage Benjamini, Krieger and Yekutieli test. *$q \leq 0.1$. ** $q \leq 0.05$

than IMD 7.5% (3.99 ± 0.88) (q =0.0759). No statistical significance was seen for Chow (3.82 ± 1.01) or FOS groups (3.48 ± 1.13) (Figure 7A). Figure 7B shows the colon weight:length ratios across all groups, where IMD 7.5% had the highest ratio (1.42 ± 0.46), which was significantly higher than IMD 15% (1.18 ± 0.53) (q=0.0056) and FOS (1.03 ± 0.18) (q<0.0001), and also tentatively higher than Chow (1.36 ± 0.33) (q=0.0928). IMD 15% group was also tentatively lower than Chow group (q=0.1000) (Figure 7B). FOS group had lowest colonic weight to length ratios (Figure 7B).

2.3.5. Effect of fiber treatments on bacterial composition in cecum and colon

A high throughput sequencing was performed in cecal and colon luminal content and community diversity (α- and β- diversity) as well as structure were assessed. A total of 9 bacterial phyla, 37 families and 84 genera were detected in cecal and colon samples.

Due to our previous findings of significantly higher IL-1β concentrations in Chow-fed males than females (Figure 4D), it was important to first examine if sex was also a variable affecting the cecal microbiota composition. A supervised multivariate analysis, partial least square discriminant analysis (PLS-DA) was used to assess the cecal and colon genera identified in males verses females of Chow control group. applied to the same set of microbiome data. This analysis successfully separated microbial communities associated with male and female sex (Figure 8A&C). The variable importance in projection (VIP) scores identified the cecal genera most significantly contributing to sample clustering (Figure 8B). Seven cecal genera (*Roseburia, Olsenella, Dorea, Blautia, Clostridium* XIVa, *Clostridium* XIVb and *Clostridium XI*) showed to

be significantly different between males and females; however, after correcting for false discovery rate, this significance was lost. Figure 8D shows the VIP scores identified for colon

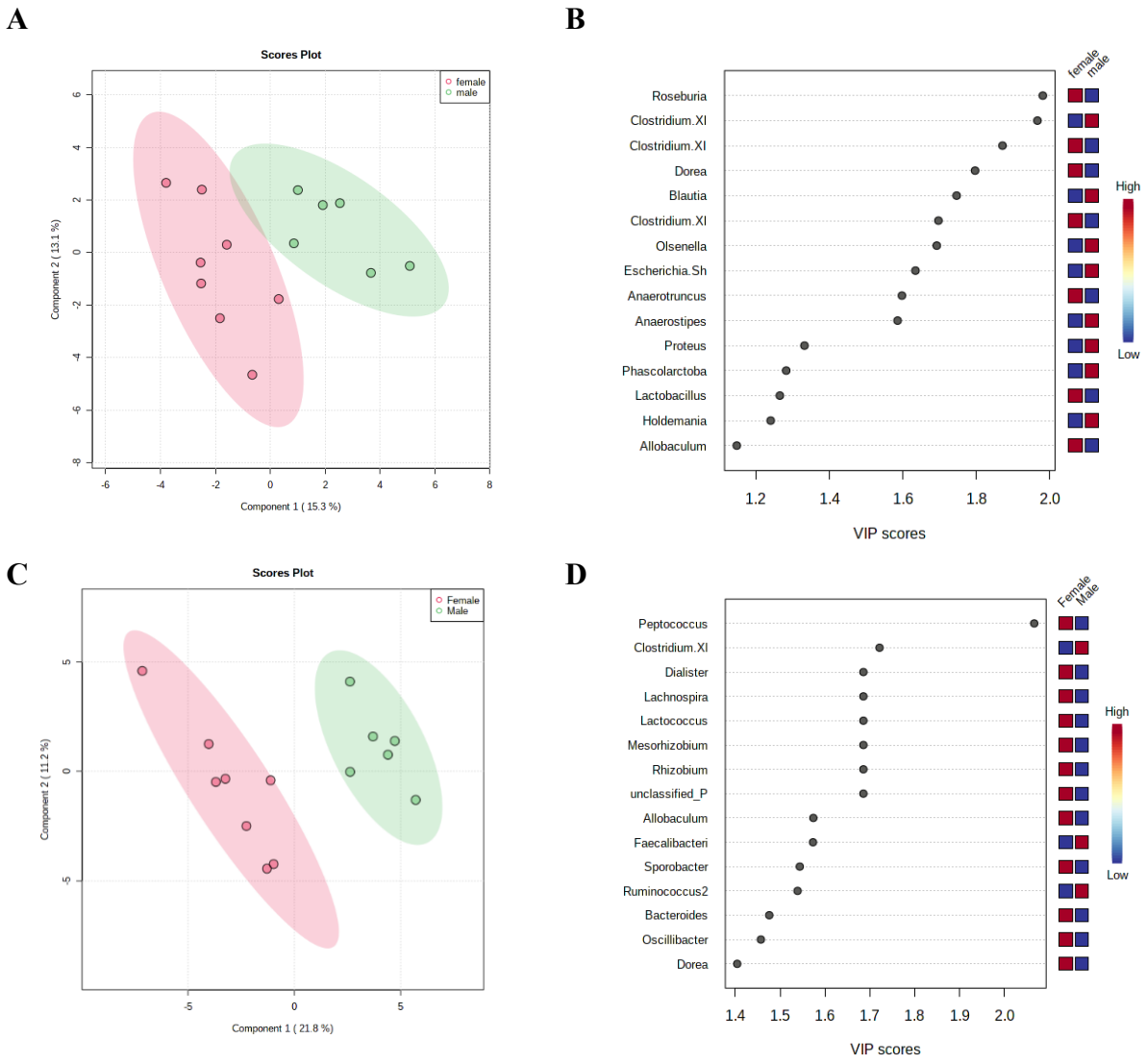


Figure 8. Chow group comparison of males vs females by partial least squares discriminant analysis (PLS-DA) of bacterial genera with displayed (A) 2D scores plot for cecum, (B) variable importance in projection (VIP) scores for cecum, (C) 2D scores plot for colon, and (D) VIP scores for colon. Males n=6 and females = 8. Bacterial composition was assessed by 16S rRNA gene sequencing (Illumina MiSeq platform). Relative abundance data was normalized using log transformation and auto scaling.

genera that most significantly contributed to sample clustering. Fifteen colon genera (*Peptococcus, Clostridium* XIVa, *Dialister, Lachnospiraceae, Lactococcus, Mesorhizobium, Rhizobium,* unclassified *Phyllobacteriaceae, Allobaculum, Faecalibacterium, Sporobacter, Ruminococcus2, Bacteroides, Oscillibacter, Dorea*) showed to be significantly different between males and females; however, once analyses were corrected for false discovery rate, this significance was lost. This lack of significant difference between the microbiotas of males and females suggests that the differences seen in inflammation between the sexes may be due to mechanisms not associated with the microbiota.

Alpha diversity described with Simpson Diversity Index was calculated for the combined sexes with metrics including the number of observed OTUs characterizing the richness and Pielou Evenness Index characterizing the evenness of species diversity. Overall, IMD diets were not associated with changes in cecal α-diversity (as Simpson Index) in comparison to Chow control (Figure 9A), nor differences in Pielou Evenness Index (Figure 9C). Only, IMD 15% was characterized with significantly lower number of observed OTUs ($q=0.0011$) versus Chow control (Figure 9B). Interestingly, FOS 15% showed significantly lower richness and evenness in comparison to Chow control. In the colon, IMD diets were again not associated with α-diversity changes (Simpson Index) in comparison to Chow control (Figure 9D), as well as differences in Pielou Evenness Index (Figure 9F). In the colon, there was significantly lower number of observed OTUs in both IMD 7.5% ($q=0.0154$) and IMD 15% ($q<0.0001$) compared to Chow control (Figure 9E). FOS again showed significantly lower richness in comparison to Chow in the colon.

Finally, in the cecum, no significant differences associated with IMD diets were seen by Bray Curtis dissimilarity, a beta-diversity index where a value of 1 represents completely

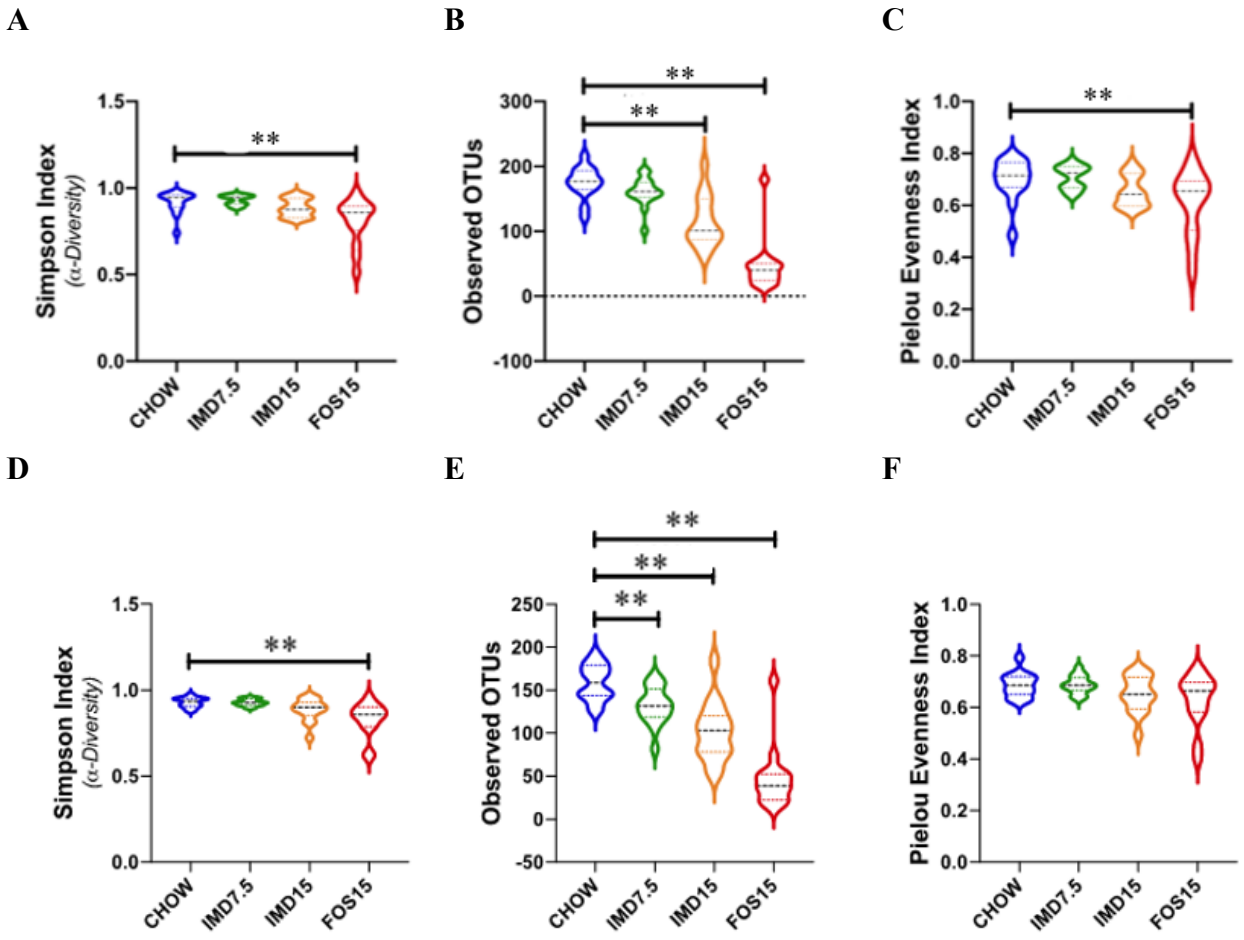


Figure 9. Alpha diversity metrics measured for cecum as (A) Simpson Index, (B) Observed OTUs, (C) Pielou Evenness Index, and colon as (D) Simpson Index, (E) Observed OTUs, (F) Pielou Evenness Index. Blue = Chow. Red = FOS 15%. Green = IMD 7.5%. Orange = IMD 15%. For all graphs, Chow n=14, FOS 15% n=14, IMD 7.5% n=16 and IMD 15% n=15. Data is represented as median ± inter-quartile range. Statistical significance was calculated between diet groups using non-parametric one-way ANOVA (Kruskal-Wallis test) and two-stage Benjamini, Krieger and Yekutieli test. *$q \leq 0.1$. ** $q \leq 0.05$

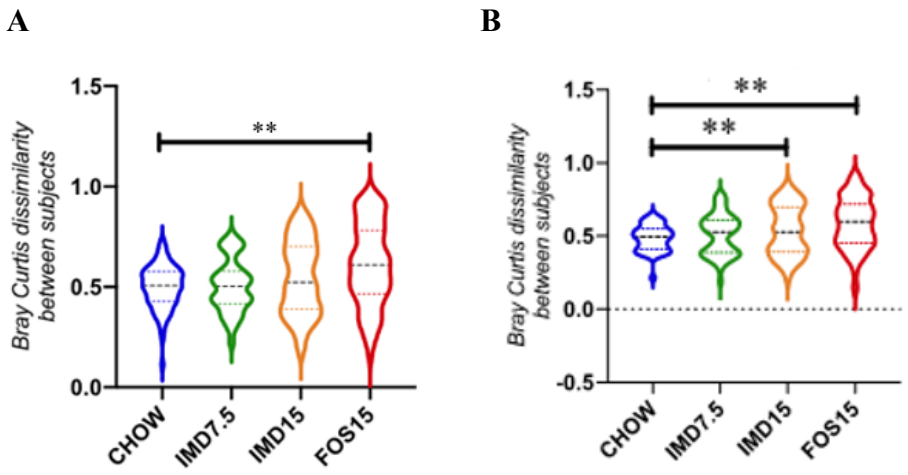


Figure 10. Beta diversity metrics measured for (A) cecum and (B) colon, as Bray Curtis dissimilarity. Blue = Chow. Red = FOS 15%. Green = IMD 7.5%. Orange = IMD 15%. For all graphs, Chow n=14, FOS 15% n=14, IMD 7.5% n=16 and IMD 15% n=15. Data is represented as a violin plot showing median and inter-quartile ranges. Statistical significance was calculated between diet groups using non-parametric one-way ANOVA (Kruskal-Wallis test) and two-stage Benjamini, Krieger and Yekutieli test. *$q \leq 0.1$. ** $q \leq 0.05$

dissimilar communities (Figure 10A). In the colon, IMD 15% showed significantly lower β-diversity than Chow control group (q=0.0055) (Figure 10B).

To determine which specific microbial groups were significantly altered by IMD treatment, one-way ANOVAs with Kruskal-Wallis test were performed at each taxonomic level comparing IMD 7.5%, IMD 15% and FOS to the negative control Chow group, in both cecum and colon samples (Table 1&2). At the phyla level in the cecum, FOS 15% treatment promoted significant increase in Actinobacteria (q<0.0001) at the expense of reduction in Firmicutes (q=0.0012) and Bacteroidetes (q<0.0001) relative abundances (Figure 11A-C). A shift in cecal Bacteroidetes relative abundance was seen only in FOS group and was not replicated in either

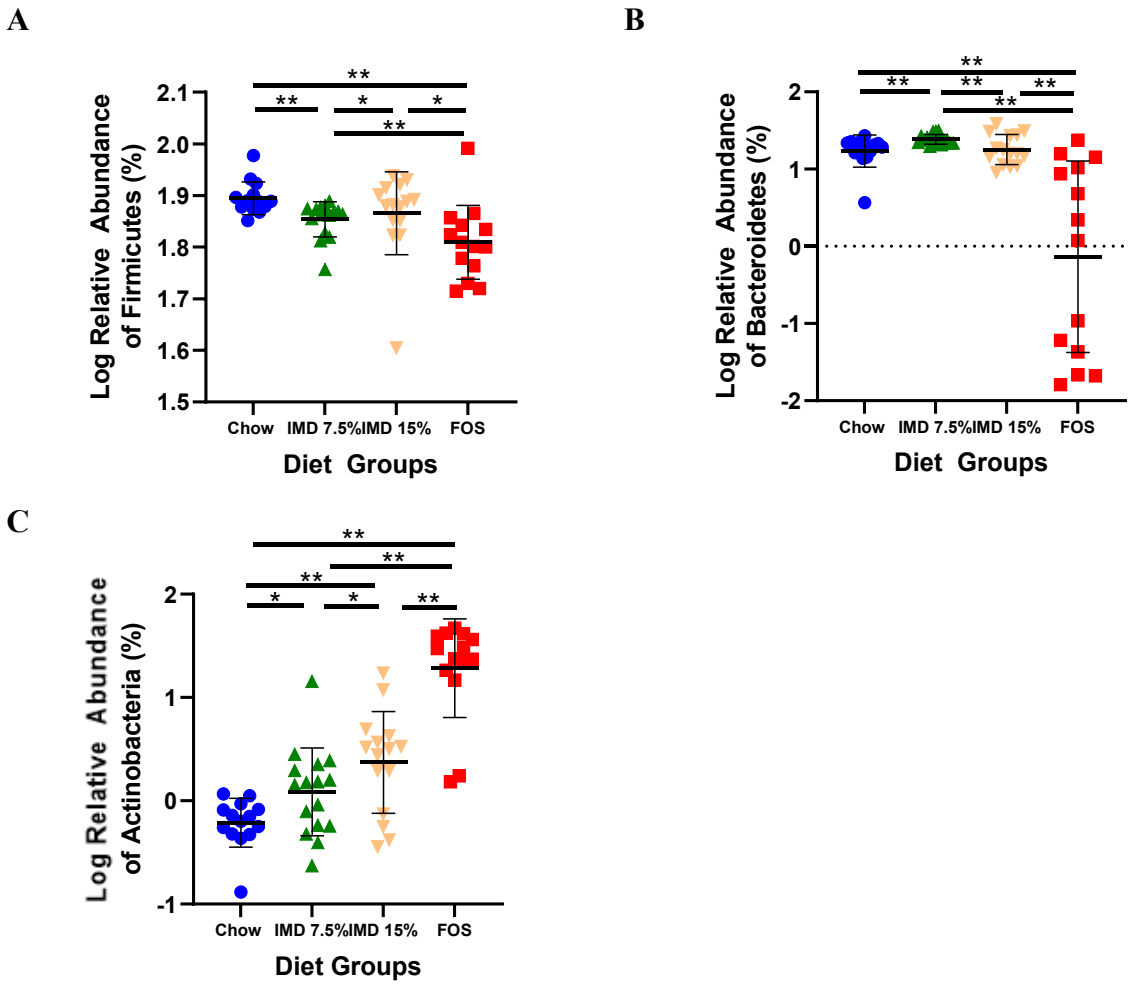


Figure 11 Effect of fiber treatments on cecal relative abundances of bacterial phylum (A) Firmicutes, (B) Bacteroidetes and (C) Actinobacteria. Blue = Chow. Red = FOS 15%. Green = IMD 7.5%. Orange = IMD 15%. For all graphs, Chow n=14, FOS 15% n=14, IMD 7.5% n=16 and IMD 15% n=15. Bacterial composition was assessed by 16S rRNA gene sequencing (Illumina MiSeq platform). Data is presented as relative abundances (%). Statistical significance was calculated between diet groups using non-parametric one-way ANOVA (Kruskal-Wallis test) and two-stage Benjamini, Krieger and Yekutieli test. *$q \leq 0.1$. ** $q \leq 0.05$

IMD groups. The IMD diets showed divergent effect on cecal microbiota composition at phylum level. For instance, IMD 7.5% was characterized with lower cecal Firmicutes (q=0.0633) versus

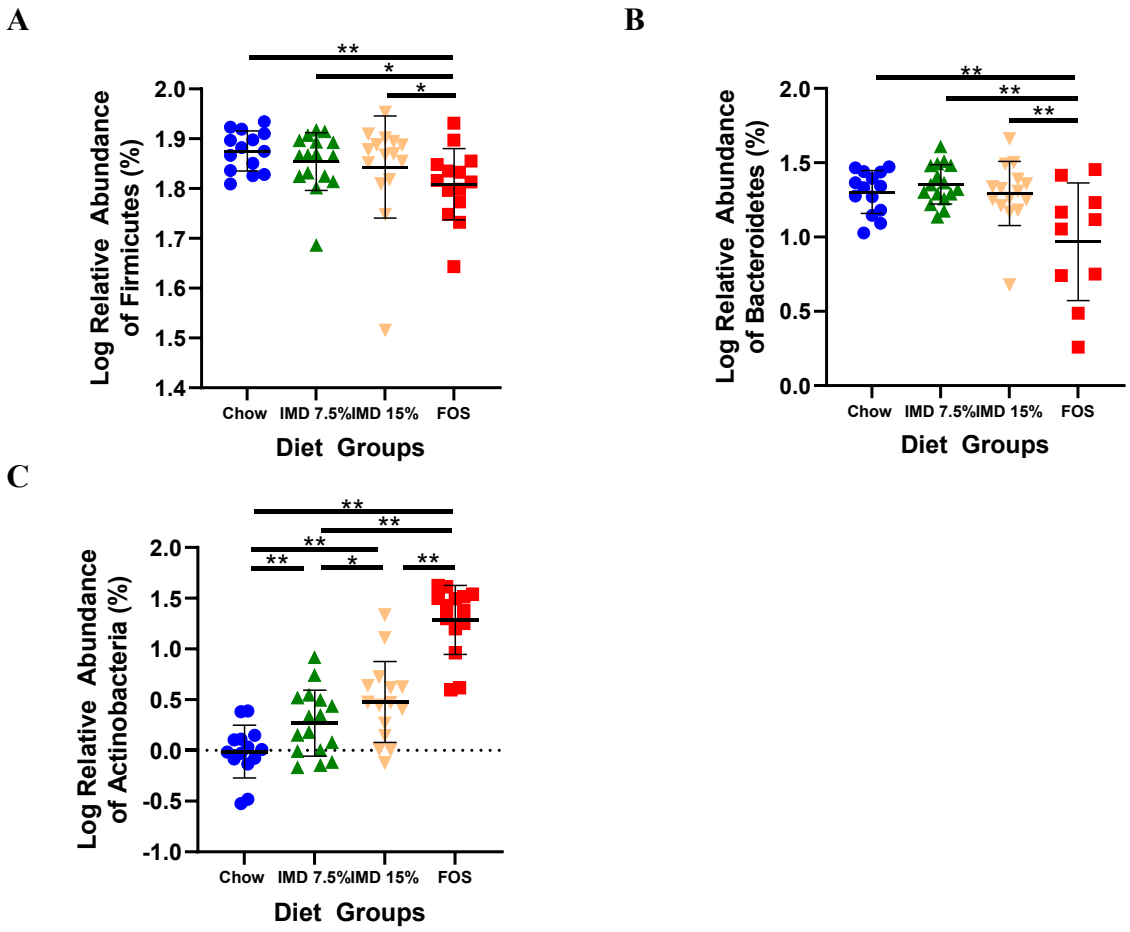


Figure 12 Effect of fiber treatments on Stool relative abundances of bacterial phylum (A) Firmicutes, (B) Bacteroidetes and (C) Actinobacteria. Blue = Chow. Red = FOS 15%. Green = IMD 7.5%. Orange = IMD 15%. For all graphs, Chow n=14, FOS 15% n=14, IMD 7.5% n=16 and IMD 15% n=15. Bacterial composition was assessed by 16S rRNA gene sequencing (Illumina MiSeq platform). Data is presented as relative abundances (%). Statistical significance was calculated between diet groups using non-parametric one-way ANOVA (Kruskal-Wallis test) and two-stage Benjamini, Krieger and Yekutieli test. *$q \leq 0.1$. ** $q \leq 0.05$

higher Actinobacteria (q=0.0227) relative abundances in comparison to Chow control (Figure 11A,C) (Table 2). In contrast IMD 15% showed a stronger significant increase in cecal Actinobacteria (q=0.0002) versus Chow control, with no difference seen for Firmicutes (Figure

11A,C). In the colon, at the phyla level, again FOS was associated with a significant increase in Actinobacteria ($q<0.0001$) at the expense of reduced Firmicutes ($q=0.0246$) and Bacteroidetes ($q=0.0448$) relative abundances (Figure 12A-C). A shift in Bacteroidetes and Firmicutes was only seen in FOS group in the colon (Figure 12A-C). Actinobacteria relative abundance in the colon was significantly higher in both IMD 7.5% ($q=0.0459$) and IMD 15% ($q=0.0030$) in comparison to Chow, and IMD 15% was tentatively more effective than IMD 7.5% ($q=0.0763$) (Figure 12C). Finally, no statistical differences were seen in the cecum and colon between groups for the phyla Proteobacteria, Tenericutes, Verrucomicrobia and unclassified Bacteria.

To determine which cecal and fecal microbial groups have the biggest contribution to IMD supplementation, multivariate supervised PLS-DA analyses were performed on Chow, IMD 7.5% and IMD 15% groups (Figures 13). The multivariate analyses by ANOVA with Kruskal-Wallist test showed separation between the three dietary groups for both cecum and colon samples (Figure 13A&C). VIP scores for cecum samples revealed the genera: *Parabacteroides, Clostridium* IV, *Acetanaerobacterium, Odoribacter, Bifidobacterium,* unclassified *Prevotella, Alistipes, Prevotella, Oscillibacter, Enterococcus, Bilophila, Olsenella, Lactobacillus, Streptococcus, Eubacteirum* (Figure 13B). In stool, VIP scores revealed the genera: *Clostridium* IV, *Alistipes, Parabacteroides, Bifidobacterium, Lactococcus, Mesorhizobium,* unclassified *Coriobacteriaceae, Escherichia Shigella,* unclassified Others, *Aerococcus, Papillibacter, Proteus, Odoribacter, Acetanaerobacterium,* unclassified *Porphyromonadaceae* (Figure 13D).

Figure 14 and 15 shows average relative abundances of cecal and fecal bacterial families that showed statistically significant differences by ANOVA across fiber treatment groups (Table

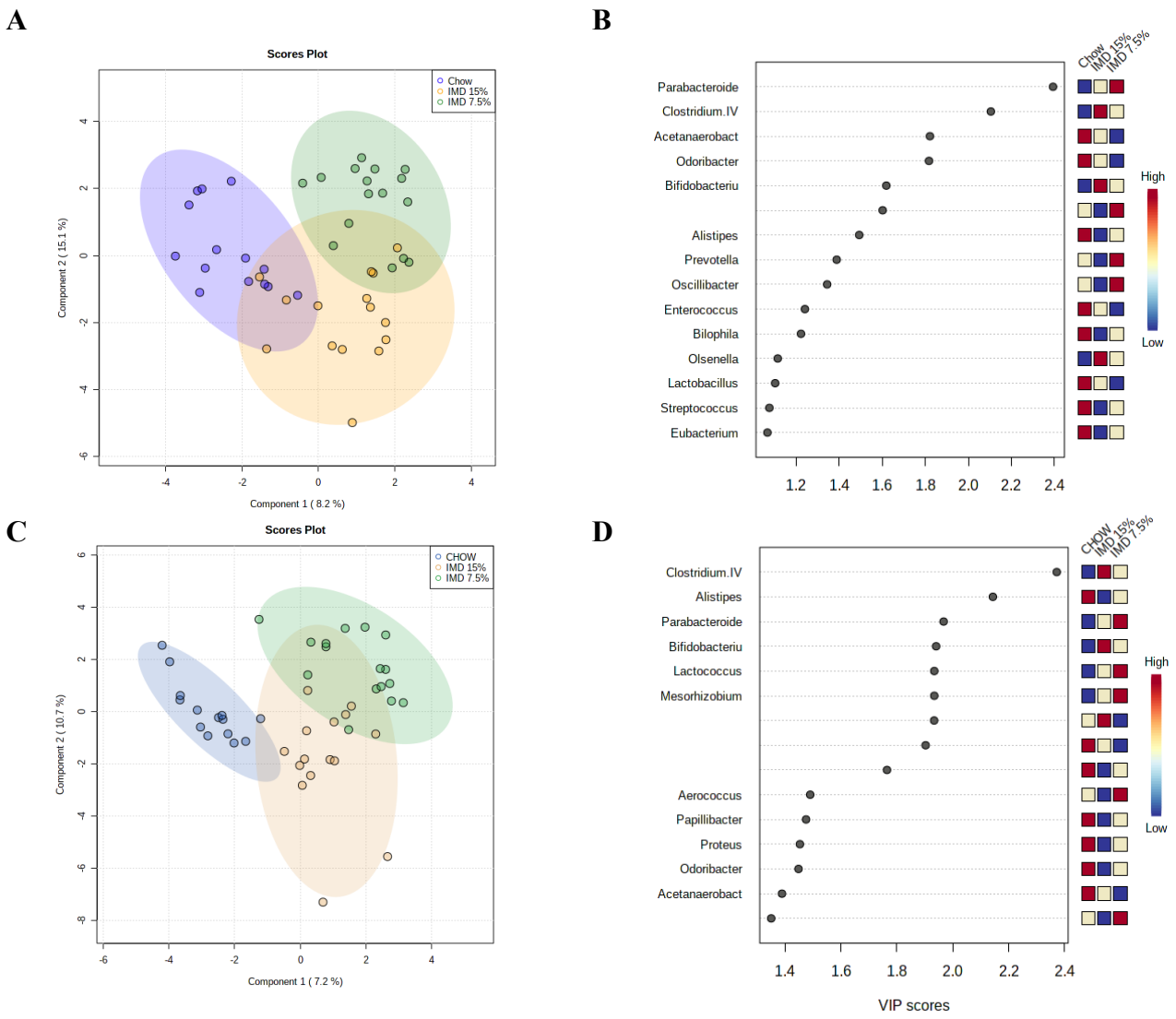


Figure 13. Comparison of Chow, IMD 7.5% and IMD 15% groups by partial least squares discriminant analysis (PLSDA) of cecal genera with displayed (A) 2D scores plot, (B) variable importance in projection (VIP) scores, as well as colonic genera with displayed (C) 2D scores plot, (D) VIP scores. Chow n=14, IMD 7.5% n=16 and IMD 15% n=15. Bacterial composition was assessed by 16S rRNA gene sequencing. Relative abundance data was normalized using log transformation and auto scaling.

1&2). Cecal *Bacteroidaceae* average relative abundance was significantly higher in IMD 15% than Chow (q=0.0477) (Figure 14A). Interestingly, FOS 15% group showed lower average relative abundance of cecal *Bacteroidaceae* than Chow (q=0.0125) (Figure 14A). Three cecal bacterial families showed a decreased relative abundance associated with IMD and FOS treatments. Cecal *Eubacteriaceae* average relative abundance was highest in Chow group and was significantly lower in IMD 7.5% (q=0.0213), IMD 15% (q=0.0061) and FOS 15% (q=0.0015) (Figure 14B). Cecal *Peptostreptococcaceae* average relative abundance was highest in Chow group and was significantly lower in IMD 15% (q=0.0016) and FOS 15% (q<0.0001). IMD 7.5% also showed tentatively lower *Peptostreptococcaceae* relative abundance versus Chow (q=0.0999) (Figure 14C). Cecal *Rikenellaceae* average relative abundance was the highest in Chow group and was significantly reduced in IMD 7.5% (q=0.0150), IMD 15% (q<0.0001) and FOS 15% (q<0.0001) (Figure 14D). Fecal *Bifidobacteriaceae* relative abundance was lowest in Chow group, which was significantly lower than all other group, IMD 7.5% (q=0.0214), IMD 15% (q=0.0154) and FOS (q<0.0001) (Figure 15A). Fecal *Lactobacilliaceae* relative abundance was significantly lower in IMD 15% than Chow control (q=0.0044) (Figure 15B). In comparison to Chow, fecal *Porphyromonadaceae* was significantly higher in IMD 7.5% (q=0.0190), unchanged in IMD 15% and lowered in FOS (q=0.0043) (Figure 15C). *Rikenellaceae* relative abundance in the stool was highest in Chow, which was significantly higher than all groups, IMD 7.5% (q=0.0082), IMD 15% (q<0.0001), and FOS (q<0.0001), with IMD 15% also being significantly more effective in reducing *Rikenellaceae* than IMD 7.5% (q=0.0472) (Figure 15D). unclassified *Bacteroidales* relative abundance for the colon was highest in Chow group compared to all groups, IMD 7.5% (q=0.0254), IMD 15% (q=0.0003),

and FOS (q<0.0001), with IMD 15% being significantly more effective than IMD 7.5% (q=0.0302) (Figure 15E). Fecal unclassified *Lactobacilliaceae* relative abundance was highest in

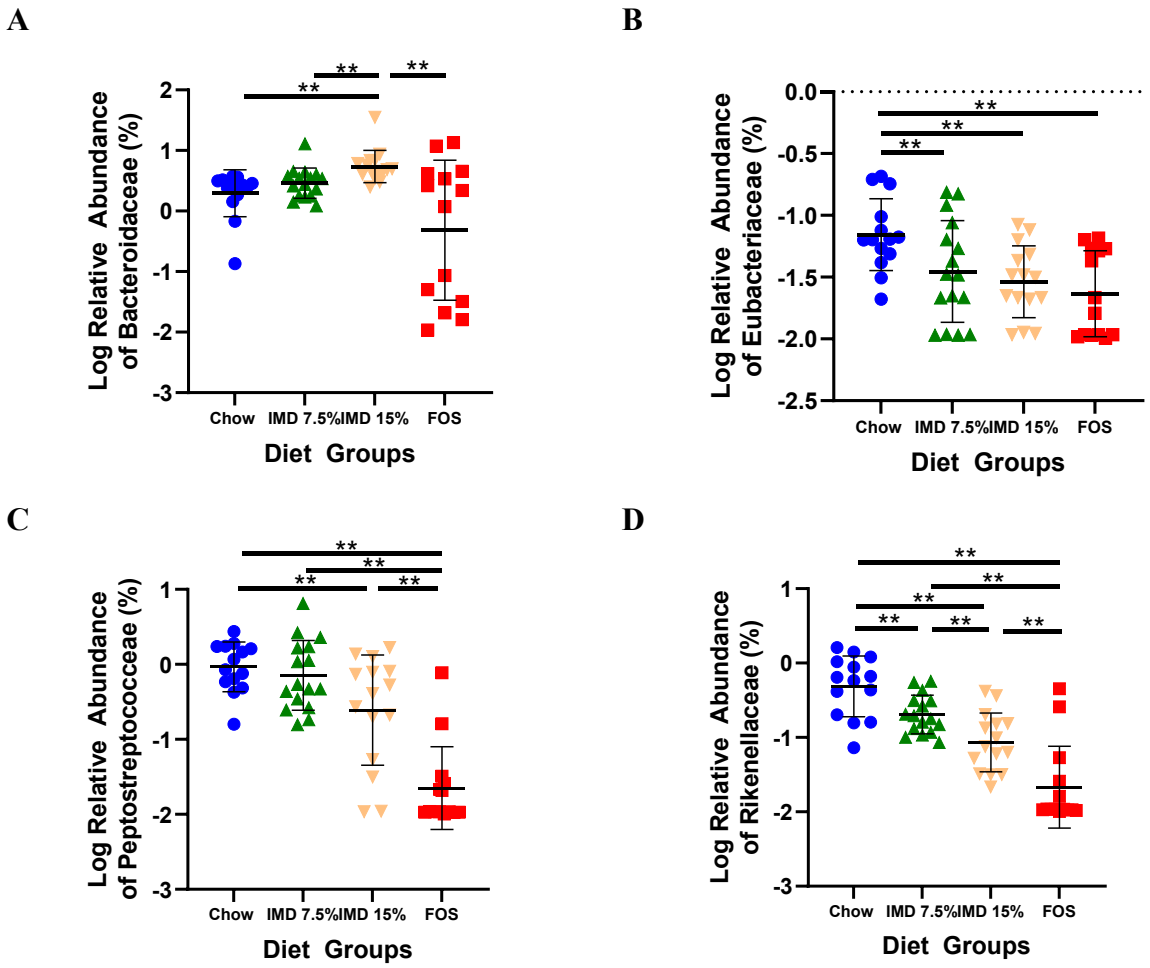


Figure 14. Effect of fiber treatments on cecal relative abundances of bacterial families (A) Bacteriodaceae, (B) Eubacteriaceae, (C) Peptostreptococcaceae and (D) Rikenellaceae. Blue = Chow. Red = FOS 15%. Green = IMD 7.5%. Orange = IMD 15%. For all graphs, Chow n=14, FOS 15% n=14, IMD 7.5% n=16 and IMD 15% n=15. Bacterial composition was assessed by 16S rRNA gene sequencing. Data is presented as log transformed relative abundances. Statistical significance was calculated between diet groups using non-parametric one-way ANOVA (Kruskal-Wallis test) and two-stage Benjamini, Krieger and Yekutieli test. *q ≤ 0.1. ** q ≤ 0.05

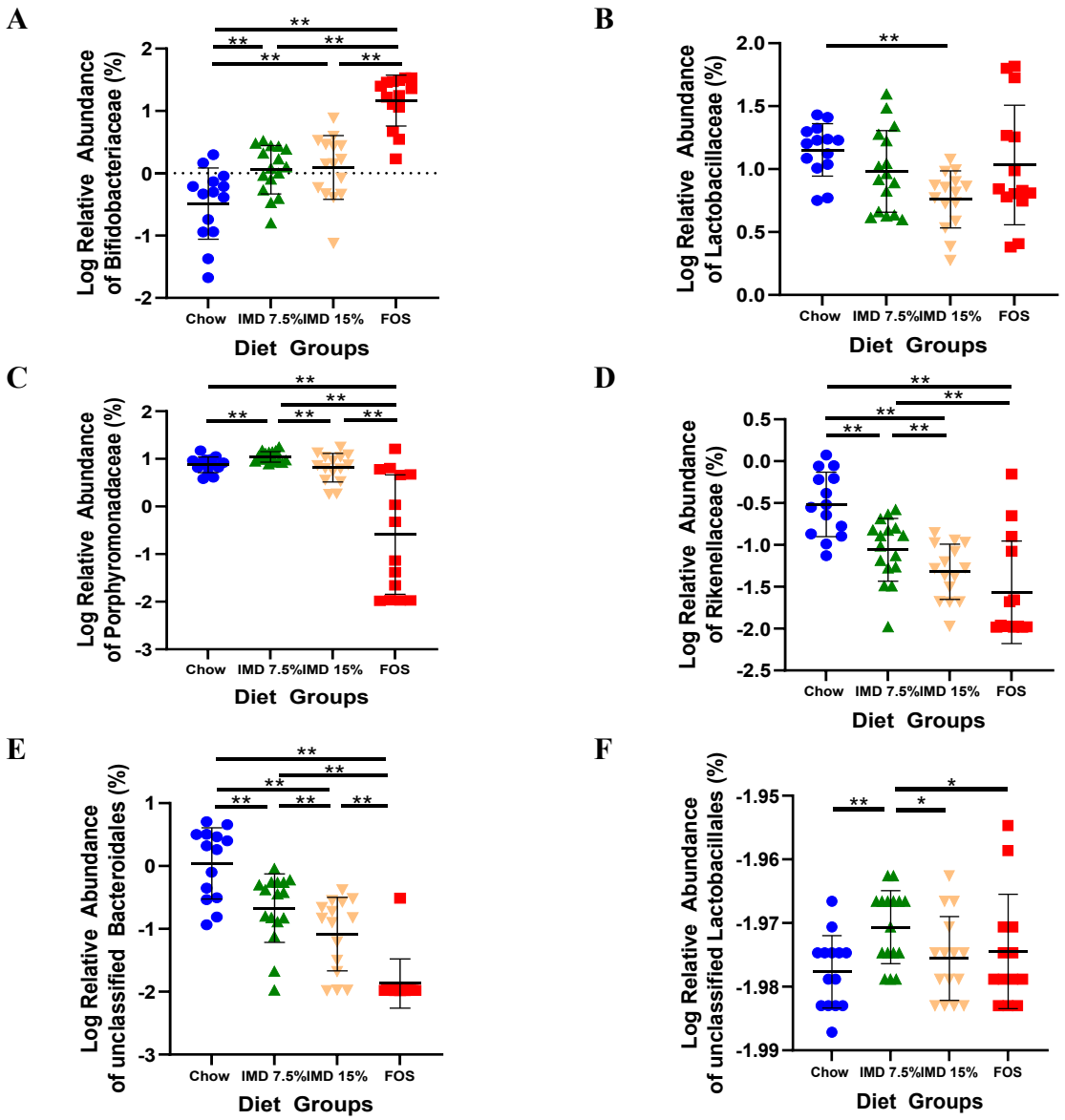


Figure 15. Effect of fiber treatments on stool relative abundances of bacterial families (A) Bifidobacteriaceae, (B) Lactobacilliaceae, (C) Porphyromonadaceae, (D) Rikenellaceae, (E) unclassified Bacteroidales, (F) unclassified Lactobacillales . Blue = Chow. Red = FOS 15%. Green = IMD 7.5%. Orange = IMD 15%. For all graphs, Chow n=14, FOS 15% n=14, IMD 7.5% n=16 and IMD 15% n=15. Bacterial composition was assessed by 16S rRNA gene sequencing. Data is presented as log transformed relative abundances. Statistical significance was calculated between diet groups using non-parametric one-way ANOVA (Kruskal-Wallis test) and two-stage Benjamini, Krieger and Yekutieli test. *$q \leq 0.1$. ** $q \leq 0.05$

IMD 7.5% group, which was significantly higher than Chow (q=0.0309) and tentatively higher than IMD 15% (q=0.0987), with no difference seen between IMD 15% and Chow (Figure 15F).

Figures 16-18 show the comparisons between diet groups at the genera level the cecum (Table 1). Figure 16 shows the cecal microbial groups that were dose dependently reduced by IMD diets compared to Chow control. Contrastingly, Figure 17 shows the cecal genera that were dose-dependently enhanced by IMD treatment versus Chow. Finally, Figure 18 shows the cecal genera that were only significant in IMD 7.5% but not in IMD 15% in comparison to Chow group. Figure 19 shows all fecal genera that were altered between diet groups.

Out of the 9 cecal genera that showed a reduction in IMD 15% group, 6 had highest relative abundance in the Chow group. *Alistipes*, *Peptococcus*, unclassified *Erysipelotrichaceae* and unclassified *Ruminococcaceae* showed highest average cecal relative abundance in Chow control group, which was significantly higher than all other treatments, IMD 7.5%, IMD 15% and FOS (Figures 16A,E,G,I). In all mentioned cecal genera, IMD 15% group showed either a significant, or tentative, reduction in abundance in comparison to IMD 7.5% group (Figures 16A,E,G,I). *Barnesiella* and *Clostridium* XIVb also were of highest abundance in the Chow fed group, which was significantly higher than IMD 15% and FOS; however, no difference was seen between Chow and IMD 7.5%, but significance was seen between IMD 7.5% and IMD 15% (Figure 16B,C). Of the 9 cecal genera that had a significantly lower relative abundance in IMD 15% versus Chow, 3 showed highest abundance in IMD 7.5% group. *Flavonifractor*, *Ruminococcus* and unclassified Prevotellaceae had highest relative abundance in the IMD 7.5% group, with Chow group showing second highest abundance (Figure 16D,F,H). For all three genera, both Chow and IMD 7.5% groups showed statistically higher abundance in comparison to both IMD 15% and FOS groups (Figure 16D,F,H).

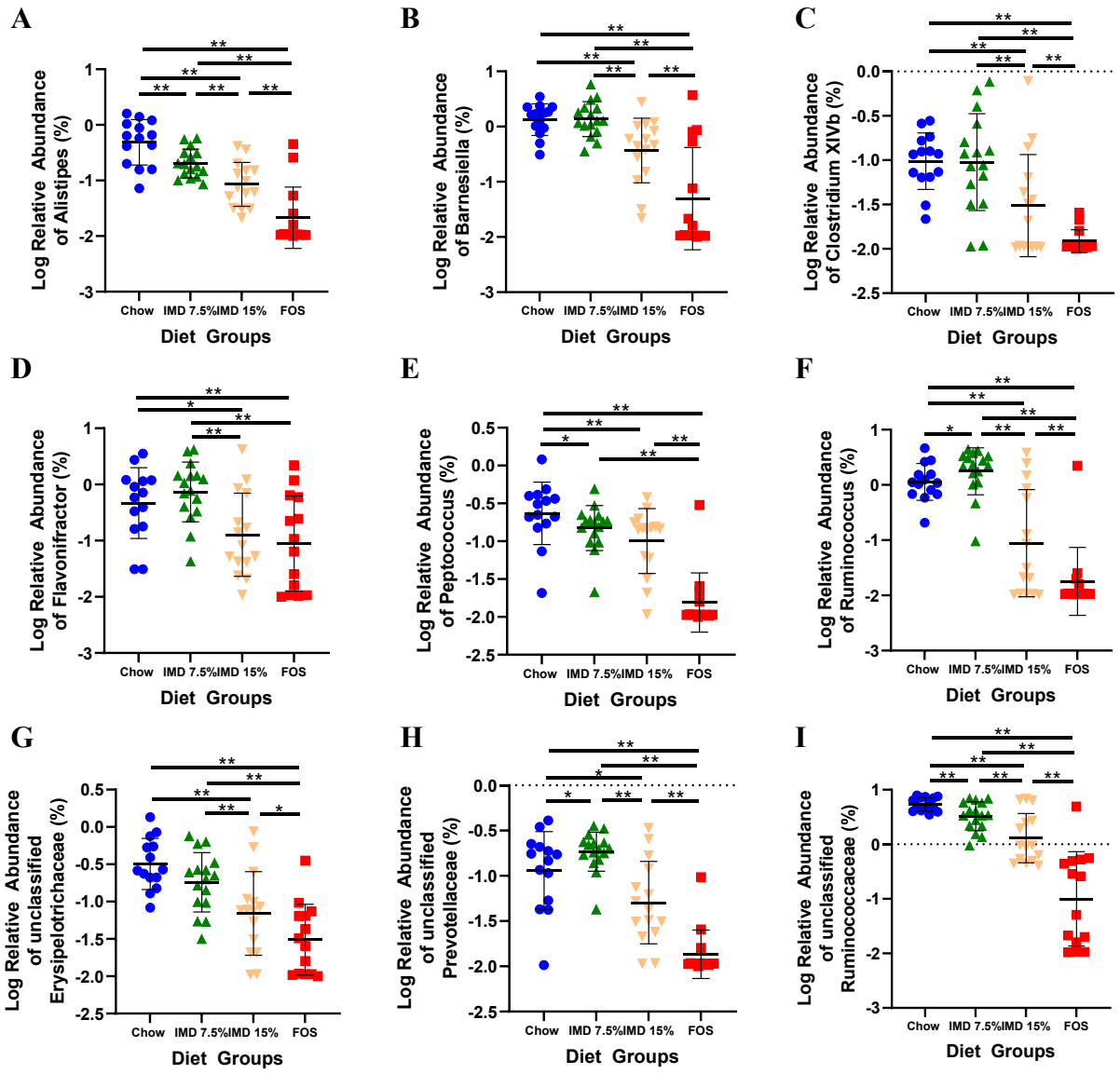


Figure 16. Effect of fiber treatments on cecal bacterial genera relative abundance where IMD 15% showed lower abundance than Chow in (A) Alistipes, (B) Barnesiella, (C) Clostridium XIVb, (D) Flavonifractor, (E) Peptococcus, (F) Ruminococcus, (G) unclassified Erysipelotrichaceae, (H) unclassified Prevotellaceae and (I) unclassified Ruminococcaceae. Blue = Chow. Red = FOS 15%. Green = IMD 7.5%. Orange = IMD 15%. For all graphs, Chow n=14, FOS 15% n=14, IMD 7.5% n=16 and IMD 15% n=15. Bacterial composition was assessed by 16S rRNA gene sequencing (Illumina MiSeq platform). Data is presented as relative abundances. Statistical significance was calculated between diet groups using non-parametric one-way ANOVA (Kruskal-Wallis test) and two-stage Benjamini, Krieger and Yekutieli test. *q $\leq$ 0.1. ** q $\leq$ 0.05

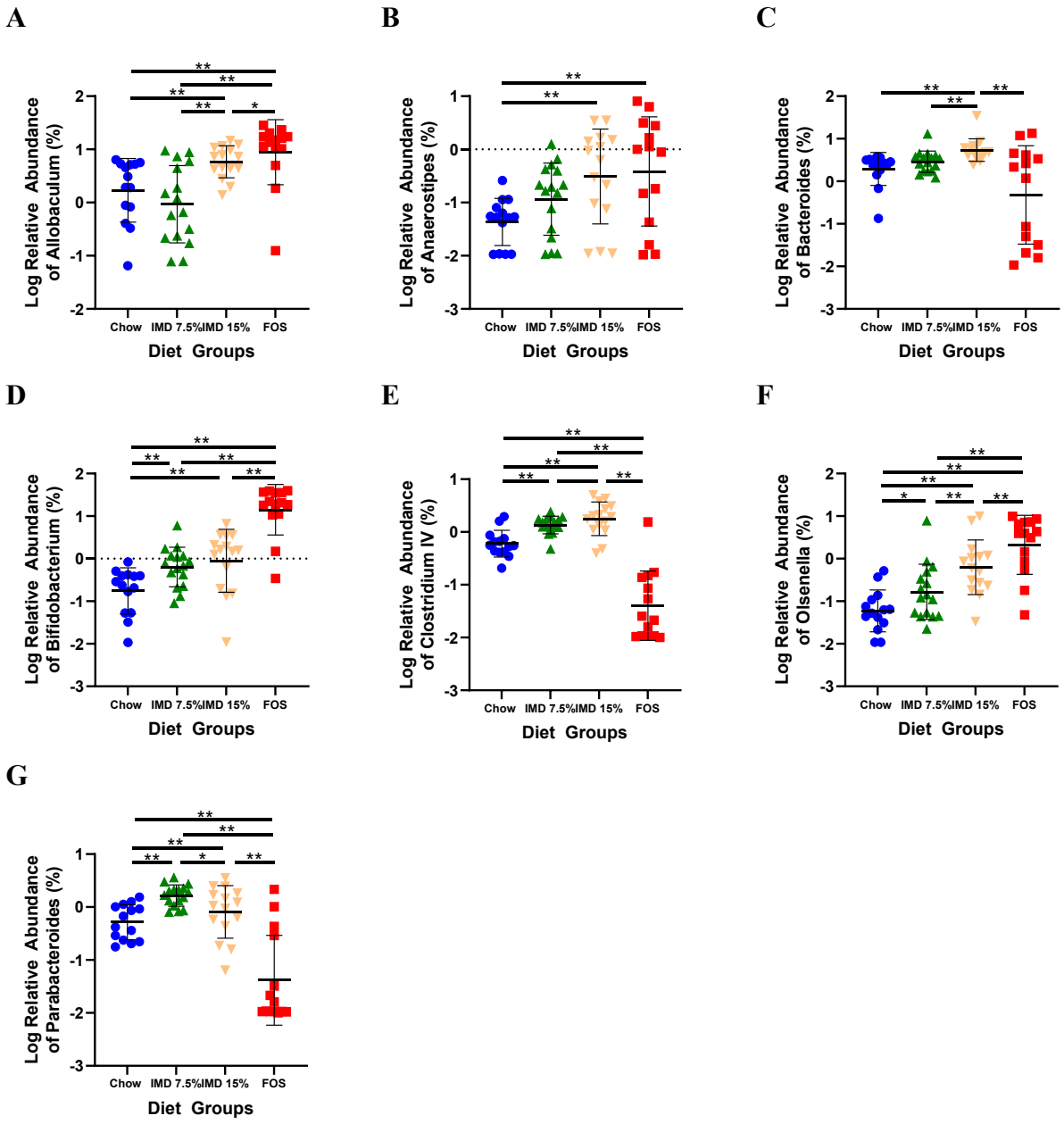


Figure 17. Effect of fiber treatments on cecal bacterial genera relative abundance where IMD 15% showed higher abundance than Chow in (A) Allobaculum, (B) Anaerostipes, (C) Bacteroides, (D) Bifidobacterium, (E) Clostridium IV, (F) Olsenella and (G) Parabacteroides. Blue = Chow. Red = FOS 15%. Green = IMD 7.5%. Orange = IMD 15%. For all graphs, Chow n=14, FOS 15% n=14, IMD 7.5% n=16 and IMD 15% n=15. Bacterial composition was assessed by 16S rRNA gene sequencing (Illumina MiSeq platform). Data is presented as log transformed relative abundances. Statistical significance was calculated between

diet groups using non-parametric one-way ANOVA (Kruskal-Wallis test) and two-stage Benjamini, Krieger and Yekutieli test. *$q \leq 0.1$. ** $q \leq 0.05$

Out of the 7 cecal genera that showed an increase in relative abundance for IMD 15% group in comparison to Chow, 3 showed lowest relative abundance in the Chow control group. *Anaerostipes*, *Bifidobacterium* and *Olsenella* showed lowest relative abundances in Chow fed group, which was significantly lower than IMD 15% and FOS groups; however, only *Bifidobacterium* and *Olsenella* showed a significant difference between Chow and IMD 7.5% as well as between IMD 7.5% and IMD 15% (Figure 17B,D,F). Three out of the 7 cecal genera that had an increased relative abundance in IMD 15% when compared to Chow, had lowest relative abundances in the FOS control group and second lowest in Chow (Figure 17C,E,G). *Bacteroides* showed statistically higher abundance in IMD 15% when compared to Chow; however, no difference was seen between Chow and IMD 7.5%, as well as between IMD 7.5% and IMD 15% (Figure 17C). *Clostiridum* IV showed significantly higher abundance in both IMD 7.5% and IMD 15% when compared to Chow, as well as a significantly higher abundance in IMD 15% when compared to IMD 7.5% (Figure 17E). *Parabacteroides* also showed significantly higher abundance in both IMD 7.5% and IMD 15% when compared to Chow; however, IMD 7.5% showed significantly higher abundance than IMD 15% (Figure 17G). Lastly, *Allobaculum* was seen in lowest abundance in IMD 7.5% group, with Chow as second lowest (Figure 17A). Both Chow and IMD 7.5% groups had significantly lower abundance than IMD 15% and FOS (Figure 17A).

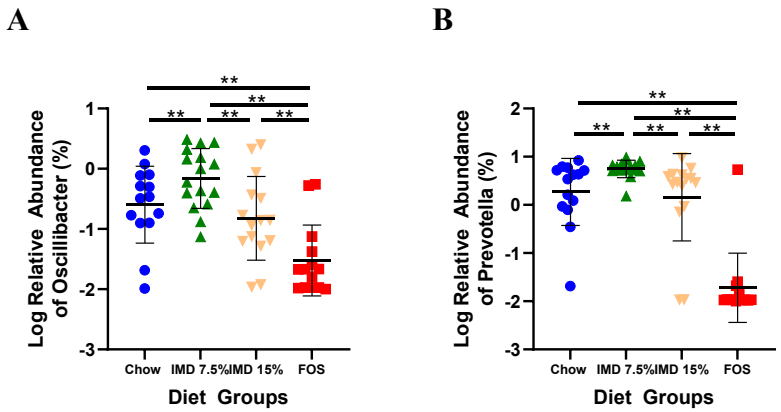


Figure 18. IMD 7.5% has a divergent effect on cecal microbiota in comparison to IMD 15%. (A) Oscillibacter and (B) Prevotella. Blue = Chow. Red = FOS 15%. Green = IMD 7.5%. Orange = IMD 15%. For all graphs, Chow n=14, FOS 15% n=14, IMD 7.5% n=16 and IMD 15% n=15. Bacterial composition was assessed by 16S rRNA gene sequencing (Illumina MiSeq platform). Data is presented as log transformed relative abundances. Statistical significance was calculated between diet groups using non-parametric one-way ANOVA (Kruskal-Wallis test) and two-stage Benjamini, Krieger and Yekutieli test. *$q \leq 0.1$. ** $q \leq 0.05$

Two cecal bacterial genera showed no statistical significance between IMD 15% and Chow control (Figure 18). Both *Oscillibacter* and *Prevotella* had lowest relative abundance in FOS group, followed by IMD 15% and Chow, with IMD 7.5% having highest abundance (Figure 18A,B). Chow group had significantly lower abundance compared to IMD 7.5% for both genera, and *Prevotella* also showed significantly higher abundance in IMD 7.5% when compared to IMD 15% (Figure 18A,B). No significant difference was seen between IMD groups and Chow for unclassified *Porphyromonadaceae* and *Clostridium* XVIII.

In the stool, 6 bacterial groups showed statistical significance between dietary groups (Figure 19, Table 2). Of these groups, only *Alistipes* showed reduction in IMD 7.5% (q=0.0078)

and IMD 15% (q<0.0001) in comparison to Chow (Figure 19A). *Bifidobacterium* and *Clostridium* IV both showed a significant increase in both IMD 7.5% (q=0.0214, q=0.0034) and IMD 15% (q=0.0154, q=0.0005) in comparison to Chow (Figure 19B,C). The remaining 3 colon bacterial groups showed divergent effects. Fecal relative abundance was significantly or

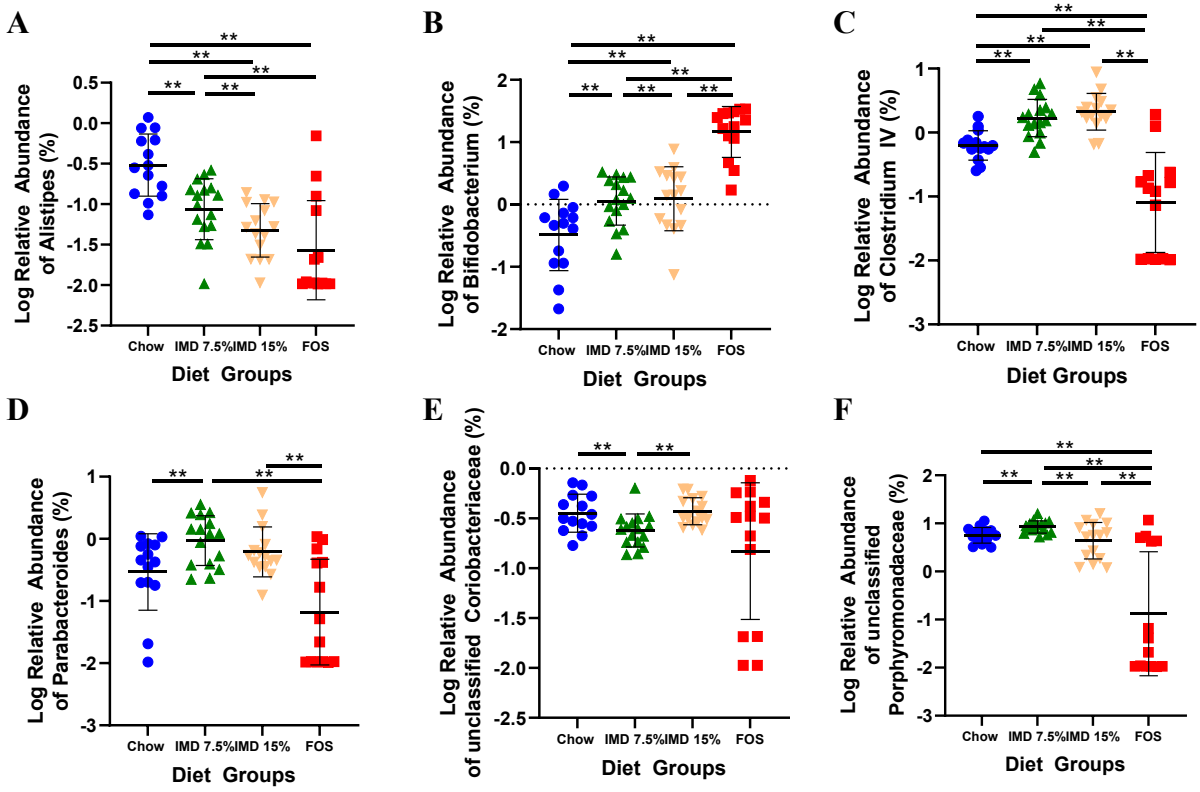


Figure 19. Effect of fiber treatments on colonic bacterial genera relative abundance for (A) Alistipes, (B) Bifidobacterium, (C) Clostridium IV, (D) Parabacteroides, (E) unclassified Coriobacteriaceae and (F) unclassified Porphyromonadaceae. Blue = Chow. Red = FOS 15%. Green = IMD 7.5%. Orange = IMD 15%. For all graphs, Chow n=14, FOS 15% n=14, IMD 7.5% n=16 and IMD 15% n=15. Bacterial composition was assessed by 16S rRNA gene sequencing (Illumina MiSeq platform). Data is presented as relative abundances. Statistical significance was calculated between diet groups using non-parametric one-way ANOVA (Kruskal-Wallis test) and two-stage Benjamini, Krieger and Yekutieli test. *q ≤ 0.1. ** q ≤ 0.05

Table 1. Significantly changed relative abundances (% ± SD) of bacterial groups in cecum contents across all diet groups. *q≤0.1 compared to Chow. ** q≤0.05 compared to Chow.

Phylum	Family	Genus	Chow	IMD 7.5%	IMD 15%	FOS
Actinobacteria			0.68 ± 0.28	2.11 ± 0.03 q = 0.0609*	4.03 ± 4.60 q = 0.0033**	26.58 ± 14.24 q < 0.0001**
	Bifidobacteriaceae	*Bifidobacterium*	0.29 ± 0.23	1.08 ± 1.39 q = 0.0242**	1.91 ± 1.81 q = 0.0045**	21.97 ± 13.39 q < 0.0001**
	Coriobacteriaceae	*Olsenella*	0.11 ± 0.15	0.69 ± 1.90 q = 0.0853*	1.74 ± 2.99 q = 0.0007**	4.12 ± 3.29 q < 0.0001**
Bacteroidetes			18.44 ± 5.52	24.47 ± 3.76 q = 0.0147**	19.70 ± 9.11 q = 0.2966	5.80 ± 11.26 q = 0.0019**
	Rikenellaceae		0.68 ± 0.49	0.24 ± 0.16 q = 0.0371**	0.13 ± 0.12 q = 0.0004**	0.06 ± 0.13 q < 0.0001**
		Alistipes	0.67 ± 0.49	0.24 ± 0.15 q = 0.0371**	0.13 ± 0.12 q = 0.0004**	0.06 ± 0.13 q < 0.0001**
	Bacteroidaceae		2.43 ± 1.07	3.47 ± 2.74 q = 0.2270	7.03 ± 7.98 q = 0.0007**	3.12 ± 4.38 q = 0.4516
		Bacteroides	2.42 ± 1.07	3.45 ± 2.72 q = 0.2270	6.99 ± 7.93 q = 0.0007**	3.09 ± 4.34 q = 0.4516
	Porphyromonadaceae	*Barnesiella*	1.58 ± 0.85	1.75 ± 1.37 q = 0.3274	0.67 ± 0.70 q = 0.0056**	0.44 ± 1.00 q < 0.0001
		Parabacteroides	0.67 ± 0.46	1.81 ± 0.81 q = 0.0032**	1.26 ± 1.02 q = 0.0912*	0.29 ± 0.61 q = 0.0375**
	Prevotellaceae	*Prevotella*	3.42 ± 2.61	5.97 ± 1.91 q = 0.0063**	3.19 ± 2.44 q = 0.1055	0.39 ± 1.44 q = 0.0004**
		Unclassified *Prevotellaceae*	0.16 ± 0.11	0.20 ± 0.08 q = 0.0750*	0.09 ± 0.09 q = 0.0566*	0.02 ± 0.02 q < 0.0001**
Firmicutes			78.65 ± 5.99	71.59 ± 5.25 q=0.0085**	74.39 ± 11.26 q=0.1720	65.33 ± 11.69 q<0.0001**
	Clostridiaceae	*Clostridium* IV	0.71 ± 0.49	1.44 ± 0.47 q = 0.0064**	2.20 ± 1.38 q = 0.0008**	0.16 ± 0.41 q = 0.0042**
		Clostridium XIVb	0.12 ± 0.08	0.18 ± 0.22 q = 0.2823	0.09 ± 0.19 q = 0.0075**	0.01 ± 0.005 q < 0.0001**
	Eubacteriaceae		0.09 ± 0.06	0.05 ± 0.05 q = 0.0481**	0.04 ± 0.02 q = 0.0224**	0.03 ± 0.02 q = 0.0041**
	Oscillospiraceae	*Oscillibacter*	0.51 ± 0.55	1.14 ± 1.01 q = 0.0606*	0.47 ± 0.79 q = 0.2280	0.09 ± 0.19 q = 0.0064**
	Lachnospiraceae	*Anaerostipes*	0.07 ± 0.07	0.27 ± 0.33 q = 0.1420	1.01 ± 1.18 q = 0.0087**	1.81 ± 2.51 q = 0.0087**
	Peptostreptococcaceae		1.16 ± 0.73	1.26 ± 1.59 q = 0.2225	0.56 ± 0.54 q = 0.0314**	0.08 ± 0.20 q < 0.0001**
		Peptococcus	0.33 ± 0.29	0.18 ± 0.11 q = 0.0661*	0.14 ± 0.09 q = 0.0110**	0.03 ± 0.08 q < 0.0001**
	Erysipelotrichaceae	*Allobaculum*	2.98 ± 2.32	2.67 ± 3.34 q = 0.2701	7.08 ± 4.09 q = 0.0306**	13.56 ± 7.90 q = 0.0006**
		Unclassified *Erysipelotrichaceae*	0.43 ± 0.35	0.26 ± 0.22 q = 0.1281	0.16 ± 0.24 q = 0.0019**	0.06 ± 0.09 q < 0.0001**
	Ruminococcaceae	*Ruminococcus*	1.47 ± 1.17	2.33 ± 1.28 q = 0.0857*	0.64 ± 1.13 q = 0.0120**	0.17 ± 0.59 q = 0.0001**
		Flavonifractor	0.94 ± 1.03	1.25 ± 1.23 q = 0.2473	0.53 ± 1.09 q = 0.0532*	0.38 ± 0.85 q = 0.0246**
		Unclassified *Ruminococcaceae*	5.64 ± 1.56	3.81 ± 2.01 q = 0.0343**	2.19 ± 2.42 q = 0.0008**	0.55 ± 1.29 q < 0.0001**

Table 2. Significantly changed relative abundances (% ± SD) of bacterial groups in stool across all diet groups. *q≤0.1 compared to Chow. ** q≤0.05 compared to Chow.

Phylum	Family	Genus	Chow	IMD 7.5%	IMD 15%	FOS
Actinobacteria			1.13 ± 0.63	2.44 ± 2.04 q = 0.0459**	4.67 ± 5.56 q = 0.0030**	23.90 ± 12.59 q < 0.0001**
	Bifidobacteriaceae		0.15 ± 0.37	1.54 ± 1.05 q = 0.0214**	2.06 ± 1.99 q = 0.0154**	19.58 ± 11.44 q < 0.0001**
		Bifidobacterium	0.58 ± 0.48	1.53 ± 1.04 q = 0.0214**	2.05 ± 1.99 q = 0.0154**	19.48 ± 12.67 q < 0.0001
	Coriobacteriaceae	Unclassified *Coriobacteriaceae*	0.39 ± 0.23	0.26 ± 0.12 q = 0.0520*	0.39 ± 0.13 q = 0.6538	0.29 ± 0.22 q = 0.2848
Bacteroidetes			21.-8 ± 6.34	23.64 ± 7.39 q = 0.4716	21.67 ± 9.39 q = 0.8398	9.06 ± 9.70 q = 0.0448
	Rikenellaceae		0.43 ± 0.35	0.11 ± 0.08 q = 0.0082**	0.06 ± 0.04 q < 0.0001**	0.09 ± 0.19 q < 0.0001**
		Alistipes	0.43 ± 3.70	0.11 ± 0.07 q = 0.0078**	0.06 ± 0.04 q < 0.0001**	0.09 ± 7.89 q < 0.0001**
	Unclassified *Bacteroidales*		1.96 ± 1.68	0.34 ± 0.26 q = 0.0254**	0.15 ± 0.12 q = 0.0003**	0.03 ± 0.08 q < 0.0001**
	Porphyromonadaceae		8.01 ± 2.98	11.38 ± 3.03 q = 0.0190**	7.89 ± 4.58 q = 0.2911	2.84 ± 4.59 q = 0.0043**
		Parabacteroides	0.49 ± 0.56	1.33 ± 1.03 q = 0.0425**	1.01 ± 1.39 q = 0.2217	0.28 ± 1.01 q = 0.1161
		Unclassified *Porphyromonadaceae*	6.02 ± 3.31	8.78 ± 2.69 q = 0.0261**	5.98 ± 4.47 q = 0.5228	2.21 ± 2.68 q = 0.0102**
	Unclassified *Lactobacillales*		0.01 ± 0.00	0.01 ± 0.00 q = 0.0309**	0.01 ± 0.00 q = 0.4926	0.01 ± 0.01 q = 0.4926
Firmicutes			75.36 ± 6.95	72.05 ± 8.82 q = 0.4283	71.28 ± 13.26 q = 0.4338	65.16 ± 10.34 q = 0.0246**
	Clostridiaceae	*Clostridium* IV	0.72 ± 0.73	2.08 ± 1.49 q = 0.0034	2.61 ± 1.99 q = 0.0005	0.31 ± 0.44 q = 0.0306
	Lactobacilliaceae		15.64 ± 6.51	12.62 ± 10.40 q = 0.1214	6.42 ± 2.84 q = 0.0044**	19.39 ± 23.03 q = 0.1214

tentatively higher in IMD 7.5% than Chow, but not different in IMD 15% for *Parabacteroides* (q=0.0425) and unclassified *Porphyromonadaceae* (q=0.0261) (Figure 19D,F). Fecal unclassified *Coriobacteriaceae* relative abundance was lower in IMD 7.5% than Chow (q=0.0520) but showed no difference in IMD 15% (Figure 19E).

2.3.6. Correlation between bacterial relative abundance and IL-1β concentration

Figure 20 shows correlations seen between cecal IL-1β concentrations and those bacterial groups found to be statistically altered by IMD and FOS treatments, as shown above. Moderate negative correlation was seen at the phyla level between Actinobacteria and IL-1β concentrations (R= -0.32, p=0.0308) (Figure 20A). At the family level, a moderate positive correlation was seen for both *Rikenellaceae* (R=0.34, p=0.0160) and *Peptostreptococcaceae* (R=0.33, p=0.0160) with

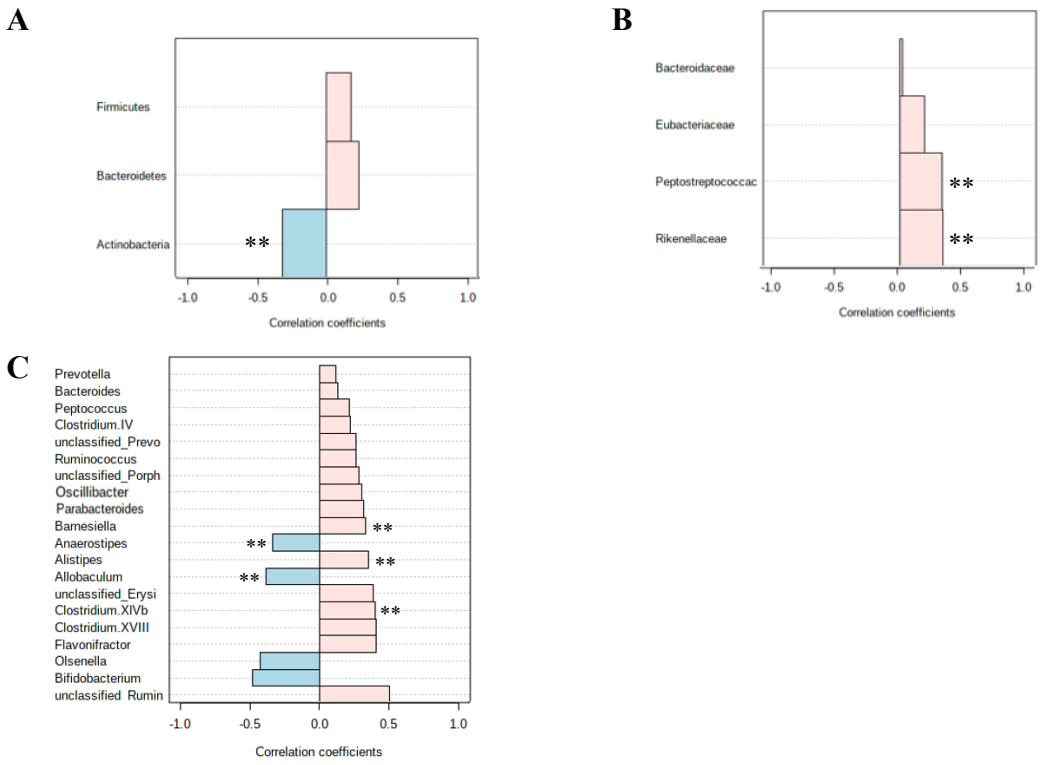
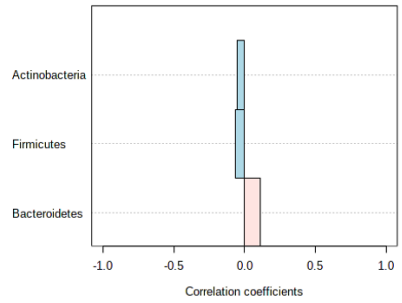


Figure 20. Correlation between cecal IL-1β concentration and cecal bacterial groups significantly altered by diet treatments, including (A) phyla, (B) family and (C) genus. For all graphs, n=59. ELISAs were used to quantify IL-1β concentrations. Bacterial composition was assessed by 16S rRNA gene sequencing (Illumina MiSeq platform). Statistical significance was calculated using Spearman Rank correlation test. **R≥0.3

IL-1β concentrations (Figure 20B). Moderate significance was seen at the genera level for unclassified *Ruminococcaceae* (R=0.5, p=0.0005), *Flavonifractor* (R=0.40, p=0.0055), *Clostridium* XVIII (R= 0.4, p=0.0055), *Clostridium* XIVb (R=0.40, p=0.0055), unclassified *Erysipelotrichaceae* (R=0.39, p=0.0063), *Alistipes* (R=0.35, p=0.0131), *Barnesiella* (R=0.33, p=0.0196) and *Parabacteroides* (R=0.30, p=0.0300) (Figure 20C). Contrastingly, cecal relative abundances were moderately negatively associated with cecal IL-1β concentrations for the genera *Bifidobacterium* (R= -0.49, p=0.0008), *Olsenella* (R= -0.43, p=0.0035), *Allobaculum* (R= -0.39, p=0.0063) and *Anaerostipes* (R= -0.34, p=0.0162) (Figure 20C).

Figure 21 shows same analyses between mucosal IL-1β concentrations in colon and fecal bacterial groups found to be significantly altered above. No significant correlations were seen at the phyla, family, or genus levels (Figure 21).

A



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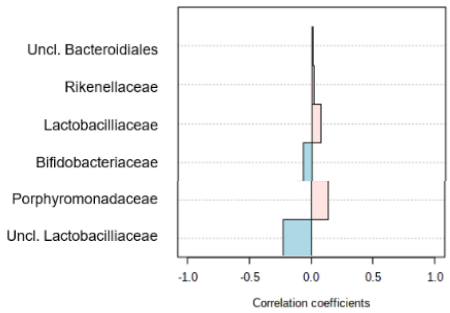

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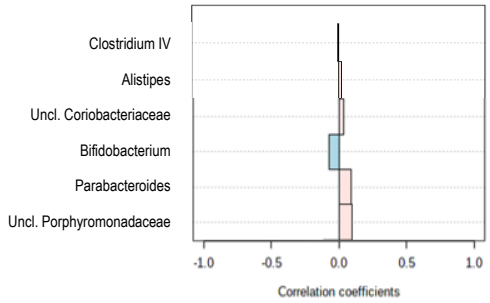


Figure 21. Correlation between cecal IL-1β concentration and stool bacterial groups significantly altered by diet treatments, including (A) phyla, (B) family and (C) genus. For all graphs, n=59. ELISAs were used to quantify IL-1β concentrations. Bacterial composition was assessed by 16S rRNA gene sequencing (Illumina MiSeq platform). Statistical significance was calculated using Spearman Rank correlation test.

2.3.7. Effect of fiber treatments on cecum and colon SCFA concentrations

SCFAs were measured to determine changes in bacterial metabolism as well as to explore possible associations between specific SCFA and inflammation. Analyses were done using ANOVA comparisons for each SCFA between dietary groups in order to determine specific changes in bacterial metabolite production associated with each diet group.

Figure 22 shows concentrations of total SCFA in cecal content (μmol/g of cecal content), as well as individual SCFA and branched SCFA as a proportion of total SCFA. Total cecal SCFA concentration in Chow group was statistically lower than IMD 7.5% (q=0.0683) and IMD 15% (q=0.0003) (Figure 22A) (Table 3). Interestingly, IMD dose-dependently increased the total SCFA production (q=0.0249) (Figure 22A) (Table 3). No difference was seen between Chow and FOS 15% total cecal SCFA concentrations (Figure 22A) (Table 3). No statistical significance was seen for acetate and butyrate across all diet groups (Figure 22B-C) (Table 3). However, proportions of propionate were highest in Chow group and diet with IMD 15% significantly reduced these proportions (q=0.0183) (Figure 22D) (Table 3). Interestingly, no significance was seen between Chow and IMD 7.5% and IMD 15% was associated with even lower propionate proportions than IMD 7.5% (q=0.0398) (Figure 22D) (Table 3). Furthermore,

Table 3. Cecal concentrations of total SCFAs (µmol/g of cecal content) and SCFAs (% proportion of total SCFAs) in each dietary group IMD 7.5%, IMD 15% and FOS, compared to Chow. *q≤0.1 compared to Chow. ** q≤0.05 compared to Chow.

SCFA	Chow	IMD 7.5%	IMD 15%	FOS
Total SCFA	78.66 ± 11.27	87.95 ± 7.32 (q=0.0683)*	96.96 ± 7.97 (q=0.0003)**	67.69 ± 26.90 (q=0.4882)
Acetic Acid	70.75 ± 8.93	75.05 ± 6.50 (q=0.4223)	74.46 ± 10.99 (q=0.4223)	76.08 ± 12.34 (q=0.4223)
Butyric Acid	14.66 ± 8.03	12.53 ± 4.94 (q=0.9672)	15.50 ± 7.63 (q=0.9672)	17.31 ± 11.04 (q=0.9672)
Propionic Acid	12.27 ± 3.30	11.01 ± 1.90 (q=0.2917)	9.08 ± 3.09 (q=0.0183)**	5.78 ± 2.86 (q<0.0001)**
Valeric Acid	0.73 ± 0.30	0.52 ± 0.25 (q=0.0616)*	0.45 ± 0.27 (q=0.0024)**	0.50 ± 0.71 (q<0.0001)**
Isobutyric Acid	0.48 ± 0.28	0.26 ± 0.08 (q=0.00436)**	0.21 ± 0.13 (q=0.0007)**	0.04 ± 0.09 (q<0.0001)**
Isovaleric Acid	0.66 ± 0.36	0.35 ± 0.11 (q=0.0118)**	0.27 ± 0.14 (q<0.0001)**	0.55 ± 0.38 (q=0.1045)

Table 4. Fecal concentrations of total SCFAs (µmol/g of stool) and SCFAs (% proportion of total SCFAs) in each dietary group IMD 7.5%, IMD 15% and FOS, compared to Chow. *q≤0.1 compared to Chow. ** q≤0.05 compared to Chow.

SCFA	Chow	IMD 7.5%	IMD 15%	FOS
Total SCFA	76.62 ± 15.43	72.15 ± 10.13 (q=0.3922)	77.99 ± 15.89 (q=0.3922)	52. 91 ± 15.34 (q=0.0009)**
Acetic Acid	81.76 ± 4.65	82.91 ± 5.63 (q=0.6657)	82.78 ± 4.85 (q=0.7487)	84.99 ± 7.53 (q=0.6182)
Butyric Acid	8.19 ± 4.39	7.18 ± 4.25 (q=0.5899)	6.21 ± 1.92 (q=0.5899)	5.96 ± 5.38 (q=0.5899)
Propionic Acid	9.27 ± 2.31	9.14 ± 2.75 (q=0.5354)	8.33 ± 3.23 (q=0.2424)	6.83 ± 3.74 (q=0.0328)**
Valeric Acid	0.16 ± 0.16	0.13 ± 0.16 (q=0.2255)	0.07 ± 0.16 (q=0.0225)**	0.07 ± 0.23 (q=0.0065)**
Isobutyric Acid	0.21 ± 0.10	0.21 ± 0.19 (q=0.9375)	0.21 ± 0.21 (q=0.6036)	0.09 ± 0.15 (q=0.0592)**
Isovaleric Acid	0.36 ± 0.09	0.31 ± 0.11 (q=0.2304)	0.31 ± 0.18 (q=0.0724)	0.27 ± 0.14 (q=0.0724)

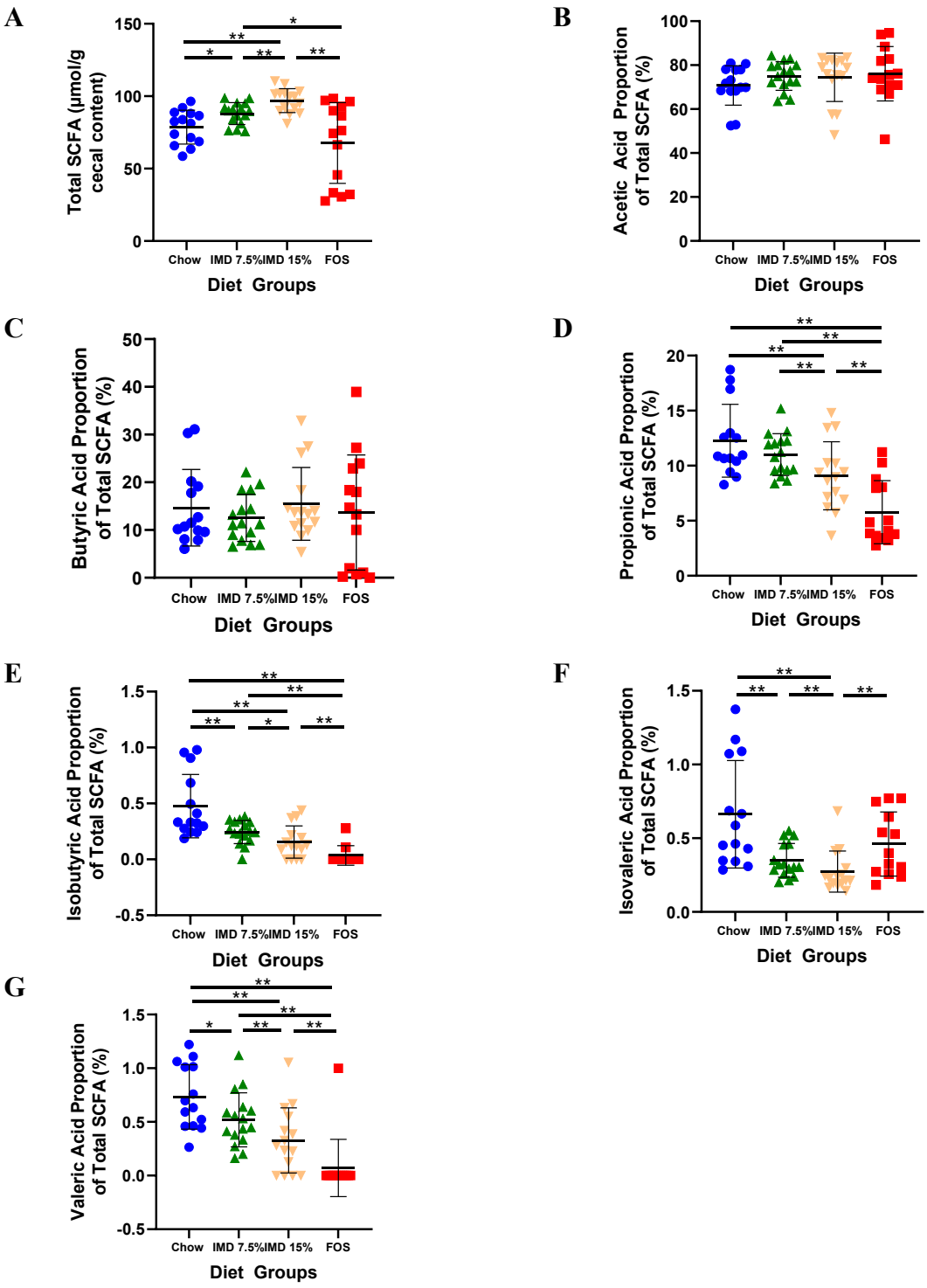
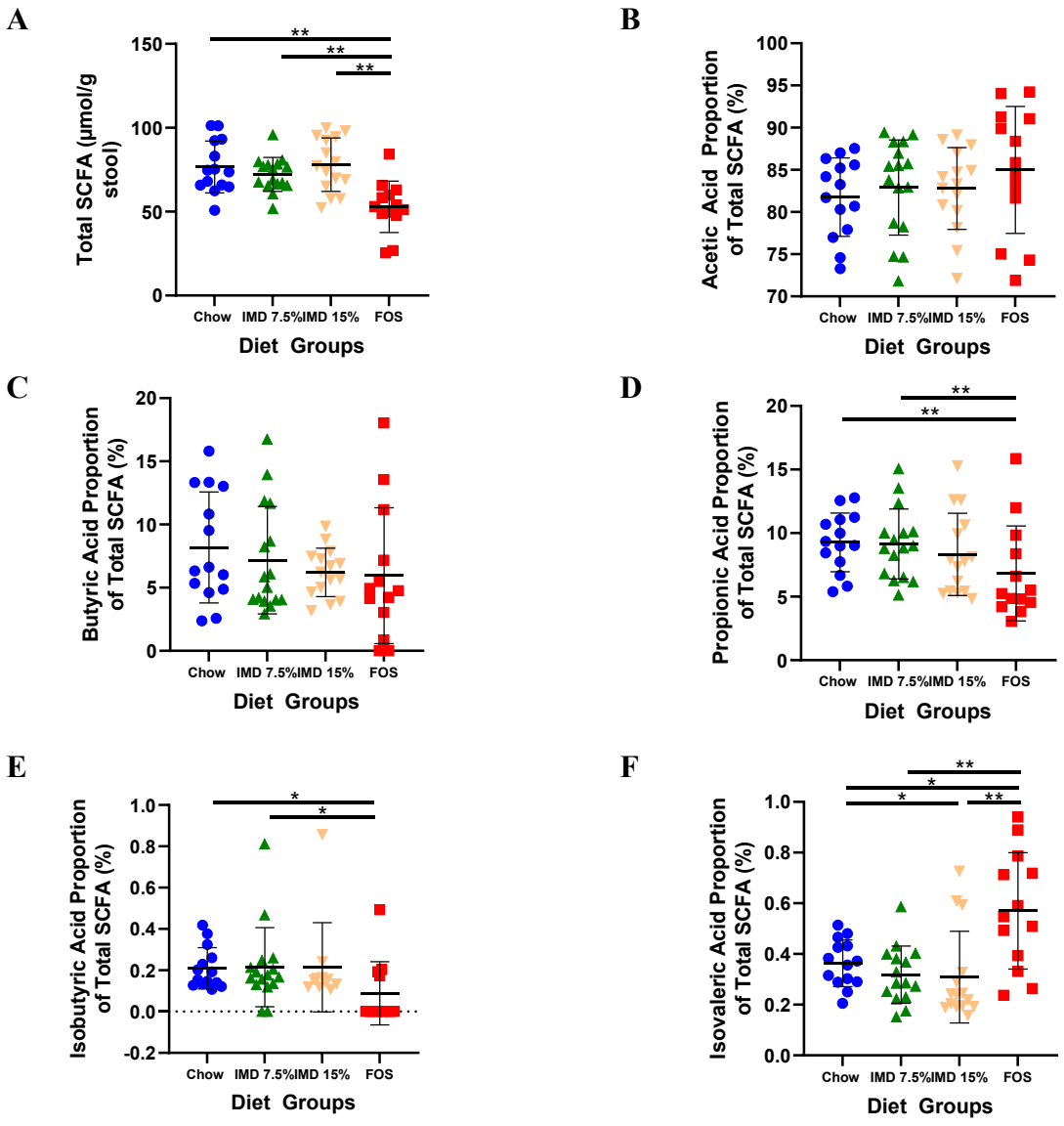


Figure 22. Effect of fiber treatments on all rats' cecal (A) total SCFA concentration, and proportion of (B) acetic acid, (C) butyric acid, (D) propionic acid, (E) isobutyric acid, (F) isovaleric acid and (G) valeric acid. Blue = Chow. Red = FOS 15%. Green = IMD 7.5%. Orange = IMD 15%. For all graphs, Chow n=14, FOS 15% n=14, IMD 7.5% n=16 and IMD

15% n=15. Fatty acid concentrations quantified using gas chromatography. Data is presented as mean ± SD. Statistical significance was calculated between diet groups using non-parametric one-way ANOVA (Kruskal-Wallis test) and two-stage Benjamini, Krieger and Yekutieli test. *q ≤ 0.1. ** q ≤ 0.05



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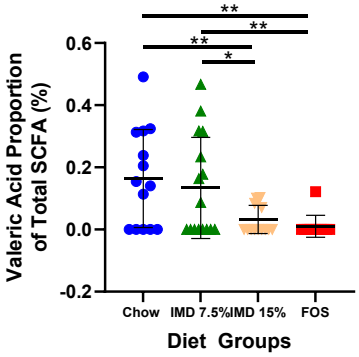

Figure 23. Effect of fiber treatments on all rats' stool (A) total SCFA concentration, and proportion of (B) acetic acid, (C) butyric acid, (D) propionic acid, (E) isobutyric acid, (F) isovaleric acid and (G) valeric acid. Blue = Chow. Red = FOS 15%. Green = IMD 7.5%. Orange = IMD 15%. For all graphs, Chow n=14, FOS 15% n=14, IMD 7.5% n=16 and IMD 15% n=15. Fatty acid concentrations quantified using gas chromatography. Data is presented as mean ± SD. Statistical significance was calculated between diet groups using non-parametric one-way ANOVA (Kruskal-Wallis test) and two-stage Benjamini, Krieger and Yekutieli test. *q $\leq$ 0.1. ** q $\leq$ 0.05

IMD dose-dependently reduced the proportions of isobutyrate, isovalerate and valerate, with IMD 15% having stronger reduction effects (Figure 22E-G) (Table 3).

Figure 23 shows total SCFA concentrations and individual SCFA proportions for stool across diet groups. Total colon SCFA concentration FOS was significantly lower than all other groups, Chow (q=0.0009), IMD 7.5% (q=0.0016) and IMD 15% (q=0.0004) (Figure 23A). Stool proportions were significantly lower in FOS than Chow and IMD 7.5% for propionate (q=0.0328, q=0.0328) and isobutyrate (q=0.0592, q=0.0592) (Figure 23D&E) (Table 4). Stool isovalerate proportion was tentatively lower in IMD 15% compared to Chow (q=0.0724), but not different in IMD 7.5% (Figure 23F) (Table 4). Valerate proportions in stool was statistically

lower in IMD 15% than Chow (q=0.0225) and tentatively lower in IMD 15% than IMD 7.5% (q=0.0890) (Figure 23G) (Table 4). No significant differences were seen across groups for butyrate and acetate proportions in stool (Figure 23B&C).

2.3.8. Correlation between SCFA composition and IL-1β concentration

SCFA composition was compared to the concentrations of IL-1 β to assess their possible correlation, either positive or negative, with inflammation. Figure 24A shows correlations seen between cecal SCFAs and cecal IL-1β concentration for the combined data across all diet groups. Moderate positive correlation was seen between cecal IL-1β concentration and propionate (r=0.46, p=0.0004), valerate (r=0.49, p=0.0003), isobutyrate (R=0.37, p=0.0059) and isovalerate (R=0.32, p=0.0148) (Figure 24A). Moderate negative correlation was seen between cecal IL-1β concentration and acetate (R= -0.39, p=0.0044) (Figure 24A). No significant correlation was seen between IL-1β and butyrate. Figure 24B shows stool correlations between IL-1β concentrations and SCFAs, where no significance was seen for any SCFA.

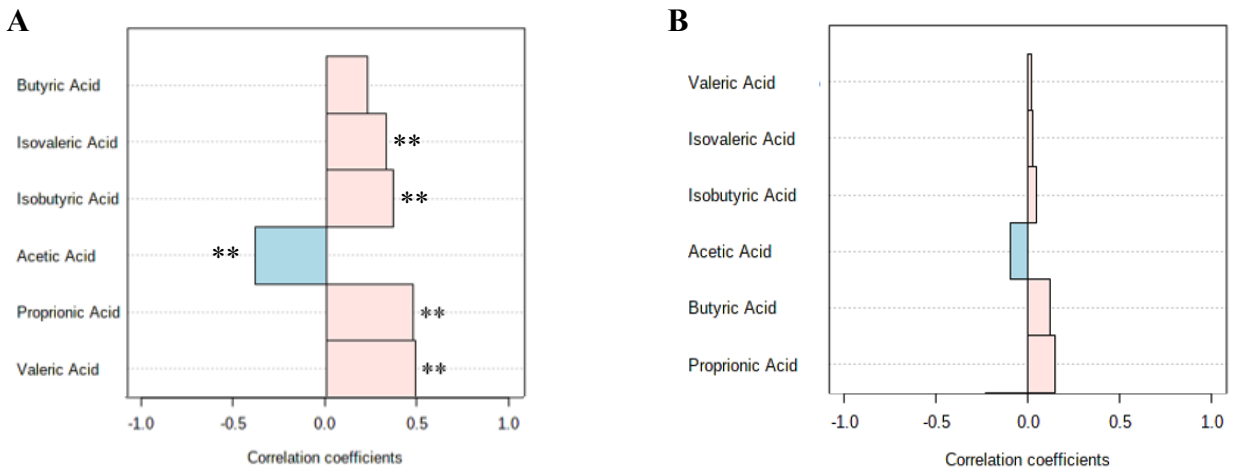

Figure 24. Correlation between IL-1β concentration and short chain fatty acid concentrations across all diets in (A) cecum and (B) colon (n=59). ELISAs were used to quantify IL-1β concentrations. Fatty acid concentrations quantified using gas chromatography. Statistical significance was calculated using Spearman correlation test. **R ≥ 0.3

2.3.9. Correlation between cecal SCFA composition and cecal bacterial composition

We then explored the correlation between the significant 12 cecal genera found in previous analyses to be moderately correlated with cecal IL-1β concentrations and the 5 significant SCFA found in previous analyses to be moderately correlated with cecal IL-1β concentrations (Figure 25). Acetate showed a moderate positive correlation to *Bifidobacterium* (R=0.49, p<0.0001), as well as a moderate negative correlation to unclassified *Ruminococcaceae* (R=-0.41, p=0.0012) and *Flavonifractor* (R=-0.55, p<0.0001) (Figure 25). Levels of isobutyrate were moderately negatively correlated with *Allobaculum* (R=-0.37, p=0.0044), *Bifidobacterium* (R=-0.30, p=0.0237), *Olsenella* (R=-0.43, p=0.0006) and *Anaerostipes* (R=-0.37, p=0.0044), while being moderately positively correlated to *Alistipes* (R=0.30, p=0.0724), *Clostridium* XIVb (R=0.36, p=0.0046), unclassified *Ruminococcaceae* (R=0.30, p=0.0586) and *Flavonifractor* (R=0.35, p=0.0064) (Figure 25). Isovalerate showed only negative correlations, which were moderate correlations to the genera *Olsenella* (R=-0.40, p=0.0017) and *Anaerostipes* (R=-0.40, p=0.0005) (Figure 25). Interestingly, both propionate and valerate, the two found in our previous analyses to be positively associated with cecal IL-1β concentrations, were associated with the same genera; a moderate or strong positive association with *Alistipes* (R=0.60, 0.78 -0.47; p<0.0001, <0.0001), *Barnesiella* (R= 0.64, 0.70; p<0.0001, <0.0001), *Clostridium* XIVb (R=0.59, 0.66; p<0.0001, <0.0001), unclassified *Ruminococcaceae* (R=0.60, 0.78; p<0.0001,

<0.0001), *Flavonifractor* (R=0.42, 0.55; p= 0.0009, 0.0006), *Parabacteroides* (R=0.70, 0.66; p<0.0001, <0.0001), *Clostridium* XVIII (R=0.47, 0.53; p=0.0002, 0.0002) and unclassified *Erysipelotrichaceae* (R=0.50, 0.64; p<0.0001, <0.0001), as well as a moderate or strong negative correlation with *Allobaculum* (R=-0.48, -0.47; p<0.0001,<0.0001), *Bifidobacterium* (R=-0.71, -0.79; p<0.0001, <0.0001), *Olsenella* (R=-0.58, -0.64; p<0.0001, <0.0001), and *Anaerostipes* (R=-0.35, -0.47, p=0.0061, 0.0002) (Figure 25).

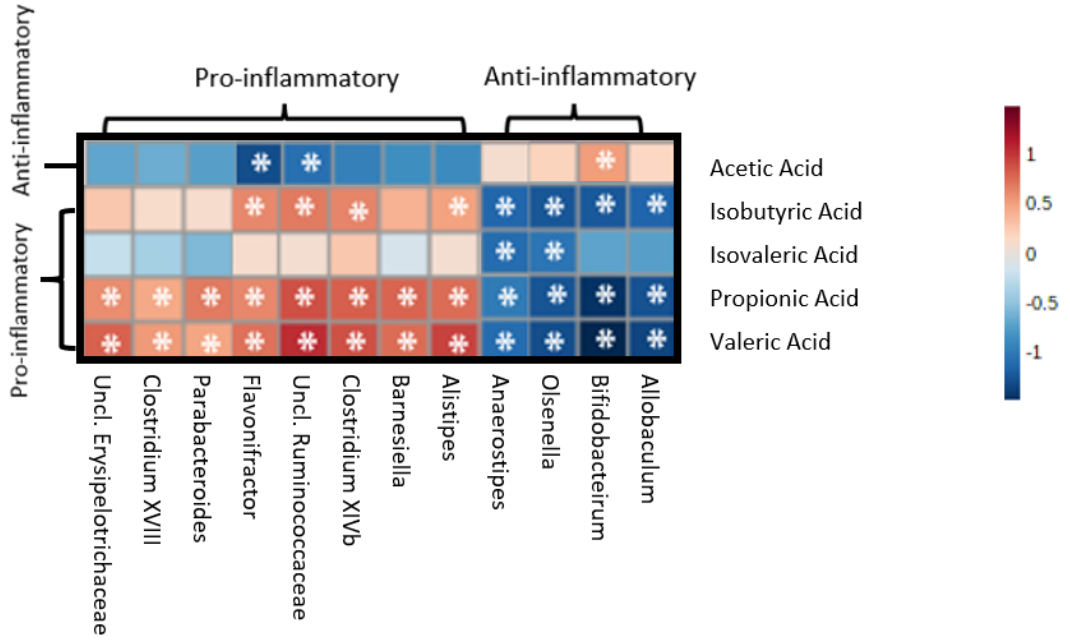

Figure 25. Hierarchical Clustering Heatmap comparing correlation of significant short chain fatty acids and significant cecal genera. n=59. Short chain fatty acid concentrations quantified using gas chromatography. Bacterial composition was assessed by 16S rRNA gene sequencing (Illumina MiSeq platform). Statistical significance was calculated using Spearman rank correlation test. *R ≥ 0.3

2.5 Discussion

The objective of this study was to determine if IMD, a novel highly branched α-glucan product, would have a beneficial effect on colitis development in HLA-B27 TG rats and, if so, to describe a possible mechanism of activity associated with shifts in microbial composition and metabolism. A previous study testing IMD, by Zhang *et al.,* compared the effects of low (3.75 *w/w*) and high (7.5% *w/w*) doses of IMD to cellulose negative control in IL-10 deficient murine colitis model [20]. The study found sex-specific differences where males showed reduced colonic IL-12p70 and enhanced microbial richness and evenness while females showed higher levels of IL-12p70 and reduction in alpha-diversity, similar to the control group [122]. Considering these previously published findings, it was important to test if HLA-B27 TG rats also show sex-specific responses to IMD.

We showed in the present study that IMD treatment greatly reduced the development of intestinal inflammation in male SPF HLA-B27 transgenic rats. Multiple methods were used in this study to evaluate colitis reduction in response to treatment, such as cecal macroscopic scoring by GGS, cecum/colon mucosal IL-1β and IFNγ cytokine concentrations and cecum/colon weight:length ratios. GGS and IL1β results demonstrated that, in comparison to the negative control Chow group, both sexes in the positive control FOS group responded with a reduction in cecal inflammation, though this reduction reached significance only for males and was just tentative in females (Figure 3B,C & Figure 4B,C). Multiple evaluation approaches revealed that treatment with IMD showed benefits only for males, and this benefit was dose-dependent, where the higher dose (IMD 15%) was even more effective than the lower dose (IMD 7.5%) (Figure 3B,C & Figure 4B,C). It is important to note that these dose-dependent findings in males were not correlated with different diet intake or relative body weight gain (Figure 2A,C). All diets,

including the control diet, were supplemented with corresponding undigestible fibers at 15% (*w/w*) concentration, thus assuring isocaloric intakes across groups (Figure 2A,C). These results are in agreement with the previously mentioned study by Zhang *et al.* that found sex-dependent benefits of IMD for males [122]. From our results, it can be then suggested that both IMD test doses, as well as FOS, are ineffective in reducing cecal inflammation in female HLA-B27 rats. Conversely, it can be hypothesized that the observed sex-specific difference in the response to the dietary fibers (IMD and FOS) can be produced by the different level of tissue inflammation in males versus females in the Chow control group. Indeed, the mean IL-1β values for males fed Chow diet was significantly higher than females on the same diet (Figure 4D). This suggests that the extent of cecal inflammation is naturally higher in male HLA-B27 TG rats than females and may explain the lack of significance seen in females. A possible mechanism for this difference in inflammation between the sexes may be the protective effect of estrogen that has previously been described in this rat model [129]. A study by Harnish DS., *et al* in male HLA-B27 TG rats given either vehicle control, estrogen, or estrogen with estrogen receptor antagonist, found that estrogen treatment improved stool character, colon histology and reduced myeloperoxidase activity (indicator of inflammation that measures neutrophil infiltration), demonstrating the protective activity of estrogen in HLA-B27 TG rats [129]. This naturally lower level of inflammation in the females may provide an explanation as to the lack of significant difference seen in colitis reduction between Chow and fiber treated (IMD and FOS) groups for females. In humans, comparison of IBD incidence between the sexes show conflicting results; in the United States and Europe, CD prevalence is higher in females than males, while the opposite in true in Asia, suggesting possible geographical effects on IBD development rates [130]. In addition, it was noticed that there was no consistent response to IMD treatment in either sex groups. For

example, males on average showed reductions in cecal inflammation (IL-1β) in both IMD groups, while the females did not (Figure 4B&C); however, 50% and 62.5% of females on IMD 7.5% and IMD 15%, respectively, responded to treatment with better reduction in cecal inflammation (IL-1β) than their average male counterparts (Figure 4B&C). Similarly, in the males, not all rats showed a beneficial response to IMD, as 25% of males in IMD 7.5% group had higher cecal inflammation than even their average Chow group counterparts (Figure 4B). We argue that though the benefit of IMD in reducing cecal inflammation may be more frequently seen in males, it is still specific to the individual and is not universal to the sex. Multivariate microbial analyses also support the lack of significance difference seen between the sexes, as no specific compositional differences were detected between male and female cecal or colonic microbiotas (Figure 8A&C). This lack of significant difference between the microbiotas of the two sexes suggests that the differences seen in inflammation may be due to mechanisms not associated with the gut microbiota. Based on these findings, subsequent analyses were performed combining the sexes, taking into consideration the inter-individual responses.

No changes were seen to colonic inflammation (IL-1β or IFNy) across any of the diet groups, including IMD and FOS treatment (Figure 5A-C & Figure 6B). As IMD and FOS are oligosaccharides (degree of polymerization, DP < 10) that are largely metabolized by the microbiota in the proximal part of the large bowel, in the case of rodents this is the cecum, it is likely that the low residual concentrations reaching the colon may have no physiological effect. Previous research on IMD has shown improvement in colonic inflammation via reduction in concentrations of colonic inflammatory cytokines (IL-12p70, TNF-α and IL-6) and, though no difference was seen in IL-1β concentrations, there was a noted suppression of the IL-1β gene expression [121,122]. However, both studies were done in murine colitis models and it is

possible that in the HLA-B27 TG models, the cecum and colon may not be equally affected by chronic inflammation. Indeed, statistical analyses comparing IL-1β in the cecum and colon of untreated Chow group found significantly lower inflammation in the colon tissues in comparison to the cecum (Figure 5D). The lack of beneficial colonic response to FOS found in our study was in conflict with previous findings from our lab reporting reduction of IL-1β concentrations not only in the cecum but also the colon in HLA-B27 TG rats [114]. It is, however, important to note that the HLA-B27 TG rat colony used for the current study was recently rederived using a different source from the one used from the past. Importantly, inflammation in this model has been shown to be triggered by specific commensals in the lumen [131,132]. Conventionalized HLA-B27 TG rats showed increased inflammatory cytokine expression while germ-free group did not, for example, rats colonized with defined bacterial cocktails that contained *Bacteroides vulgatus* showed development of colitis and gastritis, while the group given defined bacterial cocktail without *B. vulgatus* as well as the germ-free controls did not [131]. A subsequent study by the same group compared the inflammatory effects of two common commensal bacteria, *B. vulgatus* and *Escherichia coli*, to induce inflammation in mono-associated gnotobiotic HLA-B27 rats and found that colitis induced by *B. vulgatus* was significantly more severe than that induced by *E. coli* [132]. These studies show that the normal luminal bacteria are able to induce chronic inflammation in HLA-B27 TG colitis models, but the extent of the proinflammatory effect is not equal across all resident bacteria. Additionally, it has been shown through the comparison of HLA-B27 TG rats from three different facilities that inflammation and dysbiosis in HLA-B27 TG rats is highly dependent on the genetic background of the host as well as environment [133]. Taken together, since colitis progression in this model is highly dependent on the commensal

microbiota composition, this may explain the differences in the levels of inflammation seen between Koleva *et al.* and our study.

Prebiotics remain undigested by host enzymes in the upper intestine and are used as sources of energy for the gut microbiota. The end products of microbial fermentation of prebiotics lead to the production of SCFAs (acetate, butyrate, propionate), which provide numerous benefits to the host [31-33,37-42]. A reduction of SCFA producing microbes has been previously associated with IBD, suggesting its possible role in inflammation as well as making this host-microbial interaction a potentially beneficial target in IBD patients' treatment [82-84]. A minor (<5%) proportion of SCFAs produced by the gut microbiota are branched SCFAs, such as isovalerate, valerate and isobutyrate, which are by-products of protein fermentation, mainly by the *Bacteroides* genera in humans, and is seen more frequently in individuals with high protein/low fiber diets [22,23,43, 44]. Although research into the direct effects of branched SCFAs is limited, there are potentially toxic or carcinogenic products in protein fermentation such as ammonia and hydrogen sulphide [22,23]. IMD treatments either showed an overall effect or showed a dose-dependent effect on increasing total cecal SCFA concentrations as well as reducing SCFAs associated with inflammation, propionate, isobutyrate, isovalerate, and valerate (Figure 22A,D-G & Figure 24A). No changes were seen across groups for the neutral and anti-inflammatory SCFAs butyrate and acetate (Figure 22B,C & Figure 24). These findings were not duplicated in the colon for total SCFAs, propionate, isobutyrate and isovalerate, although a reduction was seen in IMD 15% compared to Chow for both isovalerate (tentative) and valerate (significant) (Figure 23D,E,F). However, a correlation could not be made between colon SCFAs and colon IL-1β concentrations to show association of these products to inflammation (Figure 24B). Previous research in HLA-B27 TG rats found an increase in proportions of the SCFAs

propionate, isobutyrate, valerate and isovalerate in response to a chemically defined diet, a more pro-inflammatory diet with sucrose as the main carbohydrate source, compared to controls [114]. The same study found a positive association between proportions of isobutyrate, valerate and isovalerate with increased colitis and IL-1β concentrations in the cecal mucosa [114]. These findings suggest that isobutyrate, valerate, and isovalerate may serve as potential biomarkers of disease in HLA-B27 TG rat colitis model. To our surprise, no significant differences were detected for acetate and butyrate proportions across diet groups in the cecum or colon (Figure 22B-C & Figure 23B-C). All of our rats were terminated around the same time of the day and did not fast, which could otherwise have been an explanation for this lack of acetogenic and butyrogenic effect. High levels of isobutyrate, isovalerate and valerate are often measured in individuals consuming high protein diets [23,44]; results from our study are unexpected as protein intake was equal across all diets. It may be possible that this increase in branched SCFA production was a result of colitis, instead of being causative, and that increased shedding of intestinal epithelial tissues provided an increased protein substrate for amino acid fermentation by specific microbes.

The contributions of the gut microbiota to inducing disease are complicated due to the lack of definitive conclusion as to whether IBD-related dysbiosis induces, or is the result of, intestinal inflammation. In this study, we analyzed the alterations to intestinal microbiota associated with the fiber treatments. Analysis of alpha diversity in the cecum measured by the number of observed OTUs showed statistically less microbial variation in IMD 15% and FOS groups, compared to Chow group, suggesting their effect in reducing species richness (Figure 9B). This was also seen in the colon, where FOS and IMD 15% as well as IMD 7.5% groups showed statistically less microbial variation when compared to Chow group (Figure 9E). This

was a conflicting finding as FOS and IMD 15% were seen to reduce inflammation, but were also associated with decreased microbial diversity, a trait previously associated in literature with IBD and disease severity [134-140]. However, the same reduction of microbial diversity after FOS administration has also been documented in both human and mice studies [141,142]. In addition, considering the reduction in diversity was seen in the IMD 7.5% group and even more significant in FOS and IMD 15%, it is possible that the reduction is due to the replacement of a substantial portion of the diet, 7.5% or 15%, with a singular fiber compound, IMD or FOS, creating a selective environment favorable for specific bacterial groups capable of utilizing one single substrate.

Indeed, the dose of IMD determined the subsequent effects on the gut microbiota. Many bacterial groups showed statistical differences between dietary groups (Figures 11-12,14-19, Tables 1-2). For example, both IMD groups induced increases in the cecum for phyla Actinobacteria, including the genera *Bifidobacterium* and *Olsenella*, where IMD 15% more closely mimicked the increase seen in FOS group (Figure 11C & Figure 17D,F). This increase in Actinobacteria as well as *Bifidobacterium* associated with IMD treatment was also seen in the colon (Figure 12C & Figure 19B). *Bifidobacterium,* in particular, is considered a hallmark bacterial group that is increased as a result of FOS treatment [143,144]. Cecal *Bifidobacterium* was moderately negatively correlated with cecal IL-1β concentrations (Figure 20C). It was also negatively correlated with valerate, propionate and isobutyrate, which were all positively correlated with cecal inflammation (Figure 24A & Figure 25). In addition, the correlation analysis indicated that low levels of cecal inflammation were associated with high acetate production exclusively by *Bifidobacterium* (Figure 24A & Figure 25). *Bifidobacterium* has been previously shown to be large producers of acetate that do not produce propionate which provide

an explanation for the significant increase in cecal acetate and decrease in cecal propionate [145]. Besides their acetate production, bifidobacteria have been shown to reduce inflammation through their effect on dendritic cell antigen sampling [146]. Dendritic cells obtained from CD patients and pretreated with the probiotic bifidobacteria mixture (*B. longum, B. breve* and *B. infantis*) prior to incubation with E. coli showed significantly improved antigen uptake and reduced proinflammatory cytokine production [146]. Intracolonic single-dose administration of *Bifidobacterium animalis* subsp. *lactis* and xyloglucan in severe UC patients showed effective mucosal healing as well as reduction in colonic symptoms [147]. The prebiotic effect of FOS, which seems to be mimicked by IMD 15%, stimulates the commensal probiotic *Bifidobacterium* which in turn induces alterations to the immune system.

Microbial communities of the gut are exceedingly complex with many microbial interactions such as interspecies cross-feeding. In the present study, many compositional shifts to the microbiota were seen with IMD 15% treatment, such as significant increases in abundance for cecal *Anaerostipes*, consistent with FOS group (Figure 17B). Cecal *Anaerostipes* was also seen to have negative correlation to cecal IL-1β concentration and was negatively correlated with the proinflammatory cecal SCFAs, valerate, propionate, isovalerate and isobutyrate (Figure 20C & Figure 25). Previous findings through mono- and co-cultures of *Bifidobacterium* spp. and *Anaerostipes* spp. supplemented with lactose or total human milk carbohydrates showed *Anaerostipes* inability to grow on these substrates except when co-cultured with *Bifidobacterium* spp. [148]. *Anaerostipes*' growth, when co-cultured, was attested to cross-feeding by utilization of lactate and acetate provided by *Bifidobacterium* spp., which was converted to butyrate by *Anaerostipes* [147]. Though significance was not seen for butyrate proportions across groups in our study, previous research does show additional benefits of *Bifidobacterium* through its key

ecological role in these cross-feeding interactions by providing substrates for other important bacterial groups in the gut, contributing to the anti-inflammatory benefits of IMD (Figure 22C & Figure 23C). Taken together, the present study found a potential mechanism for IMD's colitis reducing effects maybe similar to that of FOS, by stimulating the proliferation of *Bifidobacterium* which is associated with anti-inflammatory changes to SCFA productions and possible beneficial effects to other microbial groups.

In contrast, a significant dose-dependent reduction was also seen in the phyla Bacteroidetes, at the genus level for *Alistipe*s in both the cecum and stool (Figure 16A & Figure 19A). Previous studies have shown *Alistipes* to be highly relevant to dysbiosis and disease [149] Our data showed moderate positive correlation between *Alistipes* and cecal IL-1β concentrations (Figure 18C). In addition, it was positively associated with cecal valerate, propionate and isobutyrate, which were all SCFA with positive correlation to cecal IL-1β (Figure 24A & Figure 25). Though a member of the commensal microbiota, *Alistipes* have been seen to be particularly enriched in colorectal carcinoma samples compared to healthy [150]. Oral gavage of *Alistipes finegoldii* into IL10 KO and wildtype mice was able to sufficiently induce intestinal inflammation within only 1 week [151]. As *Alistipes* thrives in inflamed environments, this may explain its reduced abundance in IMD 15% and FOS groups, which were shown to have reduced IL-1β concentrations (Figure 4A). Or perhaps IMD may directly affect the abundance of *Alistipes* by stimulating the proliferation of other competing microbes. The abundance of cecal and fecal *Alistipes* seen were relatively low, making up only <1% of the microbiota across all groups (Table 1 & Table 2). However, this study only explored luminal bacteria and not mucosal bacteria. Previous reports have confirmed that mucous-associated bacteria differ from those associated with the lumen [152]. Mucosal bacteria have been more closely associated with

colitis, so it is possible that *Alistipes* may be seen in greater abundance in the mucous [153]. The reduction in *Alistipes* seen in this study, as well as the decrease in associated proinflammatory SCFA, by high dose IMD treatment is similar to the reduction seen in FOS group, and may be another potential mechanism for IMD's colitis reducing effects (Figure 15A).

As fibers are not solely metabolized by one microbial group, divergent compositional shifts can be seen between the IMD 7.5% and IMD 15% groups. For example, these divergent effects can be seen for the cecal genera *Oscillibacter, Parabacteroides, Ruminococcus, Flavonifractor, Prevotella* and unclassified *Prevotellaceae*, which were highest in IMD 7.5% group compared to all other groups including IMD 15% (Figure 16D,F,H & Figure 17G & Figure 18A-B). This divergence may be due to a matter of microbial competition. For example, these bacterial groups may be the initial fermenters of IMD due to their stronger competitiveness for the substrate. As such, when the substrate is low in abundance, as in the case of 7.5% IMD availability, only these groups are able to compete, thus restricting the availability of resources in its ecological niche. When the substrate is in high abundance, as is in IMD 15% diet, excess substrate is available for fermentation by less abundant groups such as *Allobaculum,* and *Anaerostipes,* for example, which were shown in increased abundance in cecum of IMD 15% group but not in IMD 7.5% (Figure 17A,B). The lower abundance of these more dominant genera seen in IMD 15% group may be produced by inhibiting effects of metabolites produced during the fiber fermentation or other physiological activities of less abundant but more competitive groups when substrate is highly available. This dose-dependent effect of prebiotics has previously been shown in our laboratory with patients with active UC treated with 7.5g/day or 15g/day inulin type β-fructans for 9 weeks [154]. The prebiotics showed a dose-dependent effect on disease activity, fecal microbiota shifts as well as production of SCFA [154]. Low

dosage increased *Faecalibacterium prausnitzii* relative abundance but failed to demonstrate health benefits to the patients, while high dosage increased *Bifidobacterium* spp. and *Roseburia* spp., and was associated with significant reduction in disease activity [154].

Future research could further aim to describe the mechanism of activity of IMD. A future study aiming to identify if isobutyrate and valerate proportions are causative of inflammation or a consequence of inflammation can be conducted by terminating rats prior to development of colitis symptoms around 6 weeks of age. This may reveal if reduced proportions of branched SCFAs in the IMD group are indeed associated with microbiota changes which suggests a microbial substrate shift from protein to carbohydrate fermentation. Conversely, if no difference in branched SCFA production is seen between the IMD group and negative control, this would suggest that inflammation may indeed be the driver of the differences in protein fermentation products. Another important focus of future IMD research could be to confirm if this α-glucan more favorably produces anti-inflammatory effects in males compared to females. To confirm this hypothesis, a well-defined study with a higher sample size for each sex would be required with a comparison between baseline and endpoint for inflammatory markers, microbiota composition and SCFA concentrations. Finally, encouraging results from our rat IBD model as well as previously tested murine IBD models shows promise for IMD as a potential adjuvant therapy in human IBD. Finding more effective prebiotic therapies will only benefit patients by providing a variety of choices that show efficacy in clinical setups in addition to safety and good tolerability.

2.6 Conclusions

In conclusion, we showed in HLA-B27 rats that oral administration of the prebiotic IMD effectively reduced the development of colitis in the cecum. These protective effects in preventing inflammation were associated with a reduction in cecal propionate, valerate, isobutyrate and isovalerate proportions. These changes in microbial metabolite production were also associated with significant shifts to microbial composition. Canada has some of the highest reported incidence and prevalence rates of IBD in the world, with costs exceeding the multiple billions [2,6]. As indicated by our study, using prebiotics as a dietary (adjunct) therapy shows potential as an easy-to-administer and cost-effective new treatment of human IBD.

Chapter 3: General Conclusions and Future Directions

3.1. General Conclusions

There is growing interest in studying prebiotics and their beneficial effects on the microbiota to prevent and/or alleviate symptoms of IBD. Data from preclinical studies and clinical trials show that prebiotic inulin-type fibers can prevent/reduce colitis; however, other dietary fibers, such as α-glucans, are understudied and it is also unknown if microbial shifts induced by α-glucans can be protective in IBD. IMD, a novel highly branched α-glucan product made by Hayashibara Co, Ltd. (Okayama, Japan), cannot be metabolized by host enzymes.

The main objective of my thesis was to determine the efficacy of IMD, in two different doses, on reducing/preventing the development of colitis and to investigate the associated protective mechanisms involving the gut microbiota composition and/or metabolism. Results showed that oral administration of IMD dose-dependently reduced the development of colitis in the cecum of male HLA-B27 TG rats, similar to that of FOS treated group. The benefits seen in males were increased for the group provided a higher dose of the prebiotic, IMD 15% group. Reduction in colitis was seen by lower cecal GGS as well as cecal IL-1β concentrations. Females did not show the same statistically significant reduction in colitis; however, we established that female HLA-B27 TG rats naturally have less cecal inflammation, which may have been due to the protective effects of estrogen seen in this model [127]. Additionally, some of our females on IMD 15% did show low inflammation markers, suggesting an individual-specific response to IMD treatment. IMD-induced reduction in cecal IL-1β was correlated with a reduction in cecal content proportions of the short chain fatty acids valerate, isobutyrate, isovalerate and propionate. Changes to diet were associated with significant changes to the microbiota composition at the phyla, family and genera levels. Key genera that were altered by IMD

administration included increased abundance of *Bifidobacterium*, which was associated with anti-inflammatory effects, and decreased *Alistipes*, which was associated with pro-inflammatory effects. Propionate, valerate, isovalerate and isobutyrate as well as *Alistipes* abundance may thus be of use in future studies as chronic inflammation biomarkers in HLA-B27 TG rat colitis models. Acetate proportions and *Bifidobacterium* abundances may be possible targets to increase in future studies as alleviators of chronic inflammation in HLA-B27 TG rats.

The results of this thesis indicate that the consumption of IMD may be protective against the development of colitis, and this protective effect may be due to its impact on the gut microbial composition and SCFA production. Based on these encouraging results indicated by our study, using IMD as a dietary therapy shows potential as an easy-to-administer and cost-effective new treatment of human IBD. Results from this preclinical study warrant future microbiota-altering intervention trials using IMD, possibly as adjunctive therapy to standard medications, in clinical IBD.

3.2. Limitations and Future Directions

Our study was limited in that microbial composition and SCFA analyses were only done for endpoint samples. No baseline samples were analyzed as to have a comparison of individual's change over time with treatment. This was done because rats' microbiotas were assumed to be fairly similar across groups, as rats came from the same few litters and were randomly assigned to groups; however, individual variation could have been due to variations in baseline microbiota and genetic traits. Additionally, studies in monozygotic twins show that concordance rates for CD and UC to be only 30-35% and 10-15%, respectively, suggesting there

is more complexity than just heritable components [12]. Indeed, we reported in our study that the effect of IMD was specific to the individual and that any beneficial or neutral response to IMD was not universal across either sex or litter. Therefore, future research should take these variations in endogenous gut microbiota across individuals into account as it is likely that not every patient will respond in the same manner. As such, the results of treatment should have been compared to baseline of each individual. Taken together, a future study could take a very similar approach to this study but alter it such that there are only 2 groups, negative control with cellulose and most optimal 15% IMD group, as this dose was seen to be more beneficial. Sample sizes for each group could then be increased and SCFA and microbial composition analyses could be done at baseline and endpoint. This would allow us to determine if certain microbial community proportions are being increased or decreased in individuals that show a response to IMD treatment compared to those that do not.

Lastly, encouraging results from this thesis study shows the potential of IMD as a therapy in human IBD. Results warrant future clinical IBD studies with IMD treatment. The best IMD dosage for efficacy as well as tolerability in humans would first have to be determined as high doses of prebiotics are known to induce flatulence and bloating. Testing for ideal dosage for safety, efficacy and adverse effects could be done as a small pilot study in a small number of IBD patients. If benefits and safety are seen in this population, and after establishing effective doses, clinical studies could move to more well-powered placebo-controlled clinical trials. In order to understand the individual response to IMD multiple longitudinal samples from the same individual overtime will be required. Such analysis will then allow not only to determine if IMD is effective in reducing the disease severity, but more importantly to determine the length of treatment required to show benefits in gut microflora. In addition to assessing clinical efficacy,

the placebo-controlled study will allow for exploration of possible mechanisms of IMD activity underlying the individual-specific response to treatment, such as individual-specific microbiota, or specific host abnormalities associated with innate and adaptive immune responses to microbiota and relation to clinical efficacy. A possible target for clinical trials could be investigating the effectiveness of IMD in preventing relapses in UC patients that are in clinical remission but have had a recent flare or have increased fecal calprotectin levels. UC may be a preferred target population, compared to CD, as this HLA-B27 colitis model has previously shown translational potential in human UC due to similarities in the gut microbiotas; In both HLA-B27 TG rats and human UC patients, FOS reduced colitis and stimulated similar protective bacterial groups (bifidobacteria and butyrate producing Firmicutes), and similar bacterial groups were shown to be associated with colitis (*Enterobacteriaceae*) [104]. Differences in microbial and inflammatory responses could then be compared to determine differences associated with patients that respond positively, neutrally and those who relapse after IMD treatment.

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